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Control of the T cell Cortex by the Septin Cytoskeleton

by

Julia Katherine Gilden

Dissertation

Submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Science

in the
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It would be impossible to overstate the importance of my advisor, Max Krummel, to the completion of this work and my development as a scientist. His enthusiasm for discovery and for the process of asking questions is truly contagious, and has always been admirable to me, even at the times when it seemed like a disease. In addition, I will always appreciate the ways Max challenged me to confront what I want from a line of experiments and from my experience in his lab, the latter being a process that has led to more personal growth than I ever anticipated from my PhD training.

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Finally, the most important thing I’ve learned in graduate school is that none of this matters without the unconditional love of Jim and Donut, each in their own way.


DEDICATION

For GMD
Control of the T Cell Cortex by the Septin Cytoskeleton

Julia Katherine Gilden

ABSTRACT

Coordination of immunity requires rapid trafficking of T cells among diverse tissues throughout the body. In their travels, cells can adopt versatile shapes and modes of motility. In lymph nodes and interstitial spaces, T cells crawl rapidly, with an amoeboid morphology characterized by leading edge protrusion and MyoII-based contraction at the trailing uropod. In this work, I have investigated the role of septin GTPases in T cell motility, and in other aspects of T cell biology. Knockdown of septins in D10 T cells results in an uncontrolled cortex with unusual and dynamic blebs and protrusion that lead to inefficient crawling in 2D. Using an osmotic stress model of cortical expansion and contraction, I determined that septins function in the contraction phase of cortical control, assembling at the plasma membrane at stretched, actin-poor regions. My data indicate that septins coordinate the cortex via recapture and contortion of plasma membrane. These findings suggest potentially important roles for septins in migration in vivo, and I have validated a conditional Sept7 knockout mouse model for use in future studies.
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INTRODUCTION

Cells of the immune system are unique in that they must rapidly patrol nearly all of the tissues in the body to perform their functions. Coordination of immunity requires that lymphocytes transition rapidly and continuously between constricted interstitial spaces, endothelial surfaces they must cling to, shear forces of rapidly pumping blood, and the packed, writhing environment of secondary lymphoid organs. All the while, they must integrate information from diverse environments, deliver information to other cells, and control the timing and mode of their motility to match functional events in their natural history. Therefore, how lymphocyte motility is regulated, and how it relates to trafficking, signaling, and effector functions, are essential aspects of lymphocyte biology.

I. MECHANISMS OF MOTILITY

Actin-Driven Protrusion

A large volume of work in epithelial cells and fibroblasts has led to a paradigm that places protrusion driven by actin polymerization as the primary force in motility. Actin filaments in the lamella and lamellipodium are oriented with their barbed ends, where new monomers are added, pointed toward the leading edge of the cell. The elastic Brownian ratchet model describes flexibility in actin filaments that allows for space to be made between the barbed end and the plasma membrane where new monomers can be added (1). As the filaments
grow, they push outward on the plasma membrane, driving the leading edge forward.

In the most extreme cases, actin polymerization for lamellar protrusion is sufficient to independently drive motility. For example, fish epidermal keratocytes crawl on molecular “autopilot,” persistently crawling forward on the power of actin polymerization alone. Evidence for this comes from studies in which the motility of intact cells was compared to that of lamellar cell fragments, and it was demonstrated that their motility was extremely similar, though lamellar fragments couldn’t achieve the highest speeds observed in complete cells (2). Remarkably those fragments lack most organelles, including the nucleus, and the bulk of cellular Myosin II (MyoII), which is concentrated at the rear of the keratocyte (3; 4). Usually, however, a component of the force generated by actin causes retrograde flow of mature actin filaments rearward with respect to the cytoplasm, with several important consequences. First, it supplies MyoII at the rear of the cell with a mature actin filament network that can be compressed for aspects of locomotions discussed in later sections. Second, the rate of retrograde flow correlates with both the rate of cellular motion and with the traction forces exerted on the substrate, particularly at focal adhesions (5; 6). Thus, coupling of actin filaments to transmembrane proteins and in turn to a rigid extracellular matrix allows retrograde flow to directly contribute to propulsion.

