ROLE OF THE CYTOSKELETON DURING LEUKOCYTE RESPONSES

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The cytoskeleton is a cellular network of structural, adaptor and signalling molecules that regulates most cellular functions that are related to the immune response, including migration, extravasation, antigen recognition, activation and phagocytosis by different subsets of leukocytes. Recently, a large number of regulatory elements and structural constituents of the leukocyte cytoskeleton have been identified. In this review, we discuss the composition and regulation of the different cytoskeletal elements and their role in immune responses.

CAPPING PROTEINS
Capping proteins bind to the growing (plus) end of microfilaments, blocking actin polymerization.

NUCLEATOR PROTEINS
Nucleator proteins bind to the plus or minus end of microfilaments and promote actin polymerization, binding to G-actin carriers and/or inducing conformational changes in microfilaments that promote its growth (nucleation).

ADAPTOR PROTEINS
Molecules that lack any known intrinsic enzymatic, DNA binding or receptor functions, but mediate protein–protein or lipid–protein interactions. Most function as flexible molecular scaffolds by regulating the spatio-temporal dynamics of specific effector molecules.

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Leukocytes carry out specific effector functions related to homeostasis as well as to the host response to infection and inflammation, and these functions are central to both innate immunity (such as phagocytosis) and adaptive immunity (for example, antigen-dependent T-cell activation). Efficient accomplishment of these functions requires a finely regulated cellular cytoskeleton to enable marked reorganization of the leukocyte membrane, receptor localization, recruitment of signalling intermediates and changes in the morphology of the cell, as well as the induction and/or inhibition of cellular programmes that lead to activation, proliferation, survival and differentiation.

Cytoskeletal proteins represent 2.8% of the human genome[1], and they form a part of complex and finely regulated polymer networks, including microfilaments, microtubules and intermediate filaments. Microfilaments, composed of filamentous F-actin, mainly control membrane plasticity (including cytoskeleton-propelled deformation and protrusion) and cell motility. The high rates of actin polymerization or depolymerization, which are regulated by many CAPPING PROTEINS, NUCLEATOR PROTEINS and ADAPTOR PROTEINS (TABLE 1), allow fast growth and deconstruction of microfilament-based structures[2]. However, microfilaments also regulate cell morphology through contraction and relaxation, which generate the mechanical force that is required for cell movement and is conferred by association with motor proteins such as myosins[3]. The key role of the actin cytoskeleton in leukocyte function has been emphasized by human conditions and animal models in which actin regulatory genes have been targeted (TABLE 2).

Microtubules are long polymers formed by the covalent association of α/β tubulin heterodimers that form hollow tubes. Many microtubule-associated proteins (MAPs), plus-end interacting molecules and motor proteins (mainly dyneins and kinesins) have been described to regulate microtubule polymerization, depolymerization and their dynamic properties[4–6]. As in all eukaryotic cells, microtubules mediate leukocyte division. Other roles of microtubules in leukocyte migration and effector functions are discussed later.

Intermediate filaments are different from actin or tubulin-based monomers. They are highly stable, do not show polarized growth and do not support motor-based cargo transport, but are involved in structural support[7]. Most leukocyte intermediate filaments are composed of vimentin. Their cellular functions remain elusive, although several lines of evidence point to a role in rigidity and structural integrity of lymphocytes during extravasation[8,9].

The regulation of cell motility and effector functions by the cytoskeleton is an active field of research that has seen a large increase in recent years. Some of the discoveries in adherent cells have translated well into leukocytes, whereas others posed specific questions referring to lineage-dependent regulation. This review focuses on the general mechanisms of cytoskeletal regulation and their specific roles in immune responses.

Table 1 | Cytoskeletal polymers in leukocytes

Protein subunit	Polymer growth	Bound nucleotide	Regulation of polymer length	Associated proteins	Motor proteins
Microfilaments					
Monomeric (G) actin	Nucleation (both sides)	ATP	Treadmilling Phosphate-based ageing and debranching Dynamic instability Post-translational modifications	Nucleators: ARP2/3 and formins Capping proteins: CAPZ and gelsolin Anti-capping: Ena/Mena/VASP Crosslinkers and lateral association: ABP1 G-actin interacting proteins: profilin and thymosin-β4 Cytolinkers: α-actinin and ERM proteins Severing: ADF/cofilin	Myosins
Microtubules					
α/β tubulin heterodimer	Nucleation (one side)	GTP	Treadmilling Phosphate-based polymer instability	Lateral association: MAPs, tau and ensconsin Plus end: CLIP170 and EB1	Dyneins and kinesins
Intermediate filaments					
Various proteins (mainly vimentin)	Not known (probably nucleation)	None	Fixed length	Cytolinkers: plectin	No

In the context of cytoskeletal polymer regulation, treadmilling defines the coordinated process of monomer loss at the non-growing pole of the polymer and monomer incorporation at the growing end of the filament. Phosphate-based ageing is a process by which ATP breakdown along the microfilament renders actin monomers bound to ADP, which promotes the association of actin-depolymerization factors and microfilament severing. Debranching refers to the splitting of microfilament branches due to ATP breakdown at branching points defined by the ARP2/3 complex. ABP1, actin-binding protein 1; ADF, actin-depolymerizing factor; ARP2/3, actin-related protein 2/3 complex; CAPZ, capping protein muscle Z-line; ERM, ezrin–radixin–moesin; MAP, microtubule-associated protein; VASP, vasodilator-stimulated phosphoprotein.

Leukocyte migration into target tissues

The sequential process of extravasation. The development of immune responses involves the migration of leukocytes from the blood to target tissues, where they carry out their effector function. Extravasation is also important during normal recirculation of leukocytes (especially lymphocytes) through lymphoid organs ('homing'). This is a sequential process that involves tethering and rolling of leukocytes on the blood-vessel wall, firm adhesion and crossing through the endothelial barrier (diapedesis). Activated endothelial cells express on their surface an array of adhesion molecules, such as chemokines, SELECTINS and their counterreceptors, and integrin ligands such as vascular cell-adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1). These proteins control the different stages of leukocyte extravasation by interacting with receptors expressed on their surface (FIG. 1).

Initial attachment (tethering) of leukocytes to the endothelium is mainly mediated by the interaction of selectins with their ligands. L-selectin, expressed by most leukocytes, interacts with sialylated ligands expressed by the endothelium and mediates rolling[10]. L-selectin cytoplasmic interaction with moesin — a member of the EZRIN–RADIXIN–MOESIN (ERM) family of proteins — is essential for its localization on MICROVILLI, whereas interaction with α-actinin, although required for L-selectin function, is not required for localization on leukocyte microvilli[11,12]. By contrast, P-selectin glycoprotein ligand 1 (PSGL1) mediates leukocyte interactions with P-selectin and E-selectin expressed by the endothelium and also supports rolling[13]. PSGL1 is anchored to the actin cytoskeleton through ERM proteins ezrin and/or moesin, and its ligation results in activation and recruitment of the tyrosine kinase SYK to PSGL1 through an IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM)-like motif in the ERM protein, which results in the transcriptional activation of genes such as *FOS*[14], and might constitute a priming mechanism to induce the production of gene products that are required after extravasation.

The next step of leukocyte extravasation is the firm attachment (arrest) of leukocytes on the surface of the activated endothelium, through the interaction of endothelial VCAM1 with $\alpha_4\beta_1$ (very late antigen 4, VLA4) and ICAM1 with β_2 integrins (in particular, leukocyte function-associated antigen 1, LFA1) expressed on the membrane of the leukocyte. The cytoskeleton regulates both sides of the endothelium–leukocyte interactions, allowing the endothelial cell to participate actively in leukocyte attachment through the generation of an actin-based docking structure that concentrates VCAM1 and ICAM1 molecules[15]. Integrins and actin filaments are interconnected through different adaptor proteins, such as talin, which might act as a scaffold to induce the formation of phosphatidylinositide-4,5-bisphosphate (PIP_2) and recruit actin polymerization machinery to the plasma membrane. Association of integrin-linked kinase (ILK) to the cytoplasmic tail of integrins also cooperates in the recruitment of signalling modules that are involved in actin polymerization such as PINCH, which can activate RAC through translocation of the RAC GTP EXCHANGE FACTOR (GEF) dedicator of cytokinesis 1 (DOCK180) to the plasma membrane, and α-PIX, which also activates RAC and CDC42, resulting in GTPase-mediated actin polymerization[16]. This indicates that integrins can nucleate actin polymerization and attachment to the plasma membrane providing a classic example of outside-in signalling. In turn, the actin cytoskeleton can modulate integrin responses by altering its association to these complexes, regulating inside-out signalling. So, transient uncoupling of LFA1 from the actin cytoskeleton induces integrin clustering and an increment of cellular adhesiveness to ICAM1

SELECTINS
Glycoproteins expressed by circulating blood cells or activated endothelial cells that support tethering and rolling through oligosaccharide-dependent interactions.

