Getting to the site of inflammation: the leukocyte adhesion cascade updated

Klaus Ley, Carlo Laudanna‡, Myron I. Cybulsky§ and Sussan Nourshargh||*

Abstract | Neutrophil recruitment, lymphocyte recirculation and monocyte trafficking all require adhesion and transmigration through blood-vessel walls. The traditional three steps of rolling, activation and firm adhesion have recently been augmented and refined. Slow rolling, adhesion strengthening, intraluminal crawling and paracellular and transcellular migration are now recognized as separate, additional steps. In neutrophils, a second activation pathway has been discovered that does not require signalling through G-protein-coupled receptors and the signalling steps leading to integrin activation are beginning to emerge. This Review focuses on new aspects of one of the central paradigms of inflammation and immunity — the leukocyte adhesion cascade.

Combinatorial specificity
Specificity achieved in a sequential cascade. If there are 3 rolling molecules, 15 chemokines and 2 integrins, theoretically, 90 ($3 \times 15 \times 2$) specificities are possible.

**Robert M. Berne Cardiovascular Research Center and Departments of Biomedical Engineering, Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908, USA.*
‡Department of Pathology and Center for Biomedical Computing, University of Verona, Verona 37134, Italy.
§Toronto General Research Institute and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto M5G 1L7, Canada.
||Centre for Microvascular Research, William Harvey Research Institute, London, EC1M 6BQ, UK.
Correspondence to K.L.
e-mail: klausley@virginia.edu or klaus@liai.org
doi:10.1038/nri2156

Leukocyte rolling, adhesion and transmigration were all described by the pathologists of the nineteenth century[1,2]. With the discovery of integrins, selectins and their respective ligands, and of chemokines and chemokine receptors, the leukocyte adhesion cascade emerged as a concept that began to explain the recruitment of leukocyte subsets to specific sites. The original model of leukocyte adhesion proposed that the cascade achieved combinatorial specificity[3,4] through the three steps of selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest. However, recent evidence suggests that additional steps occur during integrin-mediated leukocyte adhesion, which remain incompletely understood[5,6]. Transendothelial migration was first described almost 200 years ago[1], but its molecular mechanisms were only discovered recently[7] and were not included in the classical adhesion cascade[3,4].Integrin-mediated adhesion is characterized by at least two events — arrest from rolling, which is mediated by increased leukocyte avidity for the endothelium (BOX 1), and a post-binding phase of adhesion stabilization, the molecular basis of which is only now beginning to emerge, although it was correctly predicted to be important more than 10 years ago[4]. In the past decade, new insights have been gained into the structures and signalling events that underlie integrin activation[5,6], into the post-adhesion events that strengthen leukocyte attachment to the endothelium, and into the molecules that are involved in leukocyte transendothelial migration[7,8]. These insights have led to an expanded version of the original three-step leukocyte adhesion cascade, which now includes slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane (FIG. 1).

Leukocyte rolling

The role of selectins. Rolling is mediated by L-selectin, P-selectin and E-selectin[9], which interact with P-selectin glycoprotein ligand 1 (PSGL1)[10] and other glycosylated ligands. L-selectin is expressed by most leukocytes, whereas E-selectin and P-selectin are expressed by inflamed endothelial cells. P-selectin is also expressed by activated platelets. PSGL1 has a dominant role as a ligand for all three selectins, although it was originally described as a P-selectin ligand. The binding of PSGL1 to L-selectin nucleates leukocyte–leukocyte interactions, by which adherent leukocytes[11] and leukocyte-derived fragments[12] facilitate secondary leukocyte capture or tethering, terms that are used synonymously. Secondary tethering also enables leukocytes that do not express ligands for E-selectin or P-selectin to reach sites of inflammation. Although PSGL1 is expressed on almost all leukocytes, it is functional only when glycosylated correctly (BOX 2). In addition to its expression by leukocytes, PSGL1 was recently found to be expressed by certain endothelial cells[13,14]. In addition to PSGL1, E-selectin also binds to glycosylated CD44 and E-selectin ligand 1 (ESL1)[15].

Box 1 | **Integrin activation**

Integrins are 'activatable' receptors, as intracellular signalling through cell-surface molecules — such as G-protein-coupled receptors — is required to greatly increase their ligand-binding capability. The avidity of integrin-mediated adhesion should be considered a 'cellular' macroscopic event that is regulated by two 'molecular' events: integrin affinity and valency of ligand binding[116,117]. Increased integrin affinity corresponds to conformational changes of individual integrin heterodimers, which leads to increased ligand-binding energy and a marked decrease in the rate of ligand dissociation. By contrast, valency corresponds to the density of integrin heterodimers per area of plasma membrane involved in cell adhesion, which can be dependent on the lateral mobility and cell-surface expression levels of integrins. The avidity of cell adhesion depends on shifts in the equilibrium of the average affinity state and the valency of a population of integrins, yet fluctuations or oscillations of individual molecules between low- and high-affinity states probably account for the formation and dissolution of bonds, which is required for complex cellular phenomena such as migration.

The interactions of selectins with their ligands enable leukocytes to adhere to inflamed endothelium under conditions of blood flow because they bind with exceptionally high on- and off-rates (which determine the speed with which bonds are formed and broken, respectively)[16]. L-selectin[17] and P-selectin[18] actually require shear stress to support adhesion; the rolling cells detach when flow is stopped. This phenomenon is related to the catch bond character of selectins[19], which strengthens each bond as shear stress is applied, and to the transport phenomena of selectin ligands relative to selectins brought about by the rolling motion of the cell that allow new bonds to form before the old ones are broken[20].

The endothelium has an active role in the rolling of leukocytes. Endothelial cells express E-selectin and P-selectin, the most important rolling molecules. Mice lacking endothelial expression of phosphoinositide 3-kinase-γ (PI3Kγ) show more than tenfold elevated rolling velocities in a model of inflammation[21]. Selectin engagement can trigger signals in both the selectin-expressing cell and the ligand-expressing cell[22–25]. The signalling pathways involved are just beginning to emerge. In myeloid cells, spleen tyrosine kinase (SYK) is downstream of PSGL1 (REFS 24,26,27). Binding of isolated human neutrophils to E-selectin induces integrin activation through a p38 mitogen-activated protein kinase (MAPK)-dependent pathway[25]. Leukocytes can also be activated through L-selectin, but the mechanism remains unclear. Selectin-mediated leukocyte rolling allows activating signals to be transmitted through adjacent G-protein-coupled receptors (GPCRs) and directly through selectin ligation.

