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Polymerization power: effectors of actin polymerization as regulators of T lymphocyte migration through complex environments

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Abstract

During their lifespan, T cells are tasked with patrolling the body for potential pathogens. To do so, T cells migrate through numerous distinct anatomical sites and tissue environments with different biophysical characteristics. To migrate through these different environments, T cells use various motility strategies that rely on actin network remodeling to generate shape changes and mechanical forces. In this review we initially discuss the migratory journey of T cells, then cover the actin polymerization effectors at play in T cells, and finally we focus on the function of these effectors of actin cytoskeleton remodeling in mediating T cell migration through diverse tissue environments. Specifically, we will discuss the current state of the field pertaining to our understanding of the roles in T cell migration played by members of the three main families of actin polymerization machinery: the Arp2/3 complex; formin proteins; and Ena/VASP proteins.

Graphical Abstract

To perform their functions, T cell migrate through tissue environments with varying biophysical properties. Migration within these different tissues relies on actin network remodeling and the use of various motility strategies. In this review we discuss the role of actin polymerizing proteins, including Formins, Ena/VASP proteins, and the Arp2/3 complex, in enabling T cell migration modes through diverse tissue environments.

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SBT, MMW, and JJ wrote and edited the paper. SBT and JJ prepared the figures.

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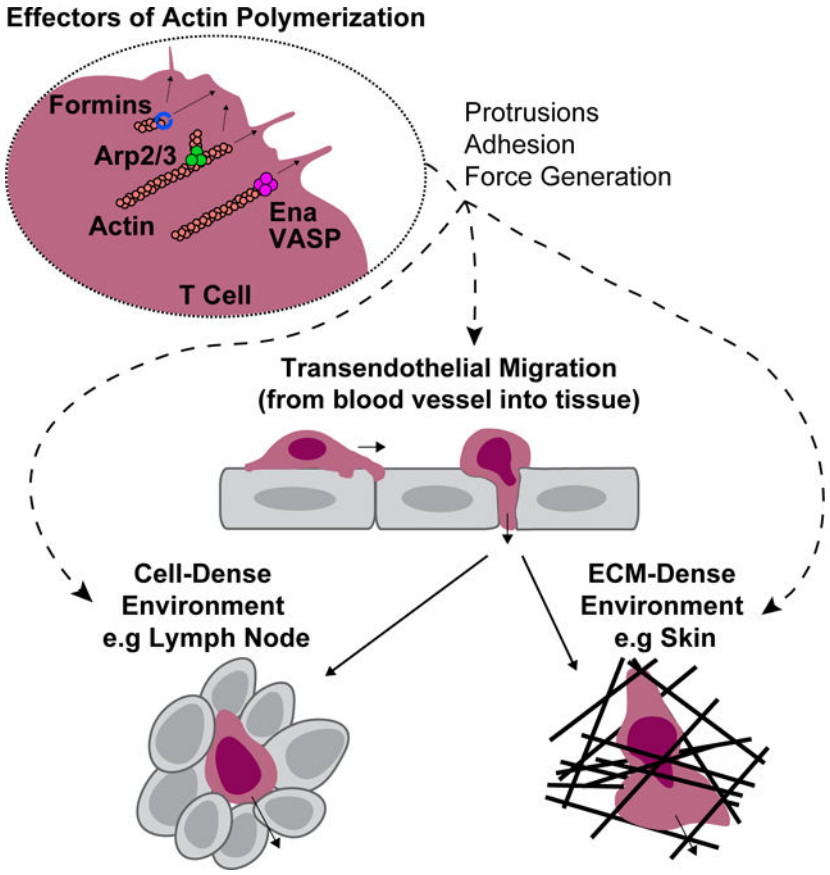

Keywords

Actin; cytoskeleton; migration; motility; extravasation; T cells; lymphocyte; Arp2/3; Formin; VASP

T cell Migration and Trafficking Overview:

A T cell's journey begins as a progenitor cell in the bone marrow which travels to the thymus to undergo development. Pre-T cells enter the thymus by exiting from the blood vasculature, a process that will be discussed in detail later in this review. However, during early embryonic development, before the thymus is vascularized, progenitor cells can also enter the thymus by a vascular independent route [1]. Interestingly, so far, there has only been one report of mutations in actin cytoskeletal regulators that prevent the entry of progenitor cells into the thymus [2], suggesting that this is a relatively permissive process.

Actin cytoskeleton rearrangements can play important roles in thymic selection by regulating both thymocyte migration as well as immunological synapse formation and T cell receptor (TCR) signaling. Within the thymus, the pre-T cell must navigate through a dense cell-packed environment to undergo the processes of positive selection in the thymus cortex and negative selection in the medulla by interacting with thymic epithelial cells (TECs) and resident dendritic cells (DCs) [1]. In the absence of autoreactivity, positively

selected thymocytes move quickly through the medulla along set migratory paths. However, as observed via multi-photon microscopy, some autoreactive thymocytes encountering a negative selecting ligand are induced to slow their speed and follow motility patterns that keep them confined to the medulla. Within these 'confinement zones' autoreactive T cells make frequent transient interactions with resident DCs, enhancing the efficiency of the negative selection process [3]. Once a T cell has matured it will exit the thymus to enter circulation in response to sphingosine-1-phosphate (S1P) gradients [4]. This can happen either by reverse extravasation into blood vessels or by entry into the lymphatics, which eventually drain into the blood stream through the thoracic duct [5,6].

Mature naïve T cells in the blood circulation will home to secondary lymphoid organs such as lymph nodes, spleen, and mucosal-associated lymphoid tissues, where they patrol for foreign antigens. To enter these and other peripheral tissues, T cells exit the blood stream through a multistep process known as extravasation or transendothelial migration (TEM) [7]. Canonically, TEM consists of four main sequential stages: rolling, adhering, crawling, and diapedesis [7].

Intravascular T cells are subjected to substantial shear stress due to the rapid flow of the bloodstream, ranging from approximately 1 dyne/cm^2 in the high endothelial venules (HEVs) of lymph nodes to greater than 6 dyne/cm^2 in the post-capillary venules of other tissues [8–10]. Transient interactions between selectins and oligosaccharides related to sialyl-Lewis^x enable T cells to resist these forces and initiate TEM by rolling on the vascular endothelial cell wall [11–13]. Low-affinity interactions between integrins and their immunoglobulin super family ligands can also facilitate rolling in some tissue-specific contexts, such as integrin alpha-4 in the Central Nervous System [14]. This rolling process enables T cells to subsequently encounter chemokines attached to the glycocalyx of the endothelium [15–17]. Signaling by these chemokines as well as shear forces acting on T cells serve to activate integrins into a high-affinity conformation [13,16–20]. Subsequent binding of high-affinity integrins to their ligands on the endothelium promotes the full arrest and attachment of T cells to the vascular wall [16,17,21].