EZRIN–RADIXIN–MOESIN (ERM). Adaptor proteins that connect plasma-membrane molecules to the actin cytoskeleton through the release of a head-to-tail autoinhibitory interaction, targeting proteins to specific regions of the plasma membrane such as microvilli and filopodia.

MICROVILLI
An actin-based protrusive structure that is mainly found in epithelial cells; it is also present in other cell types such as leukocytes, where they have a role in tethering and rolling.

IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF (ITAM). A structural motif containing tyrosine residues, found in the cytoplasmic tails of several signalling molecules. The tyrosine in the motif (Tyr-Xaa-Xaa-Leu/Ile) is a target for phosphorylation by SRC tyrosine kinases and subsequent binding of proteins that contain SH2 domains.

GTP EXCHANGE FACTOR (GEF). These proteins catalyse the incorporation of GTP into the catalytic site of small GTPases, thereby acting as their activators.

AVIDITY
Avidity is the modulation of the ability of integrins to bind to their ligands, based not on the intrinsic affinity of each individual integrin molecule for its ligand, but by integrin clustering on the plasma membrane (increased number of integrin molecules per unit of membrane area), which results in increased binding capability.

AFFINITY
Integrin affinity is defined as the individual binding capability of integrins to their ligand(s), which is modulated by external factors that directly regulate integrin conformation, not their ability to cluster and therefore modulate their density on the plasma membrane.

G-PROTEIN-COUPLED RECEPTOR (GPCR) FAMILY
The largest family of membrane receptors, they bind small molecules (including chemoattractants), have seven transmembrane domains and signal through heterotrimeric G-proteins, resulting in cell activation.

LAMELLIPODIUM
Flat, fan-shaped, actin-rich structures involved in protrusion. They are usually generated in response to extracellular signals and direct cell-body migration.

(that is, increased AVIDITY)[17,18], whereas its subsequent coupling to the cytoskeleton accounts for enhanced LFA1 binding to its ligand(s) (increased AFFINITY)[17]. Diapedesis involves LFA1 binding to ICAM1 and interaction of leukocyte integrins with junctional adhesion molecules (JAMs), which normally regulate endothelial cell–cell junctions, but they also participate in transmigration[19]. Other signals that are required include those elicited by shear stress (which modulates pseudopod extension in neutrophils[20]), and chemokines expressed on the surface of the endothelium are required for diapedesis[21]. So, leukocytes must integrate positional information with respect to the endothelial monolayer, shear stress and signals from adhesive and chemotactic receptors to undergo shape changes and extravasation. However, this process is far from completely understood, as the mechanisms by which leukocytes perturb endothelial cell–cell junctions and cross through the gap is unknown. A working hypothesis consists of sequential activation of leukocyte GTPases by microenvironmental signals. Activation of leukocyte-expressed CDC42 by endothelial-cell-presented chemokines would have a dual role, allowing cell orientation ('the chemotactic compass') and extension of exploratory filopodia into the gap. Accumulation of adhesion receptors such as LFA1 at the filopodia (the interaction of which with lateral endothelial-cell ICAM1 could act as a priming site to promote filopodia enlargement), together with shear stress would activate RAC, allowing the conversion of filopodia into broad lamellipodia, which would force the breakdown of endothelial-cell junctions. Transmission of positional information throughout the leukocyte body would result in activation of RhoA in the rear of the cell, leading to rear detachment and stretching of the non-transmigrated portion of the leukocyte to gain passage through the gap. On the endothelial-cell side, transmigration can be enhanced by shear stress, as it activates cytoskeleton regulatory molecules, such as RAC, which might induce cytoskeletal rearrangements that are required for extravasation[22], such as weakening of the cell–cell junction, as it has been shown that activation of RAC downregulates RhoA activation, which is crucial for the integrity of intercellular junctions[23].

Leukocyte polarization and migration. The directed migration of leukocytes (chemotaxis) is governed by extracellular signals, such as chemoattractant gradients (which can be soluble, displayed on the extracellular matrix or presented on the surface of other cells), adhesion signals that promote substrate-dependent migration (haptotaxis) or a combination of both. Leukocyte chemoattractants include chemokines[24], some cytokines, bacterial peptides (for example, formyl-Met-Leu-Phe) or complement-derived peptides (for example, C5a). These molecules trigger specific receptors of the G-PROTEIN-COUPLED RECEPTOR (GPCR) FAMILY[25], directing actin polymerization and contraction, and regulating the shape changes that are associated with leukocyte migration. The GPCR family also activates other signalling pathways, regulating processes such as cell activation, changes in adhesiveness and apoptosis[26].

During firm adhesion, the combination of integrin signalling and exposure to immobilized chemokines on the apical surface of endothelial cells induces a marked change in the morphology of leukocytes. Leukocytes extend a front, F-actin-rich LAMELLIPODIUM, which constitutes the leading edge, and a trailing edge or UROPOD in which both the microtubule and intermediate-filament networks are retracted during migration (FIG. 2). Some components of the immune system such as dendritic cells (DCs) and macrophages, have specific structures, similar to ring-shaped adhesive structures known as podosomes. With some exceptions (DCs and highly differentiated macrophages), immune cells do not form stress fibres or focal adhesions — a feature that might explain their higher motility rates on integrin substrates, compared with adherent cells such as fibroblasts or endothelial cells.

Actin polymerization at the leukocyte lamellipodium is regulated in a similar way to what has been described in other cell types, and is locally triggered by signal amplification of the chemoattractant gradient that constitutes the polymerization stimulus. Whereas lamellipodium extension is regulated by both actin polymerization and actomyosin-based contraction (BOX 1 and FIG. 3), rear detachment seems to depend on contraction only. So, myosin-based contraction at the

Table 2 | Genetic defects and alterations related to the cytoskeleton of immune cells

Gene	Alteration	Species	Systemic phenotype	Cellular phenotype	References
WASP	Multiple mutations (Severely truncated or no protein)	Human	Immunodeficiency, eczema and micro-thrombocytopaenia	Deficient T-cell activation Impaired phagocytosis	128
	XLT (mutated gene)	Human	Attenuated thrombocytopaenia	Partial deficiency in actin polymerization	
	Knockout	Mouse	Immunodeficiency and thrombocytopaenia	Deficient T-cell activation	
Wip	Knockout	Mouse	None	Deficient T-cell activation B-cell hyperresponsiveness	129
Vav1	Knockout	Mouse	Deficient positive selection in the thymus	Deficient adhesiveness	55
Rac1	Transgenic	Mouse	Switch in thymocyte selection	Increased adhesiveness	130
Rac2	Knockout	Mouse	Neutrophilia, increased sensitivity to infections	Deficient PMN migration, NADPH oxidase activation	131
				Deficient T- and B-cell migration and immunoglobulin production	132
RhoA	Loss-of-function	Mouse	Impaired thymus development	Unknown	133

PMN, polymorphonuclear leukocyte; WASP, Wiskott-Aldrich syndrome protein; WIP, WASP-interacting protein; XLT, X-linked thrombocytopaenia.