Integrin-mediated leukocyte rolling. Integrins also participate in rolling and mediate firm leukocyte adhesion. Cell lines expressing $\alpha_4\beta_7$-integrin roll on immobilized recombinant mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1), and lymphocytes can roll on immobilized vascular cell-adhesion molecule 1 (VCAM1) by engaging their cell-surface ligand very late antigen 4 (VLA4; also known as $\alpha_4\beta_1$-integrin)[28]. VLA4-dependent rolling is mostly seen for monocytes and monocyte-like cell lines[29,30], T-cell lines[28] and T cells[31]. *In vivo*, VLA4 supports lymphocyte rolling in venules of the central nervous system (CNS) in conjunction with P-selectin[32] or can directly mediate rapid adhesion independent of P-selectin engagement[33].

β_2-integrins also support rolling. When resting mouse neutrophils suspended in their native whole blood roll on a substrate of recombinant E-selectin and intercellular adhesion molecule 1 (ICAM1), engagement by

Slow rolling
Rolling mediated by selectins (usually E-selectin) and integrins (usually LFA1), most commonly seen in neutrophils. Typical velocity is under 5 μm per second.

Shear stress
Shear stress (dyn per cm^2) is the force exerted by the flowing blood (dyn) on each unit of area of endothelial surface (cm^2).

Catch bond
A molecular bond that becomes stronger as pulling force is applied to it. By contrast, a slip bond becomes weaker.

G-protein-coupled receptor
(GPCR). A receptor that is composed of seven membrane-spanning helical segments, which are connected by extracellular and intracellular loops. These receptors associate with G proteins, which are a family of trimeric intracellular-signalling proteins with specific β- and γ-chains, and one of several α-chains.

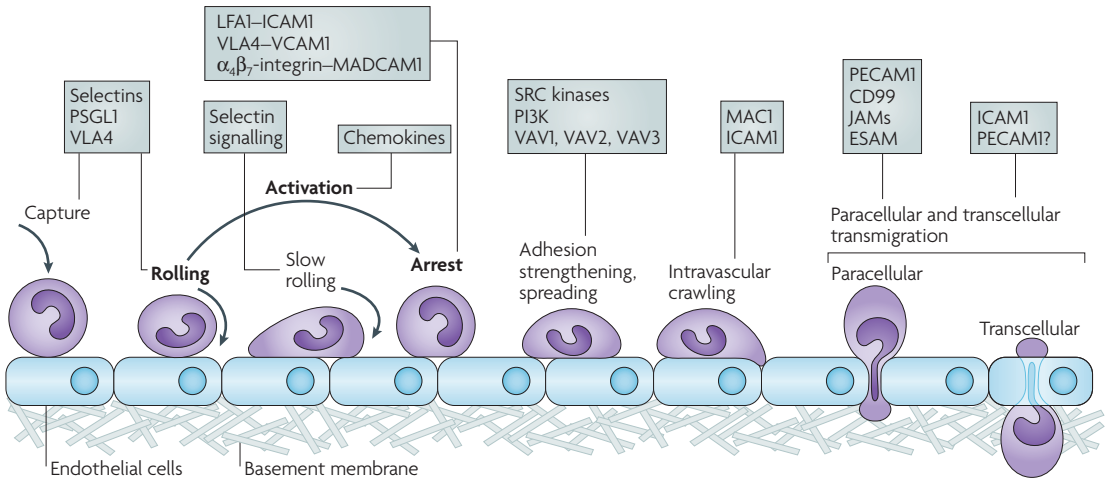

Figure 1 | **The updated leukocyte adhesion cascade.** The original three steps are shown in bold: rolling, which is mediated by selectins, activation, which is mediated by chemokines, and arrest, which is mediated by integrins. Progress has been made in defining additional steps: capture (or tethering), slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular and transcellular transmigration. Key molecules involved in each step are indicated in boxes. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as $\alpha_L\beta_2$-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as $\alpha_4\beta_1$-integrin).

Box 2 | **Selectin ligands**

Selectin binding is glycosylation dependent. All known selectin ligands require modification by the enzyme fucosyltransferase VII[118] and a sialyltransferase, such as ST3Gal-IV[119]. A minimal recognition motif for all selectins is the sialyl-Lewis X tetrasaccharide, which is sialic acid α2-3 linked to galactose, which is β1-4 linked to N-acetylglucosamine that also bears an α1-3-linked fucose. High-affinity PSGL1 (P-selectin glycoprotein ligand 1) binding to P-selectin and L-selectin also requires core 2 N-acetylglusosaminyl transferase-I[120]. Binding to P-selectin by PSGL1 is optimal only when tyrosines near the N-terminus of PSGL1 are sulphated[121]. Binding to E-selectin is further enhanced by fucosyltransferase IV[122]. L-selectin ligands in peripheral lymph nodes and at sites of chronic inflammation express *O*-glycans bearing terminal 6-sulpho-sialyl-Lewis X[123]. The scaffold glycoproteins include glycosylation-dependent cell-adhesion molecule 1 (GLYCAM1), endoglycan, podocalyxin and CD34, which are expressed by high endothelial venules in secondary lymphatic tissues[123].

E-selectin induces an intermediate affinity conformation of lymphocyte function-associated antigen 1 (LFA1; also known as $\alpha_L\beta_2$-integrin)[34], which allows it to transiently bind to its ligand ICAM1 on the substrate[35]. In at least one study, rolling of human lymphocytes was shown to be enhanced and slowed when ICAM1 was co-expressed with L-selectin ligands in a human vascular endothelial cell line[36]. Similarly, LFA1 expressed by K562 erythroleukaemia cells supported rolling on ICAM1, suggesting that these cells maintain LFA1 in an intermediate affinity state[37]. Recent structural evidence suggests that LFA1 can change its conformation and thereby increase its ligand-binding affinity under shear stress[38]. Taken together, these studies support an important role for the integrin LFA1 in its intermediate affinity conformation as a rolling and signalling molecule.

When mice are treated with tumour-necrosis factor (TNF), endothelial cells express E-selectin and increased amounts of ICAM1, and the rate of neutrophil rolling in the venules becomes slow (below 5 μm per second). Slow rolling *in vivo* was shown to require not only E-selectin[39], but also engagement of β_2-integrins[40], specifically LFA1 or macrophage receptor 1 (MAC1; also known as CD11b–CD18 and $\alpha_M\beta_2$-integrin)[41]. A role for MAC1 in mediating slow rolling in this model is consistent with an early report demonstrating activation of the adhesive capacity of MAC1 following E-selectin engagement[42].