Post-arrest, T cells respond to chemotactic cues and crawl on the endothelial luminal surface, relying on integrin-mediated adhesions for traction [16,17]. During this process T cells adopt an amoeboid morphology, with a ruffled, protrusive structure known as the lamellipodium at the leading edge of the cell and a contractile structure known as the uropod at the trailing edge [22–24]. Additionally, during crawling, micro-protrusions known as podosomes and filopodia extend from the ventral side and leading edge of T cells into the endothelium, to probe for chemokine signals and permissive sites for transmigration across the endothelium [16,21,25].

During diapedesis, the final stage of TEM, T cells translocate across the vascular cell wall. This process predominantly occurs between endothelial cells at cell-cell junctions via the paracellular route. However, in certain tissues, such as the Central Nervous System, diapedesis also occurs through the endothelial cell body via the transcellular route [25–30]. Ligation of endothelial adhesion molecules such as Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) by T cell integrins drives

signaling events within the endothelial cells that promote the internalization of VE-cadherin [30–32]. Internalization of VE-cadherin loosens endothelial cell-cell junctions and thus facilitates subsequent diapedesis of T cells [30–32]. Extension of actin-dependent protrusions, such as filopodia and podosomes, may also induce changes within the endothelial cells to initiate diapedesis [16,25,29,30]. During diapedesis T cells extrude their body through and underneath the endothelium [16,29,30,33,34]. As T cells migrate beneath the endothelium, they retract their uropod to complete the process [23,29,30,33].

The nucleus is the most rigid cellular structure and, as such, presents a significant obstacle to the completion of diapedesis [23,33–37]. It has been proposed that the segmented and deformable shape of the neutrophil nucleus facilitates the rapid migration of these cells through restrictive environments [27,35,38]. In contrast, T cells exhibit a more rigid, ovoid nucleus and rely on significant contractile forces in the uropod to transmigrate the nucleus through the endothelium during diapedesis [22,33,36]. Thus, nucleus translocation can be a rate-limiting step for T cell migration through restrictive environments and, consequently, diapedesis [39,40].

Upon entering secondary lymphoid organs such as lymph nodes, T cells once again encounter a cell-packed environment, including dendritic cell networks and stromal cells such as fibroblastic reticular cells (FRCs), which guide their migration [41–43]. Soluble and cell surface-immobilized chemokines play a key role in guiding interstitial T cell migration in part by regulating actin cytoskeleton remodeling [44,45]. Within lymph nodes T cells undergo semi-random migration to maximize encounters with dendritic cells in search for their cognate antigen [46,47]. If T cells do not detect the presence of such antigens after 12–24 hours [48], naïve T cells will leave the lymph node through the lymphatic sinuses to enter lymphatic circulation and drain to another lymph node or return to the blood circulation through the thoracic duct and continue their patrolling. If, during their meandering migration within a lymph node, a T cell encounters an antigen-presenting cell (APC) bearing its cognate antigen, the T cell will temporarily stop crawling to interact with the APC and become activated [46,47,49,50]. The specifics of immunological synapse formation, T cell activation, and the role of the actin cytoskeleton in these processes has been reviewed in depth elsewhere, for example in references [51–53]. Within the lymph node, T cell interactions with dendritic cells can occur in three distinct phases. During the first phase, T cells engage in short, serial interactions with cognate DCs (ranging from about 30 minutes to 1 hour). Gradually, in the second phase, these transition into longer, more stable interactions, in which T cells remain in contact with DCs for 8–12 hours and begin producing IL-2 and other cytokines. Finally, in the third phase, T cells disassociate from DCs, and begin migrating rapidly and proliferating vigorously, still forming transient interactions with DCs that grow shorter over time [49]. These transient interactions, known as kinapses, observed in the first and third phases of primary T cell activation demonstrate the ability of a T cell to integrate activation signals from APCs over a series of interactions [54]. T cells that are being activated will remain in the lymph node for approximately 3 days, as regulated by the relative balance of S1PR and CD69 expression [4], before reentering circulation. Effector T cells will then be recruited from the circulation to the site of inflammation by extravasating through the vasculature of the inflamed tissue.

Finally, within peripheral tissues, recruited effector T cells (and resident memory T cells) encounter a variety of environmental conditions depending on the tissue composition of stromal cells, extracellular matrix (ECM), and inflammatory state [55,56]. In these tissues T cells will migrate towards the source of inflammation following chemotactic cues and navigating through complex ECM structures with varying sizes of openings to squeeze through [57,58]. To efficiently survey tissues, particularly in inflamed sites, T cells can adopt a type of motility termed Levy walk, which is a modified random walk that entails alternate periods of confined motility and more straight migration to optimize target searching efficiency [59]. Interestingly, T-helper (Th) 1 cells and Th2 cells in the inflamed skin can use different searching strategies, with Th1 cells having more confined chemokine-dependent motility and Th2 cells relying on integrin alpha-V/beta-3 to search a larger tissue volume. Hence, intrinsic differences resulting from T cell differentiation tune effector T cell sensitivity to environmental factors, leading to T cell subset-specific migration patterns [60]. In a recent *in vivo* study, intravital imaging revealed that the homeostatic patrolling of tissue resident memory T cells in the salivary glands is both integrin and chemokine independent but relies instead on the topology of resident macrophages to serve as a frictional interface to drive motility [61]. Similarly, the ECM provides a three-dimensional (3D) scaffold that can direct T cell migration within tissues, and this process is often modulated by environmental inflammation. For example, during toxoplasma infection, Secreted Protein, Acidic, Rich in Cysteine (SPARC), is upregulated in the brain, inducing the formation of a network of ECM fibers. These fibers serve as tracks to guide T cell migration towards the source of the infection [62]. However, these ECM tracks can also be appropriated as a method of immune evasion by tumors. As observed in the lung, tumors are often surrounded by densely aligned ECM fibers, restricting T cell migration to circling the perimeter, and stopping T cell infiltration in the tumor [63].

T cell motility modes:

To adapt to different environmental conditions and optimize migration, T cells use various motility strategies that require actin network remodeling to generate mechanical forces and shape changes [22]. These motility modes include migration on flat surfaces (such as on endothelial cells), migration in cell-packed environments (as in lymph nodes), and migration through confined spaces (such as during diapedesis and motility through the ECM) (Figure 1 A–D). Furthermore, T cells can also adopt a bleb-based motility mode which is characterized by myosin-dependent hydrostatic pressure-driven round protrusions of the cell membrane which drive the cell forward (Figure 1E). Bleb-based motility in T cells has recently been suggested to be induced in response to S1P signaling [64].