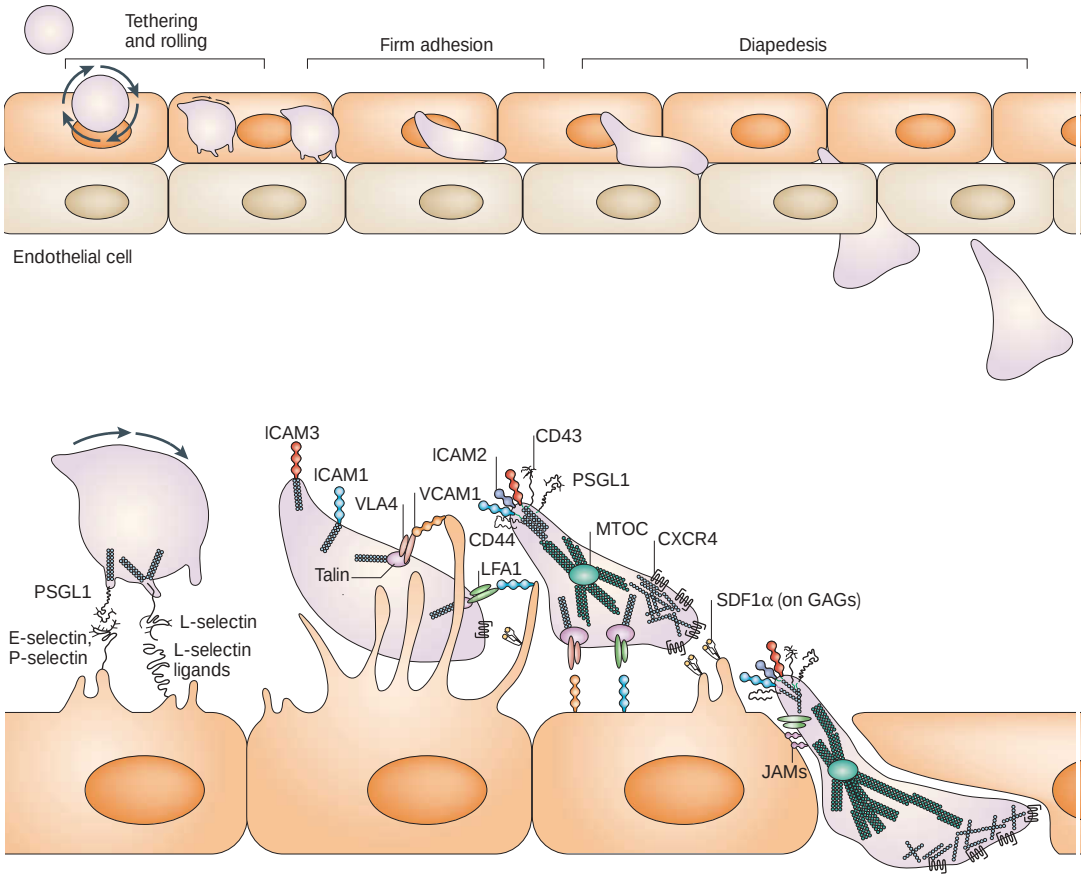

Figure 1 | **Leukocyte extravasation into target tissues.** The picture represents the sequential steps of leukocyte extravasation. Tethering of the leukocyte on the surface of activated endothelium occurs through interactions between L-selectin and its endothelial ligands, as well as P-selectin glycoprotein ligand 1 (PSGL1)-mediated contacts. Tethering and rolling precede firm adhesion, which is mediated by interaction of leukocyte integrins very late antigen 4 (VLA4, $\alpha_4\beta_1$) and leukocyte function-associated antigen 1 (LFA1, $\alpha_L\beta_2$) with endothelial vascular cell-adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1), respectively. The actin cytoskeleton has a dual regulatory role during this step (see text). Integrin-mediated cell adhesion is enhanced by interaction of G-protein-coupled receptors — for example, CXC-chemokine receptor 4 (CXCR4), the receptor for stromal cell-derived factor 1α (SDF1α), also known as CXC-chemokine ligand 12) — with chemokines immobilized on glycosoaminoglycans (GAGs) in a mechanism of receptor cross talk. During this step, leukocyte polarization occurs, with clustering of adhesion molecules in the cellular uropod. Finally, leukocytes extravasate and enter the target organ — a process that involves integrins as well as junctional adhesion molecules (JAMs) expressed by endothelial cells. For simplicity, not all of the domains of ICAM1, ICAM3 and VCAM1 are represented; they contain five, five and seven immunoglobulin domains, respectively. MTOC, microtubule-organizing centre.

leading edge is controlled by RhoA through local phosphorylation of myosin light chain (MLC) (similar to what has been described in fibroblasts[27]), which induces MLC assembly with the myosin heavy chain isoform IIA at the leading edge of migrating lymphocytes[28]. RhoA-regulated contraction and myosin activity are required for retraction of the trailing edge in monocytes[29], and is likely to involve activation of the myosin IIB isoform (FIG. 2).

Although most leukocyte subsets have common traits regarding cytoskeletal reorganization, chemotactic receptors in phagocytic cells (such as the C5a receptor in differentiated HL-60 neutrophilic cells) do not cluster at the leading edge[30], whereas chemokine receptors in lymphocytes do[31–33]. It is not clear whether this difference arises from different experimental approaches, chemotactic receptor density, or the different molecular strategies that lymphocytes and phagocytes use to navigate through a chemotactic gradient. However, both scenarios result in signalling intermediate redistribution, GTPase-regulated actin polymerization[34,35] and activation of lipid kinase(s)[36], therefore generating asymmetric pools of phosphoinositides (especially phosphatidylinositide-3,4,5-trisphosphate, PIP_3) and F-actin that result in stable cell polarization and directionality[37,38]. Other signals, such as those mediated by protein kinase C-β (PKC-β) have been shown to be crucial for LFA1-mediated polarization[39], and the RAS-related small GTPase RAP1 also controls lymphoid morphology and the distribution of chemotactic receptors[32], as well as LFA1 clustering at the leading edge through a novel effector, RAPL[40].

With regard to the uropod, it has been shown that different adhesion molecules (ICAMs, CD43, CD44, PSGL1 or CD95) cluster at this region[41] (FIG. 2). The uropod has been postulated to function as a recruiting antenna to capture bystander cells[42]. Alternatively, it

UROPOD
A slender appendage formed at the trailing, rear edge of fast-migrating cells, such as amoeba, neutrophils or lymphocytes.

IMMUNOLOGICAL SYNAPSE
A structure that is formed at the cell–cell interface between a T cell and an antigen-presenting cell; also known as the supramolecular activation cluster (SMAC). Important molecules involved in T-cell activation — including the T-cell receptor, numerous signal transduction molecules and molecular adaptors — accumulate at this site. Mobilization of the actin cytoskeleton of the cell is required for formation of the immunological synapse.

might be a store of molecules, which are not used during cell migration but might be readily mobilized to other membrane regions of the cell when required for effector functions[43]. Clustering of adhesion molecules at the uropod is an active process mediated by adaptors of the ERM family, the regulation of which depends on the phosphorylation of serine residues[44] by different kinases, including the RhoA effector ROCK (Rho-associated, coiled-coil containing protein kinase) — the importance of which has been shown *in vitro* only[45] — as well as on PIP_2 produced by phosphatidylinositol-4-phosphate 5-kinase, which seems to be the main regulatory mechanism *in vivo*[46].