Leukocyte activation and arrest

In vitro and *in vivo* studies have established that leukocyte arrest during rolling is rapidly triggered by chemokines or other chemoattractants and is mediated by the binding of leukocyte integrins to immunoglobulin superfamily members, such as ICAM1 and VCAM1, expressed by endothelial cells[43,44]. During inflammation, endothelial cells are activated by inflammatory cytokines to express adhesion molecules and synthesize chemokines and lipid chemoattractants that are presented on their luminal surface. Activated endothelial cells also transport chemoattractants from their abluminal surface[45]. Other chemoattractants can be generated by proteolytic cleavage in activated mast cells and platelets, and delivered to endothelial cells through circulating microparticles or exocytosis of intracellular granules. For example, platelets are known to deposit CC-chemokine ligand 5 (CCL5; previously known as RANTES), as well as other chemokines, such as CXC-chemokine ligand 4 (CXCL4) and CXCL5, onto the inflamed endothelium and thereby trigger the arrest of rolling monocytes[46,47].

Chemokines bind with high affinity to specific GPCRs. Chemokines can oligomerize and form heterophilic interactions that modulate their functions; for example CXCL4 amplifies CCL5-triggered monocyte arrest[48]. Many chemokines bind to glycosaminoglycans (GAGs) on the endothelial-cell surface, and this binding is required for efficient leukocyte recruitment, as chemokines with mutations in specific amino acids that mediate GAG binding induced chemotaxis *in vitro* but failed to recruit leukocytes to the peritoneal cavity[49]. Binding to GAGs may also protect chemokines from proteolytic cleavage and affect binding to high-affinity GPCRs.

Integrins most relevant to leukocyte arrest belong to the β_1-integrin and β_2-integrin subfamilies, and the most studied of these are the β_1-integrin VLA4 and the β_2-integrin LFA1. Classical chemoattractants and chemokines are the most powerful physiological activators of integrin-mediated adhesion. In particular, chemokines can rapidly regulate integrin avidity (BOX 1) in a cell-specific manner by increasing both integrin affinity and valency[5,50,51]. Ligation of specific heterotrimeric GPCRs by chemokines activates integrins by triggering a complex intracellular signalling network within milliseconds. This makes GPCRs particularly suitable to control rapid events, such as leukocyte arrest under shear-stress conditions. Indeed, integrin activation under physiological conditions can be an almost instantaneous event[51]. GPCR-triggered signalling by chemokines or other chemoattractants leading to rapid integrin activation is referred to as inside-out signalling, in contrast to outside-in signalling, which is downstream of ligand binding to activated integrins and probably contributes to adhesion stabilization and cell motility. Inside-out and outside-in signalling can occur concurrently[6].

Specificity in leukocyte arrest has traditionally been ascribed to differential expression of integrins and their ligands, and of chemokines and their receptors, as part of the qualitative output of the combinatorial multi-step model[3,4]. In addition, chemokine-triggered signalling networks can regulate distinct integrins in distinct leukocyte subtypes, thus highlighting the existence of integrin- and cell-specific signalling modules (see Supplementary information S1 (figure, table)). Diversity in leukocyte recruitment may emerge not only from the intrinsic complexity of pro-adhesive signalling networks, but also from quantitative variations of pro-adhesive parameters, such as the expression levels of chemokine receptors and their affinities for chemokines, which regulate integrin activation by selective lymphocyte subtypes[52] (BOX 3). The basal state of integrin activation

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

in subsets of circulating leukocytes and their topology in the plasma membrane, such as their expression in preformed clusters or on microvilli, are also likely to have a role in the overall regulation of inflammation. Activated lymphocytes and transformed lymphoblasts often constitutively express high-affinity forms of integrins, which makes the interpretation of arrest data obtained with such cells more difficult.

Integrin-affinity modulation triggers arrest

Modulation of the affinity of an integrin for its ligand is widely recognized as a crucial step in chemokine-induced arrest under flow[6,29,53]. Integrin clustering and valency modulation may also have a role under specific conditions[50]. The most detailed information about integrin structural rearrangement during affinity upregulation in leukocytes comes from studies of LFA1 (REF. 54). Inside-out signalling induces integrins to undergo a dramatic transition from a bent low-affinity conformation to extended intermediate- and high-affinity conformations, which leads to opening of the ligand-binding pocket[55]. In monocytes, the increase in the affinity of VLA4 and its binding to VCAM1 also involves conformational changes[29,56]. It is important to emphasize that low-, intermediate- and high-affinity integrins probably represent discrete states in a continuum of integrin conformational changes. Furthermore, chemokine-stimulated inside-out signalling induces conformational changes in only a fraction of integrins. Therefore, although the average affinity of the entire integrin population in a cell is increased, at any instant individual integrins assume a range of conformations. How this generates a discrete 'digital' output represented by leukocyte arrest is not clear and probably will need a comprehensive qualitative and quantitative analysis of the signalling events controlling integrin activation.

The intracellular signalling cascade from GPCRs to integrin activation is still incompletely understood. Based on studies in leukocytes, platelets and transfected cell lines, it can be subdivided into three stages — phospholipase C (PLC) signalling, activation of small GTPases, and induction of transitional integrin conformational changes through the association with actin-binding proteins, such as talin-1 (FIG. 2). These signalling pathways have not yet been fully established in leukocytes. Different leukocyte cell types, species and integrins probably use different intermediate signalling steps, thus providing an additional layer of specificity.

Box 3 | **Concurrency**

Concurrency, a term that originated from the information sciences to indicate the quantitative cooperation between contemporary events, has been recently included in the general multi-step model of leukocyte recruitment as a conceptual framework able to account for quantitative variation of pro-adhesive parameters[69]. Although still hypothetical, recent experimental data[52] support the consistency of this 'quantitative' view, suggesting that it will be essential in the future to generate mathematical models and computer simulations of leukocyte recruitment that enable predictive modelling of anti-adhesive pharmacological treatments. The control of leukocyte arrest probably involves quantitative regulation of signal transduction.