In terms of modulating motility, actin polymerization can only drive forward momentum in cells when it is coupled with a transfer of force to the surroundings. To date, in most cases the molecular mechanisms that regulate T cell motility modes in complex environments have been extrapolated by using *in vitro* model systems to mimic environmental conditions found in tissues. These studies show that migration on flat 2D surfaces, as with migration over endothelial blood vessel walls, requires some form of adhesion to transmit forces to the substrate to enable motility. In this context, integrins provide a clutch mechanism to resist vascular shear forces and transfer force to the substrate [29]. During this adhesion-

dependent intravascular crawling on the endothelial cell wall, the T cell cytoskeleton modulates integrin function as well as motility [17,19]. In contrast, T cell migration is mostly adhesion-independent within lymph nodes [65,66], with integrins only accounting for ~15–35% of the motility speed in this setting [67]. A mechanism by which T cells can adapt their motility mode to their environment is the modulation of actin polymerization (mainly driven by chemotactic signals) in different conditions of force transfer to the substrate. In naïve T cells, the balance between CCR7 signaling and integrin adhesiveness regulates actin polymerization rate, with an inverse correlation between retrograde actin flow and adhesion to the substrate [68]. With increased adhesion to the surrounding environment, both the actin polymerization rate and retrograde flow decrease, allowing the T cell to efficiently translate actin polymerization into forward migration. However, in complex 3D environments T cells are not strictly dependent on substrate adhesion and can use shape changes and environmental topography to transmit cytoskeletal-generated forces to the surrounding environment for motility [69]. For example, using microchannels with smooth or serrated walls Reversat et al. showed that T cells are able to migrate normally in both conditions but T cells lacking talin, and thus unable to engage integrin adhesion, only migrate in the serrated wall channels [69]. This ability to transfer forces to the substrate even in the absence of integrin adhesion can enable T cell motility in tissues such as lymph nodes [65,66]. This could also be the case in some peripheral tissues where actin filaments can push directly against the ECM or other cells in their environment as T cells navigate through the tissue (as in Figure 4). However, in certain constrictive, ECM-dense environments found in non-lymphoid peripheral tissues such as the inflamed skin, it appears that integrin-dependent adhesion is important for efficient T cell motility [57].

Various studies have imaged the motility behavior of T cells in lymph nodes as well as in some peripheral tissues in the presence and absence of antigen. However, mainly due to technical challenges, there is little direct observation of actin dynamics *in vivo*. Recently, Yan et al. have visualized actin dynamics in T cells during extravasation and migration within lymph nodes [70] using T cells from LifeAct-GFP mice [71], which enables real-time visualization of sites of actin polymerization. This *in vivo* LifeAct imaging showed that during the diapedesis step F-actin is enriched at the sides of the T cell as it squeezes through the HEV endothelial barrier in lymph nodes. In contrast, imaging during lymph node migration suggested highly dynamic actin rearrangements and protrusion formation [70]. Overall, these approaches and tools will be key in determining the molecular mechanisms of how actin polymerization effectors regulate T cell motility within tissues *in vivo*. However, in relation to the actin polymerization machinery, only mDia1 knock-out (KO) T cells have been imaged during lymph node migration thus far [72]. Thus, the relative contribution of specific actin polymerization machinery to different motility strategies within tissue environments is still mostly unknown. Another outstanding question is how T cells sense the physical characteristics of their environment (such as topology and stiffness) and signal to engage their cytoskeletal machinery to adapt their motility.

Actin Networks and Cytoskeletal Effectors Overview:

Actin is found in two forms in cells, monomeric globular actin (G-actin) and polymerized filamentous actin (F-actin). Nucleation of new actin filaments is thermodynamically

unfavorable. However, once an initial oligomer has formed, actin polymerization can proceed in a polarized fashion, with polymerization occurring 10x faster at the dynamic barbed end than the less active pointed end [73]. To control this dynamic network, cells rely on effectors of actin polymerization to efficiently nucleate and elongate actin filaments, as well as actin filament capping proteins to stop polymerization or severing proteins to break filaments. Like other cells, actin networks in T cells are comprised of branched and linear filaments and different networks can play selective functions in T cell migration [24,74]. Importantly, the amount of actin monomer available for use in actin filaments at steady state is limited; thus, branched and linear polymerization remain in competition with each other. Consequently, inhibition of one cytoskeletal effector protein can lead to deregulation and overactivity of others, complicating our understanding of individual protein functions [75]. Immediately beneath the plasma membrane, a network of actin filaments known as the cortical cytoskeleton is important for cell shape maintenance and changes. At the leading edge, a branched actin network is responsible for the generation of pseudopod protrusions. In 2D migration settings this takes the form of a lamellipodium, a large and flat membrane structure important for motility. Furthermore, the polymerization of actin at the leading edge generates force to push the T cell membrane forward [76]. In the uropod, actin filaments are cross-linked by the motor protein non-muscle Myosin-IIA (MyoIIA) to generate contractile forces at the back of the cell [23]. As discussed above, the combination of these forces when transmitted to the extracellular environment facilitates the migration of T cells.

Branched actin networks are initiated through the activity of the actin related protein 2/3 (Arp2/3) complex. Downstream of Rac and Cdc42 signaling, Wiskott-Aldrich syndrome protein (WASP) and WAVE/SCAR family proteins bind Arp2/3 to form an activated complex that can recruit profilin-bound actin monomers to nucleate new actin filaments (Figure 2). This complex binds to pre-existing acting filaments to nucleate new filaments at an angle of 70 degrees, thus developing a branched actin network [73,76]. In migrating T cells, these branched actin networks expand at the leading edge of the cell, pushing the membrane forward to create the lamellipodium [77]. Arp2/3 and branched actin networks may also be involved in initiating filopodia, thin membrane projections containing linear actin at the leading edge of cells [78]. Additionally, WASP and Arp2/3 contribute to the formation and maintenance of the immune synapse [79,80].

Linear actin polymerization is mediated by two major families in T cells: the Ena/VASP family and the formin family [81–83]. These cytoskeletal effector families can also contribute to branched actin networks by elongating filaments nucleated by Arp2/3. The Ena/VASP family is comprised by three members: Vasodilator-stimulated phosphoprotein (VASP), Ena/VASP-like (EVL), and mammalian-enabled (Mena). However, Mena is not typically expressed in lymphocytes [81,84]. These proteins facilitate monomeric actin recruitment to the barbed end of the actin filament, prevent actin filament capping, and can play a role in actin filament bundling [85–89] (Figure 2). EVL and VASP share significant structural homology and are defined by an N-terminal EVH1 domain, which regulates cellular localization, and a C-terminal EVH2 domain, which facilitates tetramerization, binds F-actin, and is thought to be responsible for actin polymerization [90–93]. Several phosphorylation sites are present in Ena/VASP proteins which regulate localization as well

as actin binding [90,94,95]. These phosphorylation sites are regulated by kinases such as PKA, PKG and PKD1 [96].

Formins remodel the actin cytoskeleton both by nucleating new actin filaments and processively elongating them via recruitment of profilin-bound G-actin and by anti-capping activity [97] (Figure 2). While mammals have 15 formin family members [98], two are highly expressed in primary T cells: Diaphanous-related formin 1 (mDia1, Diaph1) and Formin like-1 (FMNL1) [99–103]. T cell can also express the formins INF2 and FHOD1 at lower levels [104,105]. Formins form homodimers to carry out their function [97,98] and regulation of these formins is controlled by an autoinhibition mechanism [97,98,106–108]. Binding of Rho GTPases to a GTPase binding domain (GBD) disrupts an autoinhibitory interaction between the N-term and C-term domains to activate the formin [97,98,107,108]. GTPase binding has also been suggested to regulate the localization of mDia1 and FMNL1 to the cell membrane [106]. In this manner, formins serve as terminal cytoskeletal effectors for Rho GTPases to induce actin network remodeling.