The rate of formation and architecture of actin filaments can be regulated downstream of many cell-intrinsic and cell-extrinsic pathways, including growth factor receptor signaling, chemotactic signals, cell-cell adhesions, and even
manipulation by microbes. These signaling pathways converge on just a few mechanisms of control, including nucleation of new actin filaments, regulation of monomer availability, filament cross-linking, and barbed-end capping.

Actin filaments are initiated by the Arp2/3 complex, which can bind to the side of existing filaments, creating a branched actin network, or nucleate filaments de novo in the cytoplasm. The activity of Arp2/3 is controlled by the WASp/Scar family of proteins, which are natively auto-inhibited and must be activated. This activation is carried out by the binding of Rho family GTPases Rac and Cdc42, WAVE and WASP, respectively. These activators are extremely potent controllers of cellular protrusions, and overexpression of active forms of Rac and Cdc42 lead to excessive ruffling and filopodia (7; 8). The activity of these molecular switches is modulated by the binding of a huge variety of guanine nucleotide exchange factors (GEFs) that favor the active GTP-bound form, and GTPase-activating proteins (GAPs), which encourage GTP hydrolysis and return to the inactive form.

Availability of actin monomers for the growth of filaments is controlled by competitive binding of thymosin and profilin. Thymosin binds and sequesters actin monomers in the steady state, slowing polymerization, but activation of profilin by phosphorylation or by PIP$_2$-binding shifts actin-monomers into the profilin-bound pool where they are rapidly added to growing barbed ends. Existing filaments can be cross-linked to form different structures for different purposes. For instance, many cells extend filopodia at their leading edges, and these contain actin filaments tightly bundled by fimbrin. In contrast, contractile
bundles of actin are more loosely packed by $\alpha$-actinin, which allows space for
Myosin to access the fibers. A third cross-linker, filamin, is a dimer that connects
actin filaments in three-dimensions to form gels that are a dominant feature of
lamellipodia. As actin filaments grow longer, they become more flexible, so force
generation at the leading edge demands that short, branched filaments be
favored over long, straight ones. This balance is achieved by the activity of
capping proteins such as CapZ and gelsolin, which, when active, bind to barbed
ends and prevent further addition of actin monomers. Gelsolin has a further role
in limiting polymer growth, as it can also sever mature filaments and cap the new
barbed end. Working together, these systems result in a lamellipodium of short,
densely branched, dynamic actin filaments with a lamellar region of longer and
more stable bundled actin filaments behind (Reviewed in (9)).

A model of leading edge protrusion driven by actin polymerization leaves
several components of this process unexplained. First, as described above, for
actin monomers to be added to a filament, the barbed end of that filament cannot
be in direct contact with the plasma membrane, but must bend away from the
plasma membrane during addition. Therefore, at the most proximal level,
polymerization cannot generate force for protrusion (10). Second, free actin
monomers are generated by depolymerization of filaments at the rear of the cell.
It has been predicted that simple diffusion of these monomers would not result in
the measured concentrations of G-actin at the leading edge that are necessary
for rapid polymerization (11). Both of these issues can be resolved by implicating
the manipulation of local hydrostatic pressure in the cell, which is achieved by
two mechanisms: Myosin II-generated contractile force on the cytoplasm, and ion- and water-flux at the leading edge that controls local cell volume.

*Myosin II Contraction*

It has long been recognized that contractility is a major feature of amoeboid motility and the dominant contractile force in cells is provided by Non-muscle Myosin II (MyoII) (12). A gross indication of this is the observation that knockdown of MyoII or pharmacological inhibition by the drug blebbistatin results in T cells lacking defined uropods at the trailing edge (13). TIRF studies of T cells have indicated that actin-rich adhesions mature and become populated by MyoII, which then contracts, pushing the cell body forward to form a new actin ‘footprint’ (14). In addition to its role in translating the bulk of the cell forward, MyoII interacts with other aspects of amoeboid motility.