In monocytes, ligation of GPCRs results in rapid activation of PLC, which leads to intracellular Ca^{2+} flux and generation of inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). In U937 cells (a human monocyte cell line) and human monocytes, PLC-mediated calcium signalling is required for the induction of high-affinity VLA4 and monocyte arrest[57]. β_2-integrin-mediated neutrophil arrest on inflamed endothelium is also highly sensitive to the inhibition of PLC[61]. Under physiological conditions, the small GTPases RAP1 (RAS-related protein 1)[6] and RAS homologue gene-family member A (RHOA)[53] regulate LFA1 affinity. Small GTPases are activated by guanine-nucleotide-exchange factors (GEFs); thus, GEFs that are activated by Ca^{2+} and DAG, such as CALDAG-GEFI (calcium- and diacylglycerol-regulated GEFI)[58], dedicator of cytokinesis 2 (DOCK2)[59] or VAV1 (REF. 60) probably participate in the modulation of leukocyte integrin affinity. Humans[61] or mice[62] with deficient CALDAG-GEFI show reduced leukocyte arrest in response to chemokines. A link between RHOA, RAP1 and actin-binding proteins that regulate the triggering of integrin affinity has not been established, although recent data suggest that RHOA may be downstream to RAP1 in regulating LFA1 activation[63] and may also function as an antagonist of repressors, such as RHOH[64].

Talin-1, an anti-parallel homodimer, is the most studied actin-binding protein implicated in triggering integrin affinity upregulation. The F3 region of the head domain of talin-1 interacts with the cytoplasmic tail of the β-chain of platelet gpIIb/IIIa (also known as $\alpha_{IIb}\beta_3$-integrin) and triggers the transition of its affinity state[65]. The idea that the head of talin-1 wedges between the α-cytoplasmic tail and the β-cytoplasmic tail of integrins[65] is consistent with the observation that the α_L- and β_2-cytoplasmic tails of LFA1 move apart during activation[54]. Binding of talin-1 to the β_3-integrin cytoplasmic domain induces or stabilizes the high-affinity conformation of the extracellular domains[66]. At present, it is not clear whether talin-1 controls the triggering of LFA1 to its intermediate-affinity state or high-affinity state. Other actin-binding proteins, such as α-actinin and L-plastin, have been suggested to trigger the affinity transition of LFA1 (REFS 67,68).

Any linear signal-transduction pathway must necessarily represent an oversimplification of a complex signalling network. At least 47 proteins have been implicated in the regulation of integrin-mediated adhesion by chemoattractants[6,69]. These 47 proteins are the seeds defining a vast network, even when only the first (direct) interactors are considered (see Supplementary information S1 (figure, table)). The resulting network includes at least 900 proteins and more than 6,000 protein–protein interactions. This enormous complexity is probably the place where specificity and diversity in signalling mechanisms emerge.

Adhesion strengthening by outside-in signalling through integrins. It is well established that in addition to mediating adhesion, integrins generate intracellular signals that regulate various cellular functions, including cell motility, proliferation and apoptosis[70,71]. Ligand-induced

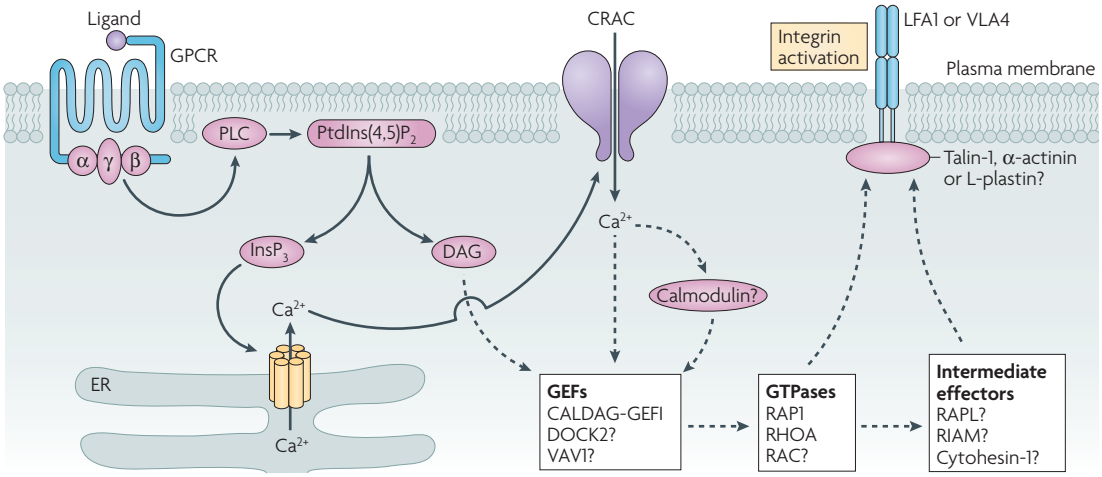

Figure 2 | **GPCR-dependent LFA1 activation.** A putative intracellular signalling cascade from G-protein-coupled receptors (GPCRs) to affinity activation of lymphocyte function-associated antigen 1 (LFA1; also known as $\alpha_L\beta_2$-integrin) or very late antigen 4 (VLA4; also known as $\alpha_4\beta_1$-integrin) in leukocytes, based on studies in leukocytes, platelets and transfected cell lines. Candidate molecules that remain to be confirmed are shown with question marks. The G-protein βγ subunit of the GPCR activates phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to produce inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ triggers Ca^{2+} release from intracellular stores in the endoplasmic reticulum (ER) , which in turn triggers Ca^{2+} influx from the extracellular space through the CRAC (calcium-regulated activated calcium) channel. Ca^{2+} activates calmodulin and may trigger one or more guanine-nucleotide-exchange factors (GEFs), such as calcium- and DAG-regulated GEFI (CALDAG-GEFI), dedicator of cytokinesis 2 (DOCK2) and VAV1. These may in turn trigger small guanosine triphosphate hydrolase enzymes (GTPases), such as RAS-related protein 1 (RAP1), RAS homologue gene-family member A (RHOA) or RAS-related C3 botulinum substrate (RAC). Finally, integrin-binding proteins activate integrins. Candidates are talin-1, α-actinin and L-plastin. Alternative intermediate effectors include regulator of cell adhesion and polarization enriched in lymphoid tissues (RAPL), RAP1-GTP-interacting adaptor molecule (RIAM), and cytohesin-1. It is likely that there are differences between leukocyte types, species (humans versus mice) and integrins.