Regulation of Migration and Trafficking by Actin Cytoskeletal Effectors:

We will now discuss what is currently known about how specific actin polymerization effectors, as well as some of their upstream regulators, modulate T cell migration during trafficking through various anatomical sites. Specifically, in this review, we focus on the roles of the direct actin polymerization machinery including Arp2/3, formins, and Ena/VASP proteins. We also include some examples of the roles played by upstream cytoskeletal regulators, particularly where direct evidence for the role of actin polymerization effectors is currently limited. A summary of this knowledge can be found in Table 1.

1. Thymocyte development and egress

While thymocyte development is not impaired in T cells deficient in mDia1, this formin has a role in thymic egress. This is evidenced by the observation that mDia1 KO mice have increased mature thymocytes in the thymus, reduced peripheral T cell populations, and that mDia1-deficient thymocytes have impaired chemotaxis *in vitro* [100,109]. However, FMNL1, the other highly expressed formin in T cells, does not appear to regulate T cell thymic development or egress, suggesting that these formins maintain distinct roles [110].

Arp2/3 complex deficiency in humans, mainly in the form of mutations in the hematopoietic-specific regulatory component ARPC1B, has been reported to cause T cell development abnormalities with reduced naïve T cells in the periphery of some patients [111,112]. However, ARPC1B and ARPC2 deficient mice appear to have normal T cell development and egress but have impaired T cell numbers and T cell functions in the periphery [113,114], possibly suggesting issues with T cell homeostasis and survival. Nevertheless, a role in thymic egress for the Arp2/3 regulator Coronin 1A has been identified in both mice and humans [115,116].

Ena/VASP proteins do not appear to be necessary for thymocyte development since double-knock out mice for EVL and VASP (the two Ena/VASP proteins expressed in T cells) do not

have defects in generating mature T cells in the thymus or in the ability of these T cells to egress into the periphery [117].

Regarding other actin filament regulators, a conditional KO of Cofilin-1, an actin depolymerizing factor, has been shown to regulate thymocyte motility in collagen matrices and promote differentiation to the single positive thymocyte stage [118]. Furthermore, mice lacking L-plastin, an actin filament bundling protein, show reduced thymic egress [119].

2. Extravasation

T cells rely on the extensive vascular network to patrol for pathogens and to reach disparate tissues throughout the body. Whether for a naïve T cell entering a lymph node or an activated T cell entering an inflamed tissue, the process of extravasation is key for the subsequent execution of their functions. As discussed above, the process of transendothelial migration (TEM) consists of 4 sequential stages: rolling, adhering, crawling, and diapedesis [7]. While the stages of TEM are similar in different tissues, differences in vascular architecture and barriers to extravasation can further define the roles and requirements for different cytoskeletal effectors. The use of multi-photon microscopy has enabled visualization of the extravasation process *in vivo*. However, in many non-lymphoid tissues, extravasation events can be rare and optical resolution limited. Thus, much of our mechanistic understanding of the function of cytoskeletal effectors in TEM comes from *in vitro* model systems of this process.

Some cytoskeletal effectors seem to have broad roles throughout the process of TEM while others have more selective functions. Formins can have important roles in T cell trafficking through various mechanisms. Transwell assays suggest mDia1-deficient T cells display impaired chemotaxis even though chemokine receptor expression levels are unaltered [72,100,109]. Likewise, integrin expression appears to be unaltered in mDia1-deficient T cells, although adhesion to integrin ligands is impaired [72,102,109]. *In vivo*, loss of mDia1 in T cells impairs trafficking to both lymphoid organs and inflamed skin [72,100]. Thus, mDia1 seems to have a role in general responsiveness to chemokines as well as adhesion, which leads to broad trafficking defects in its absence. While the role of mDia1 in the TEM process hasn't been directly analyzed in T cells, data from B cell leukemia show that mDia1 deficiency causes a defect in TEM completion [120].

Compared to the other cytoskeletal effectors, FMNL1 appears unique in that it seems specifically involved in the trafficking of activated T cells and only to non-lymphoid tissues [110]. *In vitro* models suggest that the requirement for FMNL1 is dependent on the restrictiveness of the barrier to be crossed, with FMNL1 deficiency only impairing T cell migration through narrow pores or constrictions, such as those found in non-lymphoid tissue endothelial barriers. This is consistent with *in vitro* TEM experiments where FMNL1 was only important for the diapedesis step of TEM [110]. During this process FMNL1 localized directly behind the nucleus, whose rigid structure has been proposed to be a significant barrier to cellular deformation required for completing diapedesis [35]. Indeed, in the absence of FMNL1 T cells were impaired in squeezing their nuclei through the endothelial barrier [110].

Regarding the Arp2/3 complex, its role in TEM has not been directly addressed. However, TEM studies on lymphocytes from WAS patients (characterized by mutated or deficient WASP) suggest that the Arp2/3 regulator WASP is particularly important in mediating transcellular diapedesis [25]. Interestingly, *in vivo* studies with lymphocytes from WASP KO mice showed altered trafficking to Peyer's patches but not to other secondary lymphoid organs [121]. Additionally, previous *in vitro* studies on WASP and WIP (WASP-interacting protein) support that the Arp2/3 complex plays a key role in T cell migration in 2D settings [121,122]. However, data from an actin binding mutant of WIP suggest that WIP may regulate migration through additional WASP-independent mechanisms [123]. More recent *in vitro* studies using 2D migration and transwell assays show that the Arp2/3 complex modulates mouse and human T cell migration and chemotaxis [112,124], partly through regulation of lamellipodia formation [124,125]. Mutations in Hem1, a key component of the WAVE regulatory complex, also cause defects in membrane protrusions and reduced 2D migration, and can lead to immunodeficiency [126]. Mutations in WASP and other regulators of Arp2/3 function, such as WIP and Coronin 1A, have also been shown to cause immunodeficiency, due in part to impaired lymphocyte migration and trafficking. As is the case for WIP, since Coronin 1A has additional non-Arp2/3 related functions it is possible that Coronin 1A deficiency affects motility through a different mechanism, such as actin filament disassembly [127]. Taken together these studies support a potentially broad role for Arp2/3 in regulating T cell chemotaxis and motility. However, its requirement at specific stages of TEM may be somewhat tissue dependent. Some of these tissue specific roles may depend on which WASP family members and other members of the regulatory complex are utilized downstream of tissue-specific cues and characteristics.

Ena/VASP protein deficiency selectively impairs activated T cell trafficking [117]. This is likely because loss of EVL and VASP impairs the expression and function of alpha-4 integrin, which is only expressed on activated T cells. These trafficking defects extended to both lymphoid and non-lymphoid tissues. Surprisingly, in a model of TEM, this alteration of integrin alpha-4 does not impact EVL/VASP-deficient T cell adhesion to, or crawling on, the endothelium but rather inhibits the diapedesis step [117]. This suggests that instead of affecting 2D motility, Ena/VASP proteins have either a role in providing anchoring support for the physically demanding diapedesis step, or a role in endothelial cell/T cell cross-talk via integrin ligands, which can lead to the internalization of endothelial junctional molecules [30].