Leukocyte effector functions

T-cell–APC interactions and the immunological synapse. Antigen presentation occurs when a T cell interacts with an antigen-presenting cell (APC) that expresses its specific antigen in the context of MHC molecules. Initial scanning of the APC surface by the T cell involves exploratory contacts that are mediated by the integrin LFA1 and their ligands ICAM1 and ICAM3, which culminates in specific immune recognition of antigen-loaded MHC class II molecules by the T-cell receptor (TCR). TCR-mediated signalling promotes the establishment of firm, LFA1-dependent adhesive interactions and selective redistribution of membrane molecules on the T-cell side into supramolecular activation clusters (SMACs), and the formation of the IMMUNOLOGICAL SYNAPSE[47,48] (FIG. 4a). Both microfilaments and microtubules are required for immunological-synapse formation. LFA1, present in the central SMAC (cSMAC) during its initial stage, is linked to the actin cytoskeleton through talin[49], and this interaction might direct its movement from the cSMAC to the peripheral SMAC (pSMAC). Other adhesion molecules such as the sialomucins CD43 and PSGL1 are excluded from the immunological synapse through their interaction with ERM family adaptors[43,50–52]. In this regard, it remains unknown how ICAM3, which also interacts with ERM proteins, is redistributed initially to the immunological synapse[43]. The actin cytoskeleton is also involved in the dynamics of SMAC formation, as ERM proteins interact with the cytolinker EBP50, which associates with the adaptor protein CBP in a regulatory mechanism of immunological-synapse formation through the control of lipid-raft generation[53]. Conversely, the immunological synapse also regulates the actin cytoskeleton, transmitting signals from SMACs that drive actin polymerization and/or contraction. So, TCR ligation at the contact area induces activation and recruitment of different kinases and molecules that are involved in actin polymerization, such as the scaffold adaptor NCK, which links the TCR to other actin regulatory elements such as Wiskott-Aldrich syndrome protein (WASP) and WASP-interacting protein (WIP)[54] (FIG. 4a). In addition to its role in controlling LFA1-mediated cell adhesion, the small GEF VAV1 is recruited to the TCR during immunological-synapse formation, which might result in local activation of the small GTPase RAC1 and subsequent actin polymerization[55]. WASP is also clustered at the T-cell–APC interface and activates the actin-related protein 2/3 (ARP2/3) complex, regulating localized actin polymerization[56]. In this regard, *WASP*$^{-/-}$ T cells form deficient immunological synapses and are poorly activated[57].

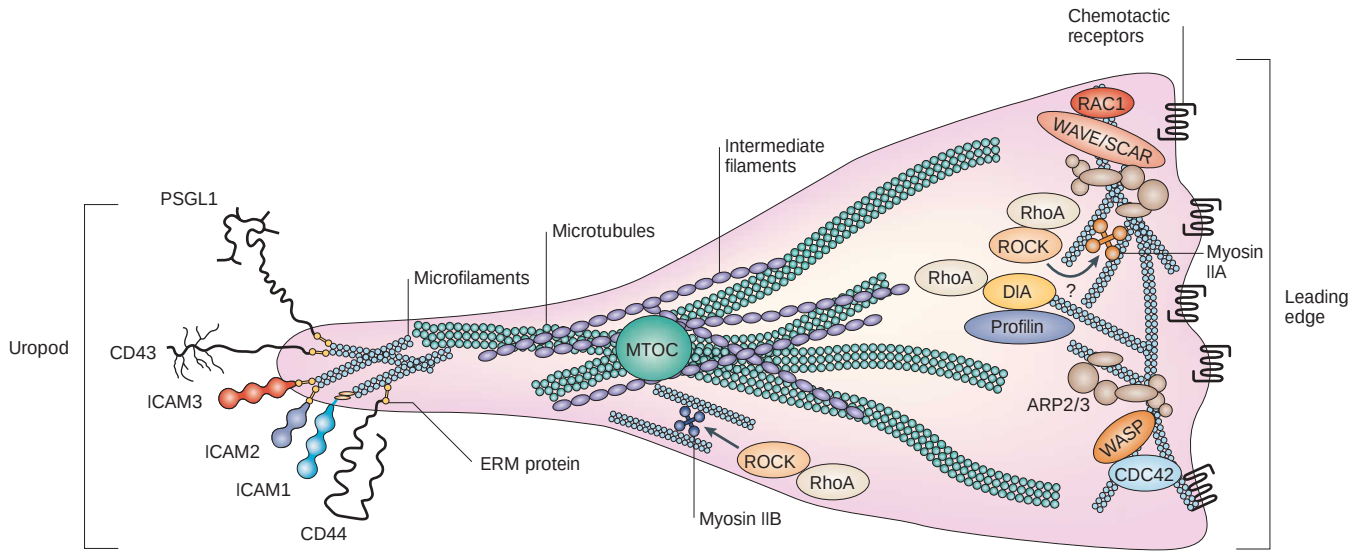

Figure 2 | **Receptor and cytoskeleton asymmetry during leukocyte migration.** After exposure to a chemoattractant source in a soluble or immobilized gradient, leukocytes develop front-to-back asymmetry, including extension of a broad, actin-based, flat lamellipodium regulated by localized activation of molecules that are involved in actin polymerization, such as the CDC42–WASP–ARP2/3, RAC–WAVE–ARP2/3 or RhoA–DIA1 pathways, guided by the local concentration of chemotactic receptors in lymphocytes. RhoA-regulated activation of myosin is represented by myosin IIA localization at the leading edge and myosin IIB localization at the rear part of the cell. The opposing pole (the uropod) is thinner, which sometimes protrudes over the plane of cell interaction with the substrate and is involved in cell–cell contacts. Actin regulates the formation of the uropod and clustering of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1), ICAM3, P-selectin glycoprotein ligand 1 (PSGL1), CD43 or CD44, using ezrin–radixin–moesin (ERM) proteins as membrane-actin cytolinkers. Other cytoskeletal polymers such as microtubules and intermediate filaments are also juxtaposed in this structure. ARP2/3, actin-related protein 2/3; MTOC, microtubule-organizing centre; ROCK, Rho-associated, coiled-coil containing protein kinase; WASP, Wiskott-Aldrich syndrome protein; WAVE, WASP family verprolin-homologous protein.

Box 1 | Rho GTPases and microfilament regulation

Many regulators of actin polymerization have been described during recent years, most prominently Rho GTPases and their regulators and effectors[114]. Rho GTPases are a subfamily (more than 10 members) of RAS-like proteins, the best-known members of which are RhoA, RAC and CDC42, which are or are not activated depending on their binding to GTP or GDP, respectively. The transition from a GDP-bound to a GTP-bound state is finely controlled by activators (GTP-exchange factors, GEFs) and inhibitors (GTPase activator proteins, GAPs and GDP-dissociation inhibitors, GDIs).

Rho GTPases directly control actin polymerization. Activation of RAC leads to conformational changes in the Wiskott-Aldrich syndrome protein (WASP)-like protein WAVE/SCAR[115,116] and activation of the actin-related protein 2/3 (ARP2/3) complex[117] (FIG. 3), which nucleates branched actin polymerization in highly protrusive areas, such as the leading edge. A similar mechanism involving activation of WASP and N-WASP by CDC42 can be inferred for filopodia formation[118]. RhoA might also control actin polymerization at the leading edge through formins such as DIA1 — a potent nucleator of actin polymerization[119] (FIG. 3), which is regulated by autoinhibition[120] and forms clusters at the leading edge of migrating T cells[121].

Microfilament stability is also regulated by Rho GTPases through the combined action of ROCK (Rho-associated, coiled-coil containing protein kinase) and p21-associated kinase 1 (PAK1) on LIM kinase[122,123], which phosphorylates and inactivates the actin-depolymerization factor (ADF/cofilin)[124] — a microfilament-severing molecule that induces structural breakdown of actin-supported structures (FIG. 3).

Actomyosin contraction is essential for cell motility. RAC and CDC42 activate their effector PAK1, which phosphorylates and inactivates both myosin light chain kinase[125] (MLCK) and myosin heavy chain II[97], leading to a loss in contractility, cell spreading and leading-edge extension in migrating cells (FIG. 3). Conversely, the RhoA effector Rho-kinase ROCK inhibits MLC phosphatase[126] and directly phosphorylates MLC[127], leading to increased actomyosin-based contraction (FIG. 3).

The role of microtubules during immunological-synapse formation and function is less obvious. Rear polarization of microtubules is rapidly and completely reversed when conjugation with an antigen-loaded APC occurs[58] (FIG. 4b). Under such conditions, the microtubule-organizing centre (MTOC) is translocated to the contact area and bounces alongside the contact surface with the APC to favour redistribution of the secretory apparatus, whereas front microtubules are bent in the opposite direction[59].