integrin clustering and allosteric conformational changes probably contribute to the initiation of outside-in signalling and the formation of signalosomes, which are required for the efficient recruitment of protein tyrosine kinases (PTKs) and the initiation of the full repertoire of signalling pathways[72]. The induction of conformational changes to the cytosolic tail of the LFA1 heterodimer upon ICAM1 binding[51,54] may have a role in the rapid arrest of leukocytes under flow. FGR and HCK (haemopoietic cell kinase), two SRC-like PTKs that are crucial transducers of outside-in signalling by LFA1 and MAC1, are not required for chemoattractant-triggered upregulation of LFA1 affinity (inside-out signalling) and rapid adhesion of neutrophils under flow. However, the lack of outside-in signalling mediated by the β_2-integrin chain greatly accelerates the detachment of adherent neutrophils under flow[70]. A similar propensity for detachment was found in neutrophils lacking the GEFs VAV1 and VAV3 (REF. 60) or PI3Kγ[73]. These data provide clear evidence for a post-arrest phase of leukocyte adhesion stabilization.

The cytoplasmic domain of the α_4-integrin chain specifically binds paxillin[74], a 68 kDa signalling adaptor molecule that contains LIM domains, which are zinc-binding structures resembling a double zinc-finger domain, and leucine–aspartate motifs, which mediate protein–protein interactions. Paxillin binds the cytoplasmic tail of the α_4-integrin chain only when serine-988 is dephosphorylated[75]. This occurs when VLA4 ($\alpha_4\beta_1$-integrin) expressed by leukocytes is in a high-affinity conformation either constitutively, such as during lymphoid development in the thymus and bone marrow, or induced transiently during recruitment to the site of inflammation by inside-out signalling by GPCRs[76]. The implication of these observations is that stable binding of ligand to high-affinity VLA4 initiates outside-in signalling through adaptor proteins associated with the β_1-integrin chain, as well as paxillin associated with the α_4-integrin chain. The role of the interaction between the α_4-integrin chain and paxillin in outside-in signalling is also relevant to leukocyte migration[77]. Topographically specific integrin phosphorylation can control cell migration and polarization by spatial segregation of adaptor-protein binding[77]. Through an association with paxillin, unphosphorylated α_4-integrin chains can recruit an ADP-ribosylation factor GTPase-activating protein (ARF-GAP) through its LD4 domain, which decreases ARF activity and inhibits RAC, thereby restricting RAC activation to the leading edge of migrating cells[78]. Recently, mice were developed bearing a Tyr991Ala mutation in the cytoplasmic tail of the α_4-integrin chain, which blocks paxillin binding. Unlike α_4-integrin-chain-deficient mice, mice with the Tyr991Ala mutation were viable, but showed impaired recruitment of mononuclear leukocytes to inflammation in the peritoneal cavity[79]. VLA4 can also engage laterally with CD44, which improves its ability to support T-cell adhesion under flow[80].

LIM domains
LIM domains are named after their discovery in developmentally regulated transcription factors LIN11, ISL1 and MEC3. Each LIM domain consists of two tandem zinc fingers separated by two amino acids. LIM domains mediate protein–protein interactions and are frequently found in multiples.