Finally, from 2D migration models, MyoIIA-driven contractile forces have been shown to contribute to the retraction of the uropod and to limit adhesion as the T cell moves forward [23,33,128]. Additionally, contractile forces generated by MyoIIA also promote transmigration of the T cell nucleus across the endothelium during TEM [33]. *In vivo,* MyoIIA deficiency impairs activated T cell trafficking to lymphoid and non-lymphoid organs [33,129]. Together, these findings suggest a role for MyoIIA in both the crawling and diapedesis steps at disparate tissue sites.

While many effectors of actin polymerization have been implicated in T cell trafficking, how they impact specific stages of TEM is still being elucidated. In combining our current knowledge from both *in vitro* and *in vivo* systems it appears some effectors such as

Arp2/3 and mDia1 have roles at multiple stages of TEM while others such as FMNL1 and EVL/VASP only seem to be critical for diapedesis (Figure 3). Consistent with the idea of diapedesis being a rate-limiting step in TEM, it seems to be particularly sensitive to perturbations in actin cytoskeletal machinery as evidenced by multiple effectors being important for this process (e.g. FMNL1, EVL/VASP, Arp2/3, and MyoIIA). While it is well established that integrin-mediated adhesion and coupling with the cytoskeleton are important for the initial attachment and crawling phases, there appears to be substantial redundancy among effectors of actin polymerization in achieving these initial TEM steps. Further use of actin polymerization reporters in conjunction with disruption of specific actin effectors or fluorescent reporters of their activity will be critical for understanding the contributions and mechanisms of individual effectors in mediating the stages of TEM.

3. Migration within tissues

After leaving the blood stream and entering tissues, T cells encounter environments with very different characteristics, including varying composition and densities of ECM as well as stromal cells with different morphology and distribution. A challenge that T cells encounter while migrating within non-lymphoid tissues is the need to navigate through small openings within the tissue parenchyma. Similarly to diapedesis, the T cell nucleus can be an obstacle and a rate limiting step for migration through small openings [35,40]. To migrate through these small openings within the tissue parenchyma, T cells need to deform their nucleus and/or mechanically enlarge the opening. Both events require force generation and the actin polymerization effectors involved in this process are only beginning to be elucidated. In dendritic cells the Arp2/3 complex has been shown to have a key role in mediating perinuclear actin polymerization to squeeze the nucleus through constrictions [130]. In T cells, using a microchannel system with constriction points, we have recently shown that actin polymerization by FMNL1 allows efficient pushing of the T cell nucleus through such constriction points [110]. An important open question is related to how T cells sense environmental cues such as the topology, stiffness, adhesiveness, and mechanical forces at play in their surroundings, a process generally known as mechanosensing. Leukocytes have been shown to be able to discriminate between constriction points of different sizes and select the more permissive ones, with front positioning of their nucleus promoting this pore selection process [131]. Interestingly, formins have the capacity to sense and respond to mechanical cues [132]. In particular, mDia1 can respond to pulling forces by increasing actin filament polymerization rate and exerting tension on actin filaments [133]. In the context of nucleus deformation during migration, we can thus speculate that formins, and specifically FMNL1, may act as a mechanosensing hub to enable T cells to squeeze their nucleus when confronted with constriction points. However, the molecular mechanism and actin structures at play for nucleus deformation and squeezing in T cells are still unclear.

Using *in vivo* imaging, efficient motility within lymph nodes has been shown to require the activity of mDia1 [72]. Although mDia1 regulates actin polymerization in response to chemokine stimulation in T cells [100], its function in T cell migration is also related to mDia1's regulation of microtubule dynamics [72]. As discussed in the section on extravasation, previous *in vitro* studies on the Arp2/3 complex and its regulators indicate that the Arp2/3 complex plays a role in T cell migration. Recent work using Arp3 knock-down

and a zebrafish transfer model shows that the Arp2/3 complex is also needed for efficient motility *in vivo*. This manifested as a switch to a bleb-based motility with reduced speed and displacement by Arp3 knock-down T cells [125]. The role of actin polymerization effectors such as FMNL1 or Ena/VASP proteins has not yet been directly assessed in interstitial motility *in vivo*. However, many other cytoskeletal effectors and upstream regulators can promote efficient T cell motility in tissues. Due to length limitations, in this review we will only discuss a few key examples. MyoIIA and its regulator Rho-associated protein kinase (ROCK) have a role in regulating motility and interaction with tissue components in lymph nodes and in the lung [23,129,134]. Rho GTPases, such as Rac1 and Rac2, and their regulators such as the guanine exchange factor DOCK2 also promote efficient T cell motility in lymph nodes [135,136]. Instead, rather than affecting chemotaxis, DOCK8 is key in maintaining cell shape and integrity, and in preventing cell death during migration in confined environments [137]. Furthermore, upstream cytoskeletal regulators such as Coronin 1A and the actin bundling protein L-plastin are important for interstitial migration of T cells [115,119].

Overall, based on currently available data, Arp2/3 and mDia1 are likely to exert their functions at the leading edge of the T cell by generating protrusions for environmental sensing and actin filament treadmilling for propulsion [124,125]. On the other hand, FMNL1 acts at the T cell rear, independently or in concert with MyoIIA [33], to generate force to push or deform the nucleus and promote passage through small openings [110] (Figure 4). Little is known about the role of Ena/VASP proteins in interstitial T cell migration; however, while important for diapedesis, this protein family is not required for 2D migration of T cells [117].

Closing Remarks and Open Questions

T cells can migrate at high speed within various environments by adapting their motility mode to the biophysical characteristics of the environments they encounter. The actin cytoskeleton through actin polymerization and acto-myosin contraction provides the shape changes and forces necessary for migration. Great strides have been made in understanding the molecular mechanisms that regulate T cell motility, especially through the use of advanced imaging techniques *in vivo* and *in vitro* (e.g. multi-photon, TIRF, super-resolution and lattice lightsheet microscopy), the ingenious use of microfabricated structures to model different environmental parameters, and the development of biosensors such as the LifeAct-GFP construct [71]. However, there is still a lack of understanding at the molecular level of the specific functions of individual actin polymerization effectors and of how these effectors may work together or through parallel mechanisms to drive motility and pathfinding within complex environments. For example, it is still unclear which specific actin polymerization effectors contribute to actin filament elongation and retrograde flow shown to be crucial for different T cell motility modes. Additionally, measurements of the forces generated by the actin cytoskeleton and transferred to the environment in T cells are still scarce. Furthermore, the actin structures and mechanisms required for nucleus squeezing through small openings in tissues or during diapedesis are only beginning to be uncovered. Mechanosensing is also emerging as a key property of T cells to efficiently navigate complex tissue environments, but very little is known about how T cells sense these mechanical cues, or how actin

cytoskeletal effectors work to respond to these cues. Finally, while small molecule inhibitors such as blebbistatin, CK-666 and SMIFH2 [138–140] have provided valuable insights into potential roles for cytoskeletal effectors in some *in vitro* contexts, the broad specificity, potential for toxicity and poor bioavailability mean these tools should be used with caution and are of limited use for *in vivo* studies [140–142].