The signalling pathways that are involved in MTOC translocation have not been fully characterized (FIG. 4b). Different kinases such as ζ-chain-associated protein 70 kDa (ZAP70) might regulate this process through α-tubulin phosphorylation, which is crucial to maintain the ratio between microtubule growth and depolymerization[60]. The focal adhesion kinase (FAK), normally implicated in adhesion-related events in adherent cells, has been shown to regulate centrosome positioning in neurons[61]. In addition to its role in regulating actin polymerization, the small Rho GTPase CDC42 controls MTOC positioning in T-cell–APC conjugate formation[58]. Furthermore, microtubules have been shown to interact with and be required for activation of different Rho GEFs[62–64], thereby indicating the existence of a bi-directional interplay between actin- and tubulin-related signalling intermediates. Finally, microtubule stability seems to be important for MTOC translocation, as HDAC6 — a histone deacetylase that induces deacetylation and dynamic instability of microtubules — regulates MTOC translocation during T-cell–APC conjugate formation (J. M. Serrador *et al.*, unpublished observations).

Cytotoxicity, granule secretion and the cytoskeleton. Cytotoxic cells such as $CD8^+$ T lymphocytes (CTLs) and natural killer (NK) cells mediate killing of virus-infected cells through polarized delivery of vesicles that contain apoptosis-inducing proteins. Fully structured immunological synapses also form between CTLs or NK cells and target cells[65,66], with specific features that include the formation of a secretory domain at the border between the cSMAC and the pSMAC, through which CTLs secrete lytic granules, usually referred to as secretory lysosomes, which contain perforin and granzymes[67]. Secretory lysosomes travel to the secretion area on microtubules, cluster around the MTOC and empty their contents through the secretory cleft into a confined space between the CTL and the target cell, inducing death by apoptosis. Such immunological synapses are short-lived and not only cause death of the target cell, but also transfer of membrane from the target to the CTL, which results in the acquisition of target membrane proteins by the CTL and its progressive transformation into a target cell itself, constituting a mechanism for the limitation of the cytolytic response[65].

Two important questions regarding the cytoskeleton arise, the first of which is the positioning of the secretory cleft and secretory lysosome polarization. This process depends on cytoskeletal connections that regulate granule transport to the plasma membrane — a process that should be directed by signals emanating from the immunological synapse and related to relocalization of the MTOC and the associated Golgi network. By contrast, secretory lysosome movement, polarization and fusion with the membrane are tightly regulated processes, and some of the components involved have been identified in patients with rare immunological disorders and skin diseases, and the corresponding deficient mice. The Chediak-Higashi syndrome is characterized by giant secretory lysosomes and is a consequence of a mutated Chediak-Higashi syndrome 1 (*CHS1*) gene (*beige* gene in mice), which mediates lysosome fusion with the plasma membrane[68]. Other defects in secretion are associated with the adaptor protein 3 (*AP3*) gene[69], which regulates the biogenesis and movement of secretory lysosomes on microtubules, probably through its interaction with kinesin-like motor proteins[70]. Finally, the Griscelli syndrome is associated with a defect in the small GTPase RAB27, and CTLs from either Griscelli patients or *Rab27*$^{-/-}$ mice have defects in lysosome polarization, although the effectors involved are not known[71,72]. Together, these results allow the establishment of a signalling map that delineates some of the components involved in granule movement and secretion, and open the door to exciting perspectives on the elucidation of the relationship of these components with molecular motors.

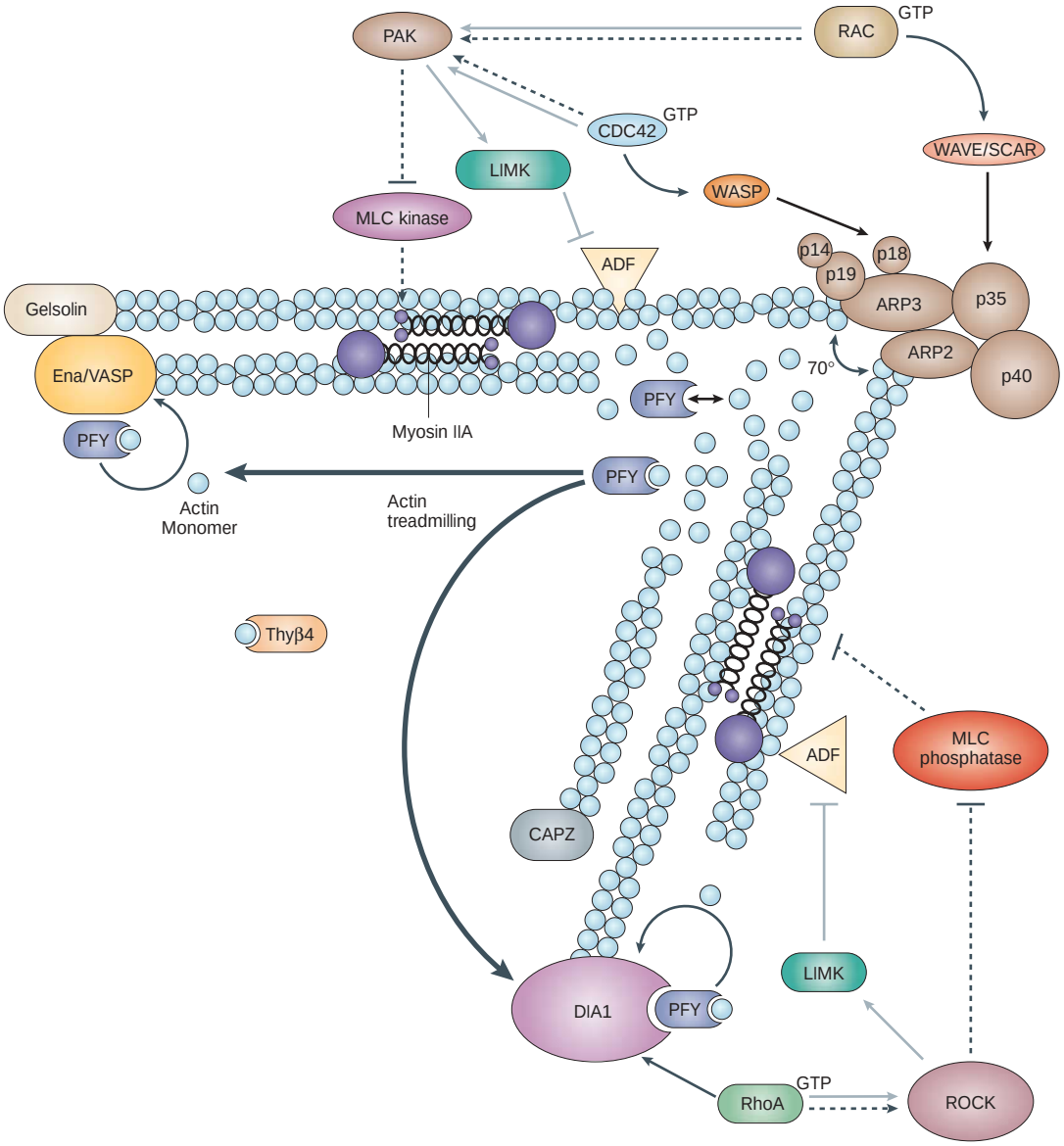

Figure 3 | **Rho GTPases and microfilament regulation.** The scheme depicts the mechanisms that are involved in the regulation of actin polymerization (black arrows), actin polymer stability (grey arrows) and contraction (dashed arrows), focusing on the regulatory role of small GTPases of the Rho subfamily. RAC and CDC42 regulate pointed-end nucleation through the activation of the actin-related protein 2/3 (ARP2/3) complex through the Wiskott-Aldrich syndrome protein (WASP)-family proteins WAVE and WASP, respectively. Barbed-end growth is limited by capping proteins such as CAPZ (capping protein muscle Z-line) and gelsolin. Capping proteins are counteracted by formins such as DIA1 (activated by RhoA), and the scaffolding protein vasodilator-stimulated phosphoprotein (VASP). Both of them induce actin polymerization at the barbed end through incorporation of actin monomers bound to profilin (PFY). This process is also limited by interaction of G-actin with thymosin β4 (Thyβ4), which sequesters actin monomers, although with lower affinity than profilin. Activation of ROCK (Rho-associated, coiled-coil containing protein kinase) by RhoA and p21-associated kinase (PAK) by RAC and CDC42 results in the phosphorylation of LIM kinase (LIMK), which in turn phosphorylates and inactivates actin-depolymerizing factor (ADF)/cofilin. Regarding actomyosin regulation, RhoA induces contraction by a dual mechanism that involves the inactivation of myosin light chain (MLC) phosphatase and direct MLC phosphorylation, whereas RAC and CDC42, acting through PAK, phosphorylate and inactivate MLC kinase and myosin heavy chain, promoting relaxation.