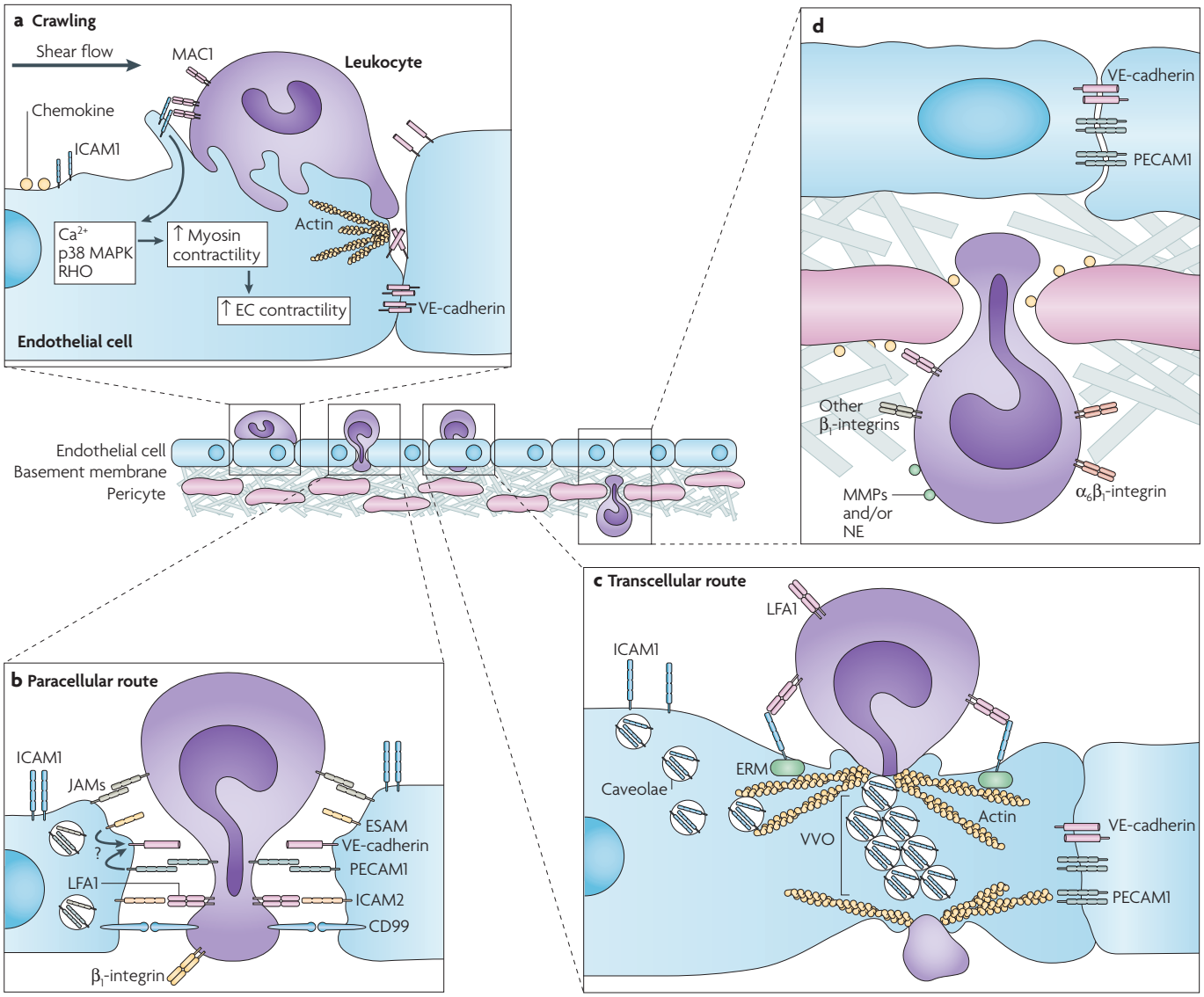

Figure 3 | **Transmigration.** Migration of leukocytes through venular walls involves penetrating the endothelial-cell barrier and its associated basement membrane and the pericyte sheath. **a** | Extension of leukocyte membrane protrusions into the endothelial-cell body and endothelial-cell junctions is triggered by ligation of intercellular adhesion molecule 1 (ICAM1) by MAC1 (macrophage antigen 1). Ligation of ICAM1 is associated with increased intracellular Ca^{2+} and activation of p38 mitogen-activated protein kinase (MAPK) and RAS homologue (RHO) GTPase, which may collectively activate myosin light-chain kinase leading to enhanced endothelial-cell contraction and hence opening of interendothelial contacts. These events may promote leukocyte migration through endothelial junctions (paracellular route), although leukocyte migration can also occur through the body of the endothelium (transcellular route). Transmigration through the endothelium can also induce cell-surface expression of members of the β_1-integrin family and proteases on neutrophils and other leukocytes that may facilitate the onwards movement of the leukocyte through the vessel wall. **b** | Paracellular migration involves the release of endothelial-expressed vascular endothelial cadherin (VE-cedherin) and is facilitated by intracellular membrane compartments containing a pool of platelet/endothelial-cell adhesion molecule 1 (PECAM1) and possibly other endothelial-cell junctional molecules, such as junctional adhesion molecule A (JAM-A). Other molecules involved in paracellular transmigration are endothelial cell-selective adhesion molecule (ESAM), ICAM2 and CD99. **c** | Transcellular migration occurs in 'thin' parts of the endothelium, and therefore there is less distance for a leukocyte to migrate. ICAM1 ligation leads to translocation of ICAM1 to actin- and caveolae-rich regions. ICAM1-containing caveolae link together forming vesiculo-vacuolar organelles (VVOs) that form an intracellular channel through which a leukocyte can migrate. Ezrin, radixin and moesin (ERM) proteins could act as linkers between ICAM1 and cytoskeletal proteins (such as actin and vimentin), causing their localization around the channel, thereby providing structural support for the cell under these conditions. **d** | Migration through the endothelial basement membrane and pericyte sheath can occur through gaps between adjacent pericytes and regions of low protein deposition within the extracellular matrix. This response can be facilitated by $\alpha_6\beta_1$-integrin and possibly proteases, such as matrix metalloproteinases (MMPs) and neutrophil elastase (NE). ERM, ezrin, radixin and moesin; LFA1, lymphocyte function-associated antigen 1.

Table 1 | **Leukocyte transendothelial cell migration**

Junctional molecule	Leukocyte ligand	References
PECAM1	PECAM1	8
JAM-A	LFA1, JAM-A (?)	97
JAM-B	VLA4	7
JAM-C	MAC1	7
ICAM2	MAC1, LFA1	124,125
CD99	CD99	8
CD99L2	Unknown	126
ESAM	Unknown	92

CD99L2, CD99-related antigen; ESAM, endothelial cell-selective adhesion molecule; ICAM2, intercellular adhesion molecule 2; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage receptor 1; PECAM1, platelet/endothelial-cell adhesion molecule 1.

Transendothelial cell migration

Crawling. Transmigration through venular walls is the final step in the process of leukocyte emigration into inflamed tissues and can occur with minimal disruption to the complex structure of vessel walls. Before crossing the walls of postcapillary venules, neutrophils and monocytes crawl inside blood vessels in a MAC1- and ICAM1-dependent manner[81,82], seeking preferred sites of transmigration. When crawling is disabled, transmigration is delayed and occurs preferentially through the transcellular pathway as opposed to the paracellular pathway[82].

Emigrating leukocytes encounter three distinct barriers (FIG. 3): endothelial cells, the endothelial-cell basement membrane, and pericytes. Leukocyte migration through the endothelial-cell barrier can be rapid (<2–5 minutes), but penetrating the endothelial-cell basement membrane can take much longer (>5–15 minutes). Transmigration is complicated by differences in phenotypes and ratios of endothelial cells to pericytes, resulting in differences in the composition of the endothelial-cell basement membrane generated by both. This structure facilitates migration in some areas and suppresses it in others.

Transendothelial cell migration can be triggered by luminal chemoattractants that may act in concert with shear flow[83], but current evidence suggests that different leukocyte subtypes may exhibit different levels of requirement for shear stress during transmigration. The interaction of leukocyte integrins with their endothelial-cell ligands (such as ICAM1 and VCAM1) may also stimulate endothelial cells in a manner that promotes leukocyte migration through the endothelium. Adherent leukocytes can induce the formation of 'docking structures' or 'transmigratory cups', which are endothelial-cell projections rich in ICAM1 and VCAM1 and cytoplasmic molecules such as ERM (ezrin, radixin and moesin) proteins and cytoskeletal components (such as vinculin, α-actinin and talin-1)[84,85]. These structures, which have only been observed in cultured endothelial cells so far, are triggered by the initial engagement of endothelial-cell adhesion molecules with their leukocyte integrin ligands. This induces their clustering and subsequent association with cytoplasmic proteins dependent on the expression of phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) and RHO family GTPases[84,85]. These projections express high levels of ICAM1 and/or VCAM1, which may initiate transendothelial cell migration through a paracellular or a transcellular pathway.

Paracellular route. Ligation of endothelial-cell adhesion molecules may lead to reduced interendothelial contacts and facilitated migration of leukocytes through endothelial-cell junctions. In support of this possibility, the cytoplasmic domain of ICAM1 has been shown to be crucial for RHO-dependent T-cell transmigration, and RHO activation in endothelial cells is involved in the opening of endothelial-cell junctional contacts[86,87]. Leukocyte transmigration is also associated with and dependent on increased levels of intracellular endothelial Ca^{2+}, a response that appears to promote transmigration through the opening of endothelial-cell contacts via the activation of myosin light-chain kinase and subsequent endothelial-cell contraction[8,88] (FIG. 3a). Inflamed endothelial cells can redistribute junctional molecules in a way that favours transendothelial cell migration. Molecules that do not support leukocyte migration, and might actually act as an obstacle to emigrating cells (such as VE-cadherin), may be distributed away from the junctional regions[89]. Endothelial junctional molecules for which leukocytes express ligands (such as platelet/endothelial-cell adhesion molecule 1 (PECAM1) and junctional adhesion molecule A (JAM-A)) may mobilize to the luminal surface, thus creating an adhesive haptotactic gradient that guides luminal leukocytes to the junctions[8].