The continued use and development of advanced imaging techniques, such as the combination of adaptive optics with multi-photon microscopy [143] and lattice lightsheet microscopy [144], paired with the use of biosensors, optogenetics, and biophysical readouts will allow the development of a more comprehensive understanding of the molecular mechanisms by which each actin effector contributes to the efficient motility and spatiotemporal localization of T cells. The ability to interrogate the role of cytoskeletal effectors in actin dynamics and motility behaviors *in vivo*, paired with the ability to image the various elements and structures of the surrounding environment will greatly increase our mechanistic knowledge of T cell motility within physiological environments. As migration is an essential component of T cell function, this improved molecular understanding of T cell migration could then be translated into therapeutic approaches for clinical intervention.

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Abbreviations:

3D	three-dimensional
APC	antigen-presenting cell
Arp2/3	actin related protein 2/3
DCs	dendritic cells
mDia1	Diaphanous-related formin 1
ECM	extracellular matrix
EVL	Ena/VASP-like
FMNL1	Formin like-1
FRCs	fibroblastic reticular cells
F-actin	filamentous actin
G-actin	globular actin
GBD	GTPase binding domain
HEV	high endothelial venules

ICAM-1	Intracellular Adhesion Molecule-1
KO	knock-out
Mena	mammalian-enabled
MyoIIA	Myosin-IIA
ROCK	Rho-associated protein kinase
S1P	sphingosine-1-phosphate
SPARC	Secreted Protein, Acidic, Rich in Cysteine
TCR	T cell receptor
TECs	thymic epithelial cells
TEM	transendothelial migration
Th	T-helper
VASP	Vasodilator-stimulated phosphoprotein
VCAM-1	Vascular Cell Adhesion Molecule-1
WASP	Wiskott-Aldrich syndrome protein
WIP	WASP-interacting protein

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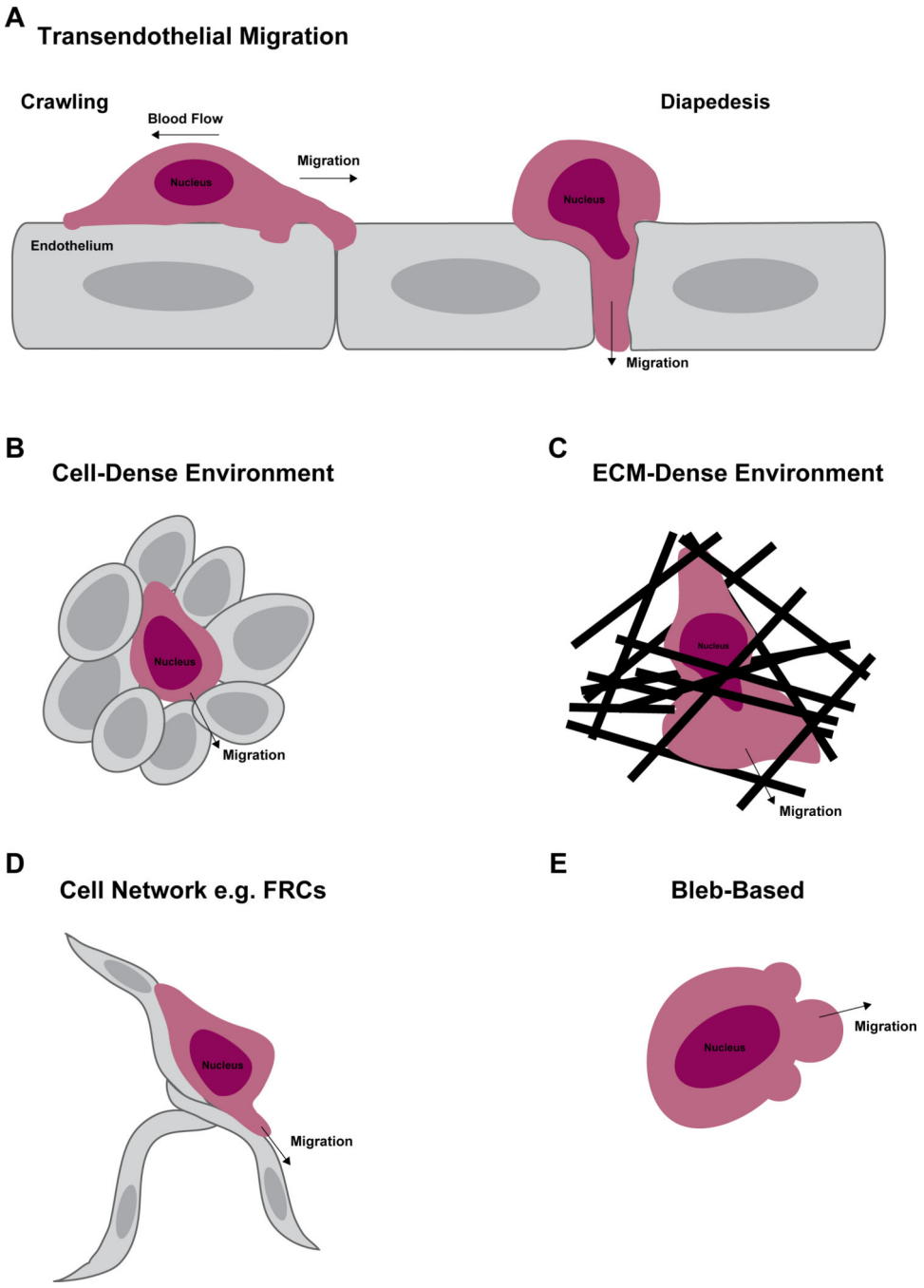

Figure 1. T cell motility modes.

During their lifetime T cells encounter many different environments and physical barriers. Depending on the context, T cells can adopt various motility strategies that facilitate their migration. Depicted here are some of the main motility modes employed by T cells. To enter tissues from the blood stream T cells undergo the multistep process of transendothelial migration (TEM) **(A)**. This process includes both the crawling phase where T cells migrate over the endothelial monolayer (a 2D type of migration) in response to chemotactic cues and the diapedesis phase where T cells squeeze across the endothelial

barrier. Within tissue parenchyma T cells may encounter a cell-dense environment **(B)**, such as in secondary lymphoid organs, or an extracellular matrix (ECM)-dense environment **(C)**, such as in the skin. ECM-dense environments may contain restrictive openings requiring substantial morphological changes to enable migration. In some tissues, T cells migrate along connected cell networks such as the fibroblastic reticular cells (FRCs) found in lymph nodes **(D)**. T cells have also been reported to be able to switch to a bleb-based migration **(E)**, where the formation of membrane blebs rather than a lamellipod or pseudopod directs migration at the leading edge.