Phagocytosis and engulfment of apoptotic cells. When professional phagocytes, such as macrophages, encounter an opsonized target, they remodel their cortical actin cytoskeleton to undergo phagocytosis. Two phagocytosis modes have been defined: type I, which is dependent on immunoglobulin receptors (FcγRs), and type II, which is dependent on complement receptors such as CR3.

Type I phagocytosis induces actin-propelled extensions that surround the target particle, closing around in a zipper-like mechanism (FIG. 5). At a molecular level, FcγR triggering results in activation of the SRC family kinases[73] and recruitment of the tyrosine kinase SYK[74]. Stimulation of VAV1 activates RAC, which promotes actin polymerization (BOX 1). Activation of FcγR also

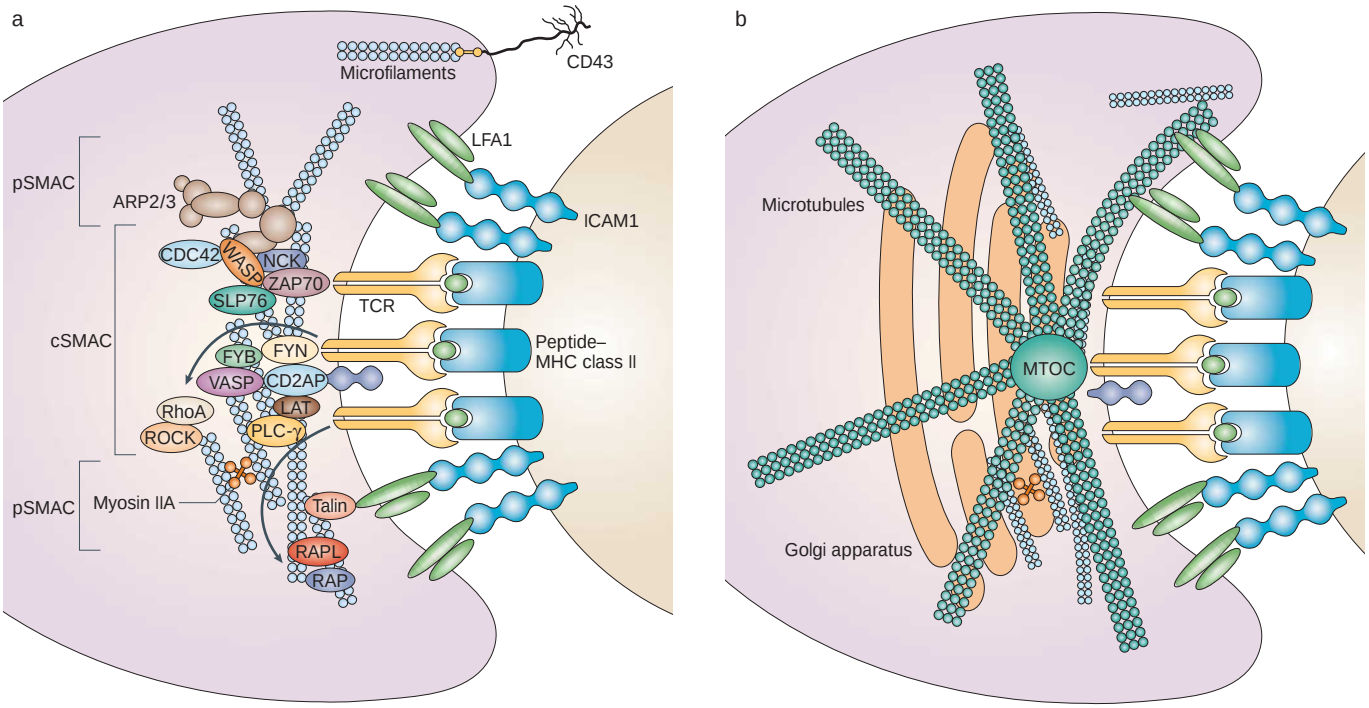

Figure 4 | **The lymphocyte cytoskeleton during T-cell–APC interactions and the formation of the immunological synapse.** Immunological synapse between a T cell and an antigen-presenting cell (APC). **a** | Formation of supramolecular activation clusters (SMACs) and regulation of T-cell receptor (TCR)-mediated actin polymerization at the immunological synapse. TCR interaction with peptide–MHC class II molecules recruits tyrosine kinases such as ζ-chain-associated protein 70 kDa (ZAP70) and FYN, adaptor proteins such as linker for activation of T cells (LAT), NCK, FYB, CD2-associated protein (CD2AP), vasodilator-stimulated phosphoprotein (VASP) or the scaffold protein SH2-domain-containing leukocyte protein of 76 kDa (SLP76) and actin polymerization-regulatory molecules such as the small GTPases CDC42 and RhoA, Wiskott-Aldrich syndrome protein (WASP), inducing localized actin polymerization at the central SMAC (cSMAC) through the actin-related protein 2/3 (ARP2/3) complex. The peripheral SMAC (pSMAC) is composed of leukocyte function-associated antigen 1 (LFA1) molecules that interact with intercellular adhesion molecule 1 (ICAM1) expressed by the APC and also regulates the actin cytoskeleton through LFA1 interaction with talin. The small GTPase RAP1 also regulates LFA1 at the immunological synapse through activation of RAPL, although the mechanism of such regulation is not well understood. Other molecules such as CD43 or P-selectin glycoprotein ligand 1 (PSGL1) are kept out of the SMACs through their interaction with ezrin–radixin–moesin (ERM) cytolinkers. **b** | Cytoskeletal distribution on a fully formed immunological synapse focusing on the reorientation of microtubule-organizing centres (MTOCs). PLC-γ, phospholipase C-γ.

recruits the small GTPase CDC42 to the PHAGOSOME, which activates WASP and N-WASP at the contact area[75]. The prominent role of WASP in this process is highlighted in WASP$^{-/-}$ macrophages, in which particle uptake is defective[76].

Although FcγR triggering has not been reported to induce actomyosin contraction, conventional and unconventional myosins are differentially distributed to the phagosome and participate in phagocytosis. Myosin II is recruited to early phagosomes, where it has a role in bending of the phagosome around the target and its subsequent closure[77]. In addition, myosin VII has an important role in phagocytosis through the regulation of adhesion to the particle[78], whereas other myosins, such as myosin Ic, myosin V and myosin IX are also recruited to the phagosome, although their precise involvement is still unknown.

Type II phagocytosis occurs in the absence of actin-process extension and involves particle sinking into the phagocytic cell membrane. However, actin polymerization that is dependent on the small GTPase RhoA but independent of RAC and CDC42 occurs at the contact area. CR3-induced activation of RhoA leads to MLC phosphorylation by ROCK either directly or through the inactivation of MLC phosphatase (FIG. 3), which causes local myosin-induced contraction at the contact area[79].