Some endothelial junctional molecules actively mediate leukocyte transendothelial migration[8,90]. These molecules include immunoglobulin superfamily members PECAM1, ICAM1, ICAM2, JAM-A, JAM-B, JAM-C and endothelial cell-selective adhesion molecule (ESAM), as well as the non-immunoglobulin molecule CD99. Whereas ICAM1 and ICAM2 interact with LFA1 integrin, PECAM1 and CD99 support homophilic interactions, and the JAMs are involved in both homophilic and integrin interactions (FIG. 3b). The evidence for the involvement of these molecules in leukocyte transendothelial migration has largely been obtained by the use of neutralizing antibodies and knockout mice *in vitro* and *in vivo* (TABLES 1,2). Different molecules appear to mediate leukocyte transmigration in either a stimulus-specific or leukocyte-specific manner. For example, PECAM1, ICAM2 and JAM-A mediate leukocyte transmigration in response to interleukin-1β (IL-1β) but not TNF[91]. Direct activation of leukocytes by TNF, N-formyl-methionyl-leucyl-phenylalanine (fMLP) or leukotriene B_4 (LTB_4) appears to bypass the need for these molecules. The endothelial-cell junctional molecule ESAM does not show a stimulus-specific role but appears to mediate neutrophil rather than T-cell transmigration[92]. Leukocyte subtype-specific pathways may be related to the levels of leukocyte ligands that are directly or indirectly involved in recruiting a specific endothelial-cell junctional molecule in the process of transmigration, which may govern leukocyte migration

Pericytes
Pericytes are cells that are ~150–200 μm long and ~10–25 μm wide. They express smooth-muscle-cell α-actin and form a discontinuous network wrapped around endothelial cells of almost all post-capillary venules, and exhibit large gaps between adjacent cells.

Table 2 | **Leukocyte transmigration in knockout mice of key endothelial cell junctional molecules**

Mouse strain	Inflammatory model	Leukocyte transmigration response	References
Pecam1$^{-/-}$ (C57Bl/6 strain)	Peritonitis: induced by local thioglycollate	Normal neutrophil and monocyte infiltration but indications of neutrophil arrest at the level of the basement membrane	127
	Air pouch model: induced by local IL-1β, *Staphylococcus aureus* and CCL3	Normal neutrophil infiltration	
	Stimulated cremaster muscle	Transient reduced leukocyte transmigration through the basement membrane as induced by IL-1β but not TNF	128
	EAE	Early increased CNS infiltration of leukocytes	129
Pecam1 $^{-/-}$ (FVB/N strain)	Peritonitis: induced by local thioglycollate	Reduced neutrophil and monocyte infiltration	130
	Croton oil-induced dermatitis	Reduced leukocyte infiltration	
Pecam1$^{-/-}$ (DBA/1 strain)	Collagen-induced arthritis	Loss of suppressive effect of PECAM1 on T-cell activation during disease onset	131
Icam2$^{-/-}$	Lung allergic inflammation	Delayed eosinophil infiltration in the airway lumen	132
	Peritonitis: induced by local IL-1β, TNF or thioglycollate	Reduced neutrophil infiltration induced by IL-1β but not thioglycollate or TNF	133
	Stimulated cremaster muscle	Reduced leukocyte transmigration (but not adhesion) induced by IL-1β but not TNF as observed by IVM	
Jama$^{-/-}$	Peritonitis: induced by local thioglycollate	Reduced neutrophil infiltration	134
	Heart ischemia	Reduced neutrophil infiltration	134
	Hepatic ischemia	Reduced neutrophil infiltration	135
Esam$^{-/-}$	Delayed-type hypersensitivity reaction	Normal T-cell infiltration	92
	Peritonitis: thioglycollate-induced	Delayed neutrophil infiltration	
	Peritonitis: induced by co-injection of local IL-1 and CCL19	Reduced neutrophil but not lymphocyte infiltration	
	Stimulated cremaster muscle	Reduced leukocyte transmigration (but not adhesion) induced by IL-1β- or TNF as observed by IVM	

CCL, CC-chemokine ligand; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ESAM, endothelial cell-selective adhesion molecule; ICAM2, intercellular adhesion molecule 2; IL, interleukin; IVM, intravital microscopy; JAM, junctional adhesion molecule; PECAM1, platelet/endothelial-cell adhesion molecule1; TNF, tumour-necrosis factor.

in different inflammatory models *in vivo*. Other endothelial-cell molecules have also been implicated in leukocyte transmigration. These include poliovirus receptor (PVR; also known as CD155), a member of the nectin family[93], several ectoenzymes such as vascular adhesion protein 1 (VAP1) and CD157 (REF. 94), and leukocyte-specific protein 1 (LSP1)[95], but their mechanistic role is unclear.

Leukocyte transmigration can be regulated by targeted translocation of PECAM1 to endothelial junctions from a cell-surface-connected vesicular compartment[96]. Signalling through junctional molecules may also promote leukocyte migration by causing the loosening of adhesive contacts between VE-cadherin on apposed cells. For example, nectins JAM-A and PECAM1, through interactions with common cytoplasmic partners (such as catenins), are connected with the cadherin system[97]. ESAM, through the activation of RHO, is also linked to enhanced permeability through reduced VE-cadherin interactions[92]. Blocking CD99 was found to suppress monocyte and neutrophil transmigration through cytokine-stimulated human umbilical vein endothelial cells (HUVECs) at a stage distal to PECAM1 (REFS 98,99). This suggests that numerous molecular interactions must occur in sequence for transmigration to be successful. Whether PECAM1-mediated leukocyte migration through the endothelium promotes the CD99-mediated step is currently unknown, but blockade of PECAM1 and CD99 exert an additive effect on monocyte and neutrophil transmigration[98,99].