As cells move forward, new adhesions form with the substrate and old ones must be dissolved so that the rear of the cell can also translate. The retraction of the rear is achieved by MyoII-generated force, which contracts the trailing edge and tears adhered membrane off the substrate. This function was first demonstrated in Dictyostelium, where MyoII-mutant cells had a greater defect in crawling speed while crawling on coverslips coated with higher concentrations of poly-L-lysine (15). In crawling T cells, knockdown of MyoII results in cells with unable to retract their uropodos or with generally larger adhesion footprints, depending on the conditions, and that favor a sliding style of
motility over the dominant “walking style” of control cells (14; 16). These data suggest a similar role for MyoII in retracting adhesions in this system.

Sustained actin-based protrusion also depends on delivery of G-actin to the leading edge by MyoII contractility. Zicha et al. measured actin monomer transport to the leading edge of a migrating fibroblast and found that it was too fast to be explained by diffusion alone. Rather, experiments using inhibitors of MyoII activators ROCK and MLCK demonstrated that MyoII activity is required for rapid transport of G-actin (11). Similarly, direct tracking of inert quantum dots introduced into fish keratocytes demonstrated forward fluid flow in migrating cells that could be abolished with blebbistatin treatment (17).

Hydrostatic Control of Leading Edge Volume

Cells make space for the addition of actin at the leading edge by locally expanding cell volume. Though in general cell volume change can be achieved by several mechanisms, the most rapid, and the one that is most likely utilized at the leading edge of migrating cells is osmotic expansion. Indeed, several studies have found measurable increases in the cell volume of neutrophils upon chemotactic stimulation with fMLP (18; 19). Aquaporins, which allow passive transport of water across cell membranes, have been observed at the expanding edges of many cell lines and primary cells, likely allowing for a net influx of water leading to local volume expansion. The most relevant aquaporin to amoeboid motility is AQP9, which is highly expressed on neutrophils and T cells (20).
Chemical blockade of AQP9 in neutrophils resulted in impaired motility and the loss of motility-associate protrusions (21). This is consistent with data from studies of other aquaporins in other cell types. Among them, knockout of AQP1 inhibited migration of aortic endothelial cells (22), and AQP3 knockdown or inhibition abrogated EGF-induced migration of human skin fibroblasts (23).

Of course, aquaporin expression alone cannot drive the influx of water. The physiological environment of a migrating metazoan cell is isotonic with the cytoplasm, and therefore no osmotic force exists to push water in through aquaporins. Also at the leading edge of migrating cells, however, are the active ion transport proteins, NHE1, which exports one proton out of the cell in exchange for one sodium ion, and AE2, which exchanges bicarbonate and chloride anions (24). Together, these transporters can raise the local intracellular ionic strength at the leading edge, driving in water through aquaporins, and creating space for the polymerization of actin. Many studies have demonstrated reduced motility in the presence of NHE1-inhibitors, though it is important to consider pleiotropic effects of NHE1 when interpreting those data (Reviewed in (25)). NHE1 activity elevates local intracellular pH, which directly enhances actin polymerization. Further, NHE1 binds ERM proteins, which link the actin cytoskeleton to the plasma membrane, and serves as a scaffold for a signaling complex that regulates motility. An elegant study using NHE1 mutations that selectively abolished either its ion transport activity or its ERM-binding activity demonstrated that both of these functions are required for polarity and motility in fibroblasts (26).
Interestingly, hydrostatic leading edge volume regulation shares mechanistic similarities with the formation of plasma membrane blebs. In blebs, local hydrostatic pressure exceeds the adhesive force between the cortical actomyosin cytoskeleton and the plasma membrane, and a small patch of that membrane is pushed out (27). This process can be driven by manipulation of either force; experimental increases in hydrostatic force by local application of hypotonic media and disruption of the membrane-cytoskeleton adhesion by knockdown of filamin expression can both lead to an increase in plasma membrane blebbing (28). Under what circumstances blebbing is instrumental in motility is not yet clear, although leading edge blebbing has been observed in several cell types, including *Dictyostelium discoideum*, zebra fish primordial germ cells, and metastatic tumor cells under conditions where proteolytic extracellular matrix degradation is inhibited (29-31). A major contributor to this blebbing appears to be contractile flow of cytoplasm from the back of the cell.