Other forms of phagocytosis include the engulfment of apoptotic bodies, which is crucial for the maintenance of cellular homeostasis. 'Eat me' signals (which, with exception of phosphatidylserine, are poorly defined) are recognized by phagocytic receptors that belong to different superfamilies, such as integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$), lectins and scavenger receptors[80]. Integrin activation induces phosphorylation of the SH2-SH3 domain-containing protein Crk-associated substrate (CAS), which recruits the adaptor complex CrkII–DOCK180 (CED2–CED5 in *Caenorhabditis elegans*) to the membrane, where it collaborates in the activation of RAC1 (CED10), thereby inducing actin-mediated phagocytosis and control of other pathways required for phagosome closure such as those involving phosphatidylinositol 3-kinase (PI3K)[81–83]. A second pathway (not well delineated in mammalian cells)

PHAGOSOME
An actin-based structure that engulfs a phagocytosed particle induced by the activation of Fc receptors, which cluster many signal transduction molecules that are involved in actin polymerization and regulation.

TYPE III SECRETION SYSTEM
A set of secreted proteins that induce membrane and cytoskeleton changes used by bacteria to force their entry into cells.

includes the activation of phosphatidylserine receptor(s) (CED1 in *C. elegans*[84]), further recruitment of human CED6 (REF. 85) and as-yet-unknown cytoskeletal rearrangements to accomplish engulfing.

Effector-independent cytoskeleton functions

In addition to its role in immune effector functions, the cytoskeleton is also crucial for other cellular processes, such as apoptosis. However, different pathogens have evolved strategies that target the cytoskeleton, so enabling them to survive and replicate in the host. The next section delineates these effector-independent processes.

Role of the actin cytoskeleton during apoptosis. Apoptosis is a key step in the homeostasis of the immune system, acting in two specific contexts; first, during the clearance of antigen-reactive T cells after infection, and second, during thymic selection to eliminate autoreactive cells. Apoptosis can be induced by different signals, for example, interaction of cell-surface receptors such as CD95 (APO1/FAS) with its ligand CD95L. CD95 is widely expressed, particularly by T cells, and constitutes a typical mechanism of T-cell apoptosis[86]. It is interesting to note that CD95 clusters at the uropod of T cells during migration[87], which raises speculation about the role of positional information during the induction and outcome of apoptosis, the extent of which has not been sufficiently explored.

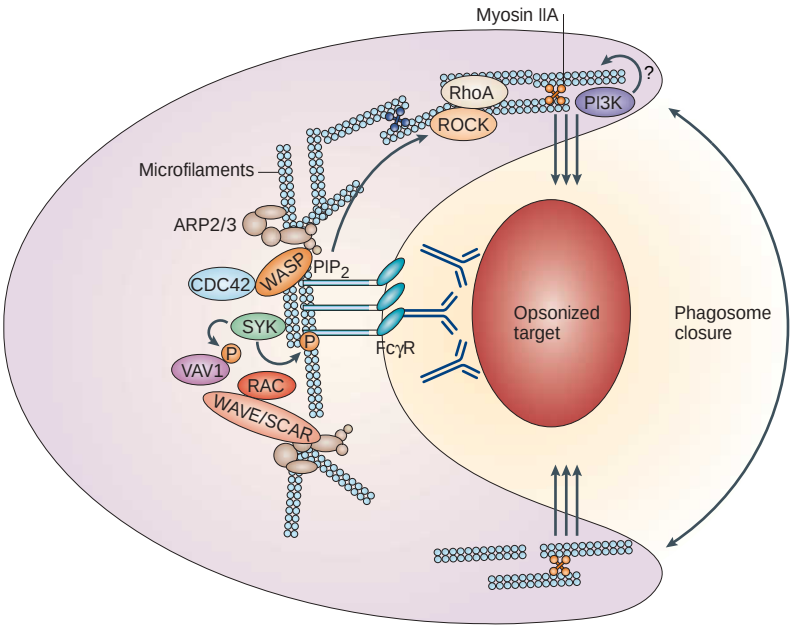

Figure 5 | **Type I phagocytosis.** Signalling events associated with activation of Fcγ receptors (FcγRs) on phagocytic cells are shown. Early kinase signalling mediated by SYK allows the formation of a phagocytic signalosome, including the GTP exchange factor VAV1, the small GTPases RAC and CDC42 and their effectors Wiskott-Aldrich syndrome protein (WASP) and WASP family verprolin-homologous protein (WAVE/SCAR), which, in cooperation with phosphoinositides such as phosphatidylinositide-4,5-bisphosphate (PIP_2), are involved in actin-dependent membrane protrusion and particle engulfing. Other signalling intermediates, such as phosphatidylinositol 3-kinases (PI3Ks) and RhoA- or ROCK (Rho-associated, coiled-coil containing protein kinase)-regulated unconventional myosins are involved in particle squeezing and phagosome maturation and closure. ARP2/3, actin-related protein 2/3.

Apoptosis can also be induced by stress signals and, in the thymus, by the absence of TCR signalling in developing lymphocytes ('death by neglect') or TCR recognition of self peptides with high affinity (negative selection)[88]. Apoptosis includes the cleavage of cellular components that are essential for structural integrity, and cell division, for example. Such cleavage is mainly mediated by cysteine proteases that belong to the caspase family. Among the targets of caspases, there are key regulators of the actin cytoskeleton. Caspase-mediated cleavage of cytoskeletal linkers to the plasma membrane such as filamin results in a decrease of tension on the plasma membrane and leads to the imbalance between inward and outward pressures on the plasma membrane, causing cell contraction and membrane blebbing[89]. Caspase 3, which is activated during thymic negative selection[90], also cleaves and activates ROCK1 (REFS 91,92), leading to myosin phosphorylation and actomyosin-dependent contraction that is required for bleb formation. However, cells undergoing blebbing are not necessarily committed to apoptosis, as this requires further disassembly of the actin cytoskeleton[93], which occurs through direct caspase-dependent cleavage of actin monomers[94] and modulation of Rho GTPase signalling. Caspases directly cleave CDC42 and RAC1 during CD95-induced apoptosis[95], therefore blocking downstream signalling and causing cytoskeletal disorganization and inhibition of anti-apoptotic signals. In addition, caspase-mediated cleavage of PKN — a serine/threonine protein kinase that has a catalytic domain highly homologous to PKC — induces actin disassembly through phosphorylation of α-actinin[96]. By contrast, cleavage of the RAC- and CDC42-regulated serine/threonine protein kinase PAK renders it active and destabilizes the actomyosin ring through phosphorylation of myosin heavy chain[97], which is confirmed by the fact that PAK is required for completion of apoptosis induced by CD95 (REF. 98). The fact that apoptotic signals provided by caspase cleavage are apparently conflicting in terms of cytoskeletal regulation (for example, ROCK versus PAK) highlights the complexity of this process, and suggests that, although contributing to the intermediate phenotypes that are observed during apoptosis, signalling induced by caspase-dependent activation/inactivation of cytoskeletal regulators finally causes the collapse of the cytoskeletal structure, cellular breakdown and formation of apoptotic bodies.

Intracellular pathogen subversion of the cytoskeleton. Most intracellular pathogens have developed elaborate systems to manipulate the cytoskeleton of the host cell[99]. A classic example is provided by some Gram-negative bacteria of the genus *Salmonella* and *Shigella*, which use a TYPE III SECRETION SYSTEM to enter the host cell and manipulate the actin cytoskeleton (FIG. 6). *Salmonella*, which mainly infects epithelial cells of the intestine and macrophages, first loosens microfilament attachment to the plasma membrane through the bacterial inositol phosphatase SigD, which also mediates pathogen-loaded vesicle fission from the membrane[100]. Then, *Salmonella* induces RAC and CDC42 activation

through the bacterial protein product SopE, which mimics signalling through endogenous GEF proteins[101], whereas the related product SptP prevents GTPase signalling through its GTPase activating protein (GAP) activity after bacterial entry has occurred[102]. Two other *Salmonella* proteins, SipC and SipA, bind to actin and modulate its polymerization and stability. SipC is part of the pore complex and directly nucleates cortical actin polymerization in a similar way to the endogenous ARP2/3 complex[103], whereas SipA stabilizes microfilaments and reduces the critical concentration for actin polymerization[104] (FIG. 6a). This mechanism is crucial for pathogen persistence in infected macrophages, as it has been shown that actin polymerization is essential for stability of the vacuoles that contain the pathogen, forming an actin-rich cocoon required for *Salmonella* survival[105]. Other bacteria directly stimulate endogenous actin polymerization. For example, the protein ActA from *Listeria* directly binds to the ARP2/3 complex, initiating actin nucleation and supporting intracellular pathogen motility[106] (FIG. 6b). Although it is well stated that actin-based propulsion is required for *Listeria* survival and growth, the precise mechanism is unknown. *Listeria* infects macrophages though active phagocytosis but persists inside cells through leaky phagosome–lysosome fusion[107], which allows pathogen survival inside cells. So, a probable mechanism through which *Listeria* avoids lysosome fusion would comprise actin-rocketed 'self-propulsion' out of the phagosome–lysosome fusion pathway.