Transcellular route. Until recently, leukocyte transendothelial cell migration was thought to occur through the paracellular pathway only, but leukocyte migration through the transcellular route (FIG. 3c) occurs in the central nervous system (CNS) and in various inflammatory scenarios[100,101] and *in vitro* models[83,85,102,103]. Transcellular leukocyte migration represents the route of emigration for only a minority of emigrating cells (~5–20% of transmigrating cells through cytokine-activated HUVECs)[85], although the response can be very rapid (<1 min)[83]. Vesiculo-vacuolar organelles (VVOs) are small continuous membrane-associated passageways that are often detected at sites of neutrophil adhesion to endothelial cells, suggesting that such structures can act as a gateway for leukocytes through the body of the endothelial cell[104]. So far, VVOs have not been observed *in vitro*. Leukocyte migration starts with the extension of membrane protrusions into endothelial cells[83,102]. Ligation of ICAM1, especially under conditions of high

Vesiculo-vacuolar organelles (VVOs). Focal clusters of vesicles and vacuoles in the form of 'bunches of grapes' within the cytoplasm of microvascular endothelial cells. In response to VEGF or histamine they can provide a direct link between the vascular lumen and extravascular space. This is thought to be part of the mechanism behind increased vascular permeability.

ICAM1 expression, triggers cytoplasmic signalling events that lead to the translocation of apical ICAM1 to caveolae- and F-actin-rich regions and to the eventual transport with caveolin-1 to the basal plasma membrane[83,102]. These responses collectively result in the formation of channels through which leukocytes can migrate, the endothelial-cell membrane being stabilized under these conditions by actin[102] and vimentin[103]. *In vivo*, transcellular neutrophil migration is associated with areas of endothelial-cell thinning, a factor that could potentially facilitate leukocyte migration through this route by shortening the transmigration pathway[100]. The conditions and leukocyte types that use transcellular leukocyte migration may be governed by the type of vascular bed. A greater number of transcellular migrating T lymphoblasts were noted through TNF-stimulated human dermal microvascular endothelial cells (~30%) compared with HUVECs (~10%)[102].

The same molecules that mediate migration through endothelial-cell junctions may also be involved in transcellular migration[101]. Membranous structures bearing endothelial junctional molecules just below the plasma membrane of endothelial-cell borders (as reported for PECAM1 (REF. 96)) could potentially participate in the process of leukocyte engulfment and transport through the body of the endothelium[8].

Migration through the endothelial basement membrane and pericyte sheath. Having penetrated the endothelial-cell barrier, leukocytes then need to migrate through the endothelial basement membrane (FIG. 3d) and, in most venules, the pericyte sheath. The endothelial basement membrane is composed of two protein networks composed of the vascular laminins, such as laminin-8 and laminin-10, and collagen type IV, which are connected by interactions with molecules such as nidogen-2 and the heparin sulphate proteoglycan perlecan[105].

Analysis of unstimulated mouse cremasteric venules identified regions of low expression of matrix proteins within the endothelial basement membrane where the expression of certain basement-membrane constituents (such as laminin-10 and collagen IV) is lower than average; these constituents also appear to be permissive to emigrating neutrophils[106] and T cells[107]. Neutrophil migration through IL-1β-stimulated cremasteric venules resulted in transient enlargement of these sites[106]. Low-expression sites are co-localized with gaps between pericytes and so neutrophil migration occurs specifically at regions of least resistance, that is, gaps between adjacent pericytes and regions of low protein deposition within the extracellular matrix[106]. As pericytes can contribute to the generation of the endothelial-cell basement membrane, the patchy expression profile of pericytes in venular walls may be directly responsible for the generation of the low-expression regions. Low-expression sites may be more permissive to chemoattractants generated in the extravascular tissue and hence create a chemotactic gradient towards these regions. Of interest, the heparin-sulphate components of the basement membrane have been reported to bind chemokines and may therefore act as a depot for guidance molecules[108].

Transendothelial migration has an important role in altering the phenotype of leukocytes in a manner that aide their onward movement through the vessel wall, as well as their migration and behaviour in the extravascular tissue[109]. Ligation of PECAM1 on leukocytes can lead to activation of members of the β_1-integrin, β_2-integrin and β_3-integrin families[110]. PECAM1 is capable of inducing mobilization of the integrin $\alpha_6\beta_1$-integrin from intracellular stores to the cell surface of transmigrating neutrophils[111]. As this integrin is the main leukocyte receptor for laminin, PECAM1-mediated increased expression of $\alpha_6\beta_1$-integrin on the surface of transmigrating neutrophils enables neutrophil migration through the endothelial basement membrane[111]. Engagement of the β_2-integrin chain also induces expression of other β_1-integrins that are involved in neutrophil migration in the extracellular-matrix environment[112]. These responses may be facilitated by cell-surface-expressed leukocyte proteases, which expose binding sites within matrix-protein constituents with which leukocytes can interact or generate chemotactic fragments by selective cleavage of basement-membrane constituents[113]. Transmigrating neutrophils express cell-surface neutrophil elastase both *in vitro*[114] and *in vivo*[115], and there is evidence for some cooperative interaction between $\alpha_6\beta_1$-integrin and neutrophil elastase in the regulation of neutrophil transmigration *in vivo*[106,115].

Conclusion

Since its conception, the traditional three-step leukocyte adhesion cascade has been augmented by several more steps, including slow rolling, adhesion strengthening, intraluminal crawling, and paracellular and transcellular migration. The integrin-activation step has been refined by adding a second, GPCR-independent, pathway in myeloid cells. The signalling network linking chemokine receptors with integrin activation is beginning to emerge. Cell-type-specific differences remain to be discovered. This will be facilitated by the wider availability of and better techniques for using primary cells with minimal manipulation. More detailed signalling studies in neutrophils, lymphocytes and monocytes may identify new targets for potential therapeutic interventions. Many GPCRs have already been targeted by drugs, and antagonists for chemokine receptors are now being tested in preclinical and early clinical settings. Natalizumab is a monoclonal antibody specific for VLA4 and is used clinically in patients who have not responded to, or cannot tolerate, other treatments for multiple sclerosis. Another US Food and Drug Administration (FDA)-approved monoclonal antibody, efalizumab, which is specific for LFA1, is used for the treatment of chronic moderate to severe psoriasis. These integrin-blocking approaches carry significant risks related to impaired host response. Future combinatorial therapies with higher selectivity for certain leukocyte subsets promise improved approaches for treating acute and chronic inflammatory disorders.