II. MULTIPLE MOTILITY MODES IN AMOEBOID CELLS

Several idealized models of motility can be postulated, such as one based primarily on actin polymerization, such as that seen in the fish keratocyte, or one based on blebbing motility and a leading edge devoid of actin. Amoeboid cells, however, change shape by definition, and with this plasticity of shape comes a diversity of possible motility modes that can be adopted depending on environmental or experimental conditions. Recently, some of the physical
determinants of amoeboid motility modes have begun to be sorted out, and this section will summarize those findings.

2D versus 3D motility

Leukocytes seeded on two-dimensional surfaces share some morphological features with migrating fibroblasts, including a thin lamellipodium-like region at the front, high-affinity integrins clustered in an adhesive zone posterior to the leading edge, and a tightly constricted rear (32). Many of these features, however, may not be relevant to cells crawling in more physiological three-dimensional environments. For instance, a study of T cells embedded in collagen matrices failed to find enrichments of F-actin or integrins associated with individual collagen fibers, challenging the relevance of the haptokinetic model to 3D leukocyte motility (33). Indeed, it has been shown in the 3D collagen system and a variety of others that integrin-mediated adhesion is dispensable for efficient motility when cells can propel themselves by exerting traction forces against rigid substrates in three dimensions (33-36).

While actin-coupled strong adhesions are probably not a major mechanism of motility for leukocytes in vivo, it is not entirely clear what mechanisms cells do use. Several modes of motility have been proposed, but differentiating between them in vivo, and determining what factors might favor one over another remain challenges. Among these possible modes are leading edge blebbing, either accompanied by adhesions on those blebs that could
sequentially anchor the cell forward, or without adhesion, where blebs could intercalate with the extracellular matrix and supply traction for locomotion; ‘chimneying’ in which cells exert traction forces in several directions at once, like a rock climber squeezing himself up between two rock faces; and motility based on actin-polymerization-driven protrusions providing traction (37). It is likely that cells switch between several of these mechanisms depending on their environments. For instance, during interstitial motility in vivo, dendritic cells can achieve peak velocities when either adhesion-dependent or myosin-dependent motility modes are blocked (34).

Spatial confinement

Different motility pathways are differentially utilized depending on the degree of 3D spatial confinement cells experience. At the extremes, a 2D surface could be considered a low-confinement setting, where haptokinetic mechanisms dominate motility, and an endothelial cell tight junction, with cell-cell contact on every surface, could be considered a high-confinement setting, where MyoII contractility is essential (38). Confinement of T cells in microchannels with carefully controlled widths has lead to identification of a MyoII-dependent “sweet spot” in T cell motility. Activated T cells in channels 7-9 μm in diameter crawl around 30% faster than they do in smaller or larger channels, and this effect is abrogated by blebbistatin treatment, suggesting that cells use MyoII to efficiently push against the walls of channels with diameters similar to their own and
generate traction forces. While the magnitudes of cell speeds are lower when integrin-based adhesion is blocked, the effects of both confinement and blebbistatin persist, demonstrating that the rapid MyoII-dependent motility mode is not adhesion dependent (38).