The scenario is more complex in the case of viruses, which use different cytoskeleton-based strategies to favour their entry into host cells, nuclear targeting of their genome or exit from the cell after efficient replication. For example, the envelope protein gp120 from HIV-1 induces the clustering of CD4 receptor and CXCR4 co-receptor in macrophages to favour viral infection by a mechanism that is likely to involve the actin cytoskeleton[108] (FIG. 6c). In a similar manner, polarization of the T-cell actin cytoskeleton is required for the formation of viral synapses and cell-to-cell transfer of human T-cell leukaemia virus type 1 (HTLV1)[109] (FIG. 6d). Vaccinia virus uses actin-based motility to replicate inside cells[110], mimicking tyrosine-phosphorylation events related to actin polymerization[111]. In addition, microtubules have a crucial role in vaccinia-virus motility and transport to the plasma membrane, where it switches to actin-based motility through the phosphorylation of SRC[112] (FIG. 6e). Finally, HIV-1 also uses microtubules and dynein motor proteins to move

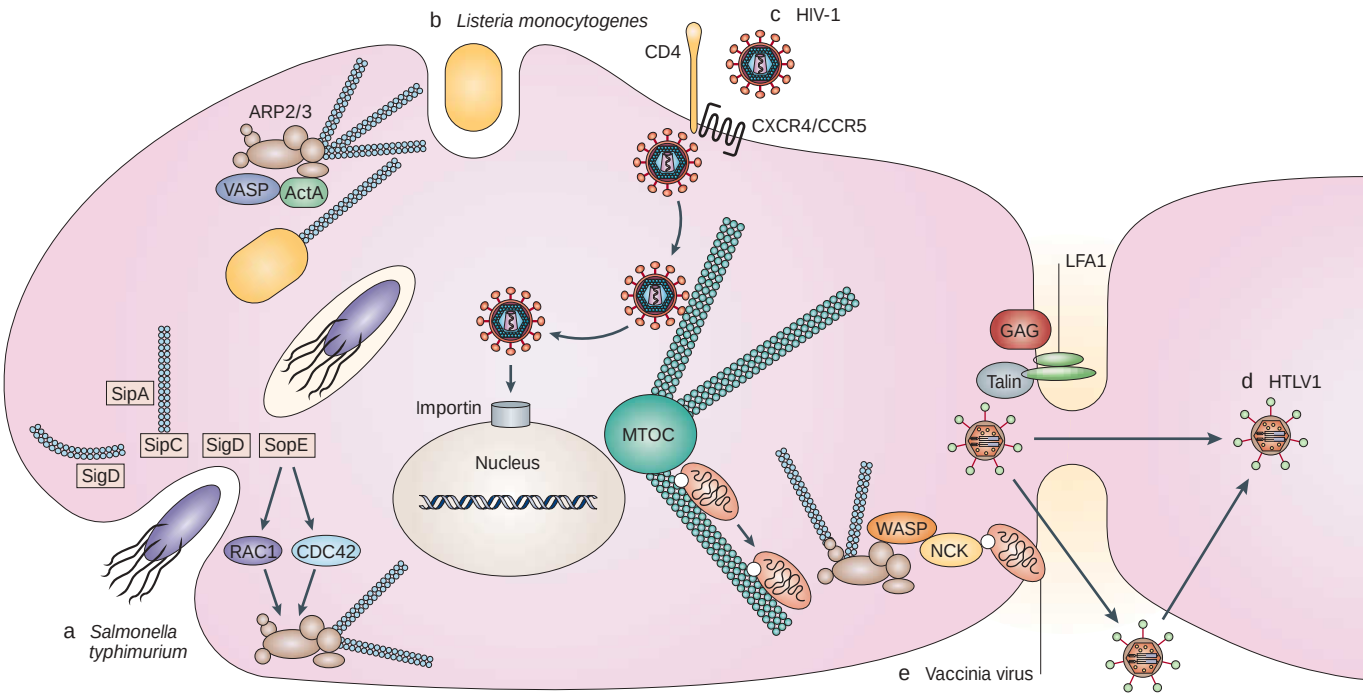

Figure 6 | **Cytoskeletal subversion by pathogens in immune cells.** Five different mechanisms of pathogen subversion are shown. **a** | *Salmonella typhimurium* encodes its own array of proteins in the type III secretion system to mimic actin regulatory elements, including SigD to break down phosphoinositides, dissociate the actin cytoskeleton from the cell cortex and induce vesicle fission; SipA and SipC, which induce actin polymerization and SopE, which activates the small GTPases RAC and CDC42. **b** | Actin-polymerization-driven *Listeria monocytogenes* intracellular motility. Bacterial ActA protein associates to vasodilator-stimulated phosphoprotein (VASP) and activates the actin-related protein 2/3 (ARP2/3) complex, which promotes directed actin polymerization and actin comet-based pathogen propulsion within the cell. **c** | HIV-1 infection. HIV proteins cluster CD4 and co-receptors CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4), in a mechanism aimed to facilitate viral entry into the cell, then the virion switches to the tubulin cytoskeleton, through which it is transported to the perinuclear area by a dynein-dependent mechanism. Finally, the viral genome is imported into the nucleus by a nuclear pore (importin). **d** | Human T-cell leukaemia virus type 1 (HTLV1) recruits integrins and actin-associated proteins such as talin, as well as viral proteins such as GAG, to intercellular junctions in a mechanism that favours the spreading of virus from cell to cell. **e** | Vaccinia virus slides on microtubules to the vicinity of the cytoplasmic membrane, and then switches to actin-based motility through the recruitment of NCK and associated proteins, inducing the formation of an actin pedestal to exit the cell. MTOC, microtubule-organizing centre.

within T cells and macrophages, but in this case to move inside infected cells towards the nucleus during early post-entry steps of the viral life cycle[113].

Concluding remarks and future directions

The leukocyte cytoskeleton has a pivotal role in achieving immune functions, ranging from cell migration to inflammatory foci to activation of the immune response, cytotoxicity and other specific tasks. Although much is known about the regulation of the actin and tubulin cytoskeleton in leukocytes, the spatio-temporal regulation of such mechanisms is not fully understood. Essential questions remain unsolved, such as the dynamic interplay between leukocyte adhesion molecules, chemotactic receptors and their ligands on the endothelial cell and their integration into a coordinated network, which results in actin-dependent cell changes. It seems unlikely that the function of every actin-regulatory protein in leukocyte migration, synapse formation and maturation has been assessed. The contribution of microtubule regulation to cell polarity and extravasation in lymphocytes and neutrophils is far from understood. Also, the mechanisms and biological significance underlying MTOC translocation remain elusive, as well as the cytoskeletal mechanisms by which the cell delivers secretory granules that result in either cell death or targeted activation. An intriguing issue is the different cytoskeletal response that is triggered by the same molecules depending on the cellular context, for example, actin polymerization can induce lamellipodial growth in migrating leukocytes, microfilament rimming along the immunological synapse or formation of a phagosome. The regulation of such processes and the composition of alternative signalosomes, depending on the extracellular signal and the leukocyte lineage involved in the response, is a challenge for the near future. Finally, the deciphering of the human genome and the emergence of proteomics are proving useful for the identification of new cytoskeletal partners, to solve existing controversies regarding the control of actin and tubulin polymerization and dynamics, and specific requirements for common and lineage-committed leukocyte functions.

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Competing interests statement

The authors declare that they have no competing financial interests.

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