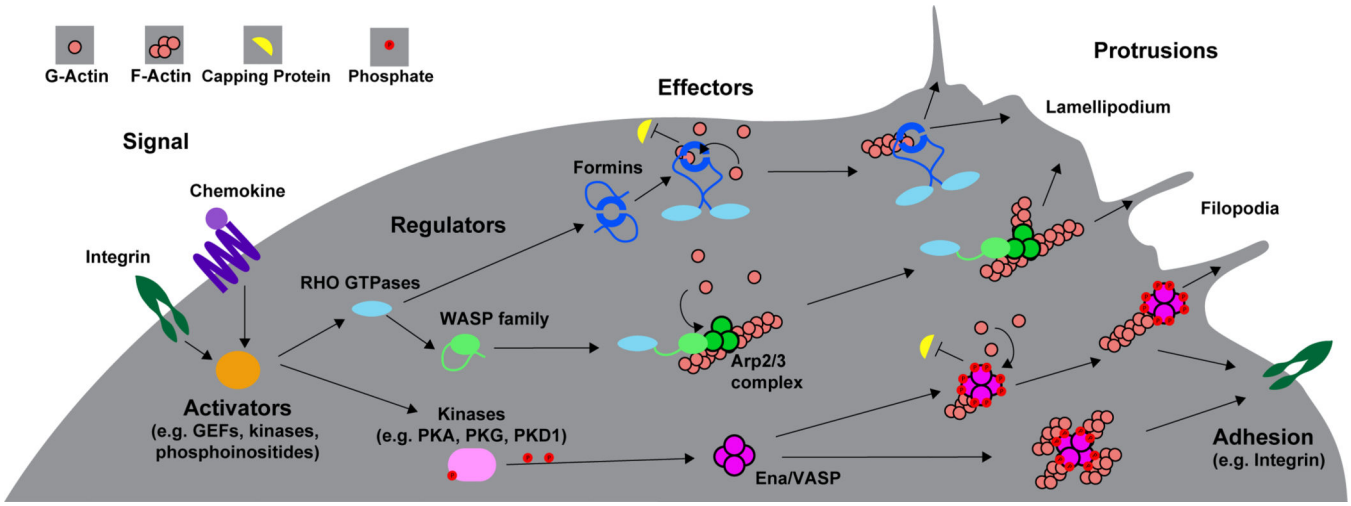

Figure 2. Regulation of actin polymerization effectors and their functions.

Upon binding their ligands, chemokine receptors and integrins induce local phosphoinositide production, kinase activity and guanine nucleotide exchange factor (GEFs) activity. The GEFs can in turn activate Rho GTPases which orchestrate cytoskeletal rearrangements through the activation of terminal effector proteins that directly interact with the actin cytoskeleton. In the case of formins, direct binding by Rho GTPases disrupts an autoinhibitory interaction enabling formin homodimers to nucleate new actin filaments (F-actin) and processively elongate them via recruitment of actin monomers (G-actin) (typically bound to profilin, not depicted) and by anti-capping activity. Rho GTPases can also disrupt the autoinhibitory regulation of WASP family members enabling them to associate with the Arp2/3 complex causing conformational changes that allow Arp2/3 to bind to F-actin. This complex recruits G-actin to nucleate a new F-actin branch from the existing actin filament. Kinases such as PKA, PKG and PKD1 downstream of integrin or chemokine signaling can phosphorylate Ena/VASP family members at multiple sites enabling them to bind actin. Ena/VASP proteins can then promote actin filament elongation via recruitment of G-actin as well as anti-capping activity. The tetrameric structure of Ena/VASP facilitates bundling of multiple actin filaments together. Actin polymerization by these various effectors pushes on the cell membrane, driving membrane protrusion formation including the broad fan-like structure of the lamellipodium and long thin membrane protrusions such as filipodia. Additionally, actin polymerization and crosslinking can affect integrin adhesion or transmit force to the surrounding environment.

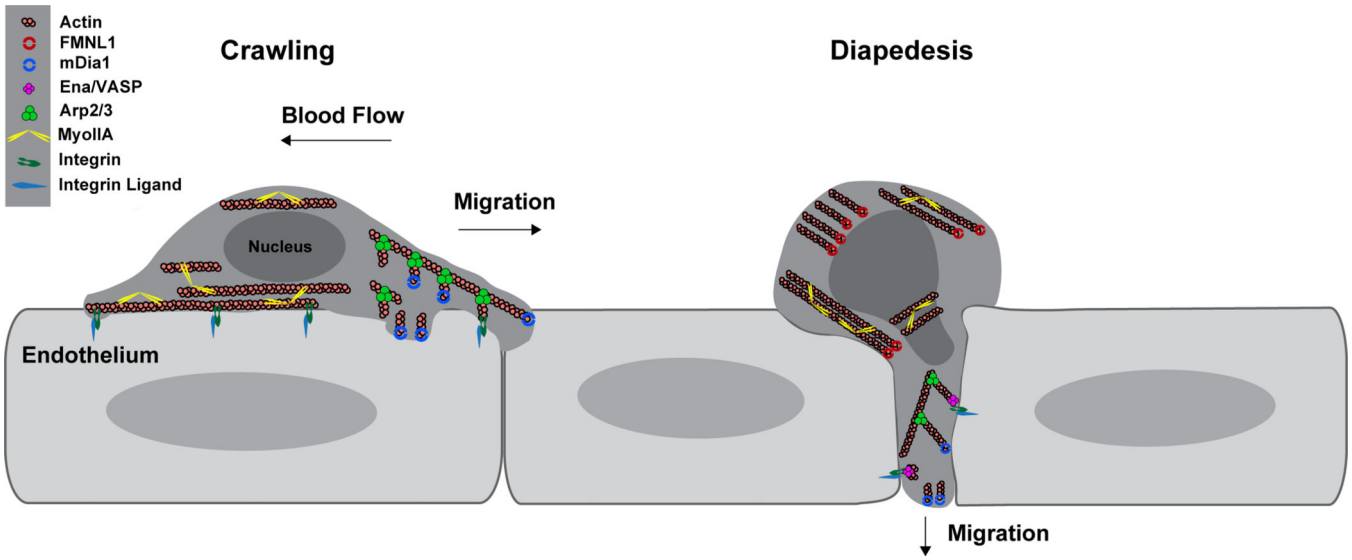

Figure 3. Model of the actin polymerization machinery involved in T cell transendothelial migration.

During the crawling phase of diapedesis (left half of figure), actin polymerization at the leading edge of the cell by Arp2/3 and likely mDia1 promote expansion of the lamellipod, driving the T cell forward. In this 2-dimensional migration setting, adhesion to the substrate, typically through integrins, is critical to couple actin polymerization into forward motion. Linear actin filaments extended by mDia1 may also be involved in generating membrane protrusions and providing adhesive connections to the endothelium and sensing of the environment. At the rear of the cell, contraction of actin filaments by MyoIIA provides force and detaches the uropod from the endothelium facilitating forward migration. During the diapedesis step (right half of figure), actin polymerization at the leading edge by Arp2/3 and mDia1 drives extension of the cell body through the endothelium. Through regulation of α4 integrins, EVL/VASP proteins may provide anchoring support or crosstalk with the endothelium. Transmigration of the rigid structure of the nucleus through the endothelial barrier is a rate-limiting step in diapedesis. Contraction of cortical actin by MyoIIA deforms the nucleus enabling passage through the narrow space in the endothelial barrier. FMNL1 also promotes nucleus passage during diapedesis, either by providing actin filaments for MyoIIA to contract or by providing independent force generation via actin polymerization at the rear of the cell.

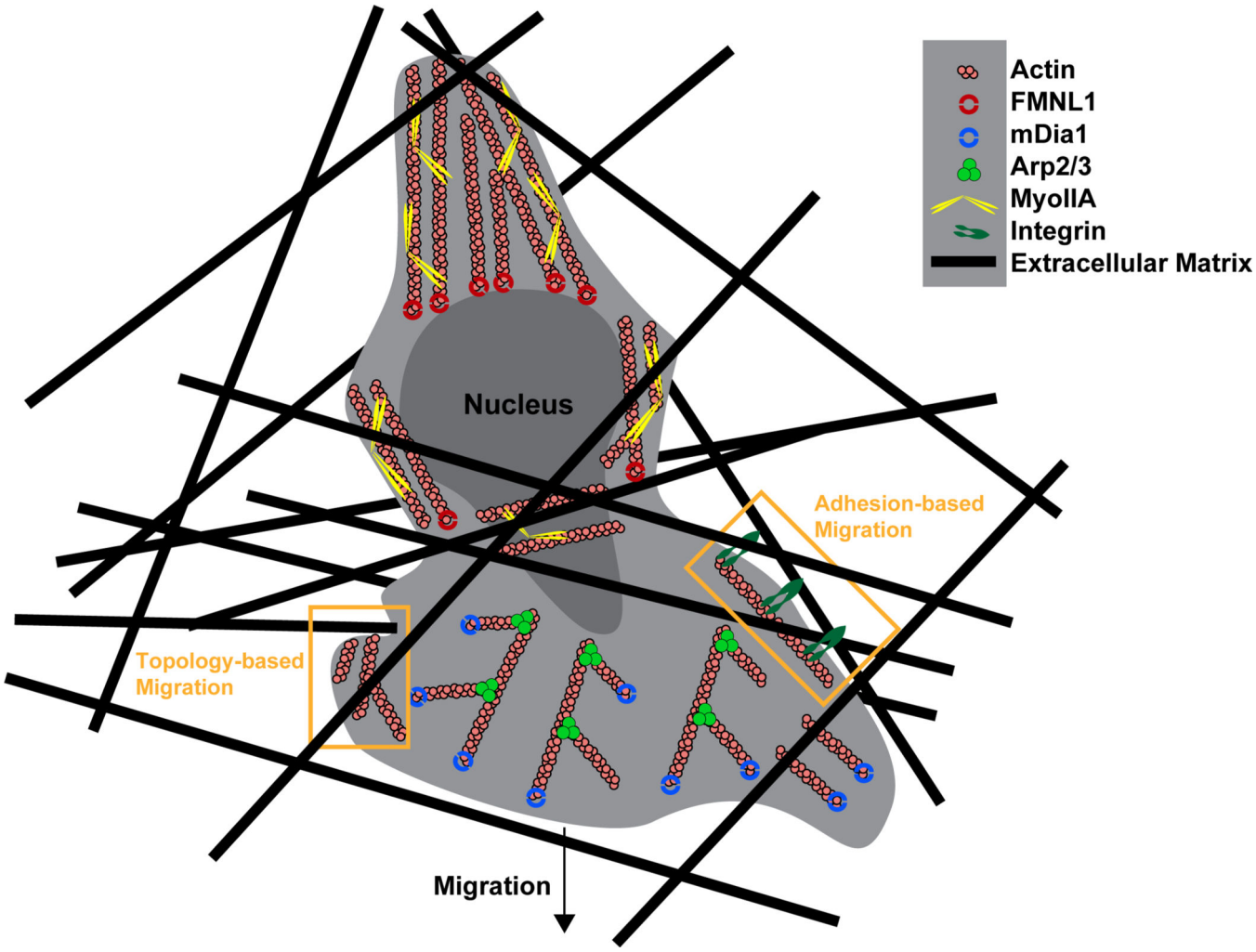

Figure 4. Model of the actin polymerization machinery involved in T cell interstitial migration.

During interstitial migration actin polymerization by Arp2/3 and mDia1 at the leading edge of the cell promote expansion of the leading edge and formation of protrusions to facilitate environmental sensing. Depending on the tissue environment, actin polymerization can enable motility in the absence of adhesions, in that actin structures may create a sufficient frictional interface by pushing directly against the surroundings, such as the ECM or other cells (topology-based migration). However, in constrictive ECM-dense environments such as the skin, integrin adhesion can play an important role in interstitial migration, as greater force transfer may be required to enable migration. During this adhesion-based migration, adhesion receptors such as chemokine-activated integrins, which are coupled to actin structures, can bind to the ECM, serving as a clutch to engage the ‘motor’ of actin polymerization, and provide the traction force necessary to move cells forward. At the rear of the cell, actin filament contraction by MyoIIA allows cellular and nucleus deformation for navigating small openings. Similar to its role in diapedesis, FMNL1 is likely also involved in facilitating squeezing of the nucleus through constrictive barriers within tissues.

Table 1

Summary of the roles in T cell migration and trafficking of actin polymerization effectors and Myosin-IIA

Effector	Arp2/3	FMNL1	mDia1	EVL	VASP	MyoIIA
Localization	enriched at leading edge	enriched at uropod, perinuclear	enriched at leading edge	unknown	unknown	enriched at uropod
Cellular Role	branched actin filaments, lamellipodia	linear actin filaments	linear actin filaments, lamellipodia	linear actin filaments	linear actin filaments	actin crosslinking, contraction
Role in Thymus	thymic egress (based on upstream regulator phenotype)	dispensable	thymic egress	dispensable	dispensable	Germline KO is embryonic lethal
Role in trafficking	naïve T cell trafficking to lymphoid tissues (based on upstream regulators)	effector T cell trafficking to restrictive non-lymphoid tissues	effector and naïve t cell trafficking to lymphoid and non-lymphoid tissues	effector T cell trafficking to lymphoid and non-lymphoid tissues (redundant with VASP)	effector T cell trafficking to lymphoid and non-lymphoid tissues (redundant with EVL)	effector T cell trafficking to lymphoid and non-lymphoid tissues
Role in Transendothelial Migration	chemotaxis	diapedesis, nucleus passage	adhesion, chemotaxis	diapedesis, α4 integrin function (redundant with VASP)	diapedesis, α4 integrin function (redundant with EVL)	diapedesis, nucleus passage
Role in Interstitial Migration	general motility, cellular integrity	unknown, likely involved in constrictive environments	motility in lymph nodes, unknown in other tissues	unknown	unknown	motility in lymph nodes, unknown in other tissues
Primary References	[78,111–116,121–126]	[110]	[100,102,109,120,145]	[117]	[117]	[23,33,129]