MyoII-dependence of constricted movement has also been observed in less-precise, but more physiological systems. Particularly, Lammermann et al. found that MyoII was critical for cells squeezing through very dense collagen networks, and similarly, transmigration through endothelial cell layers is MyoII-dependent due to the need to compress the rigid nucleus for transit through tight spaces (34; 38).

Adhesion to the substrate

Though under some circumstances a partial integrin-dependence of 3D or in vivo motility has been observed, it seems that adhesion to the substrate plays the greatest role when cells migrate in a 2D setting (34; 39; 40). Here, it seems that the ability of cells to tightly adhere determines both the efficiency of motility and the mechanisms at work. Dendritic cells deficient in integrins or Talin, which links integrins with the actin cytoskeleton, accelerate their rate of actin polymerization at the leading edge and retrograde flow such that they maintain normal crawling speeds and compensate for slippage against the substrate (37). Further, cells crawling on surfaces that vary in their adhesive properties maintain their speeds across transitions in adhesiveness, demonstrating that the
mechanics of cell motility can be rapidly tuned to the environment (37). Jacobelli et al. demonstrated that T cells can switch migratory modes, from a continuous-adhesion 'sliding' mode on ICAM-1 coated surfaces to a 'walking' mode of discreet actin protrusions and MyoII contractions (14). Together, these observations suggest that cells have some mechanism of sensing the adhesive properties of their surroundings and modulating their motility modes and rates for optimal movement through a space.

III. THE SEPTIN CYTOSKELETON

Properties of Septin Polymers

Septins are a family of GTP-binding proteins that assemble into filaments and constitute an emerging non-canonical cytoskeleton (41). All septin proteins contain a conserved GTP-binding domain, and N-terminal proline-rich and C-terminal coiled coil domains that vary between family members. There are 13 septin genes in the mouse genome and Septin proteins have been divided into four groups on the basis of homology in the proline-rich and coiled-coil regions. Members of these four groups can partially compensate for one another functionally (42). A member from each of three groups is required to build the minimal septin complex in mammals, a hexamer, although the size of the basic subunit varies between phyla. A crystal structure exists for one such minimal hexamer, a rod-shaped complex of septins 2, 6, and 7 in a 2:2:2 ratio (43). These hexamers arrange end-to-end into higher order structures both in cells and in
vitro, resulting in elaborate networks of rings, gauzes, and filaments. As will be described in the following sections, these diverse septin superstructures can associate with actin, microtubules, and membranes, or can be free-floating in the cytoplasm.

Septins were initially identified as cell division mutants in yeast, where they play a role in scaffolding cytokinetic machinery at the bud neck and in segregation of material between the mother and daughter cells. Septins appear to function in animal cell division as well (44-48), but septins are also highly expressed in post-mitotic cells such as spermatozoa and neurons, suggesting septin functions beyond cytokinesis. Recent studies have demonstrated roles for septins in motility, polarity, metastasis, and exocytosis.

*Interactions with the Actomyosin Cytoskeleton*

Among the earliest observations of mammalian septins was that in interphase cells, they frequently align on actin stress fibers (44), and the interplay between septins and the actomyosin cytoskeleton in animal cells has continued to be of great interest. In vivo as well as in vitro data suggests that septins are recruited by certain bundled actin structures. Dissolution of stress fibers by treatment with latrunculins or cytochalasins results in septin redistribution to puncta, rings, and diffuse cytoplasmic localization (44; 49; 50). Likewise, purified septins in vitro spontaneously assemble into rings in the absence of actin (50). What precisely recruits septins to actin structures remains somewhat unclear, as
actin and septins do not seem to have any direct affinity for one another. In vitro, actin bundled by anillin, but not by fascin, filamin, or, α-actinin, was able to recruit septins fibers, and in vitro, overexpression of an anillin domain can lead to septin mislocalization (50). It is unlikely, however that anillin is the relevant physiological recruiter of septins to actin structures, as it is sequestered in the nucleus during interphase.

Interestingly, there also appears to be some dependence of actin on septins for maintenance of stress fibers. Sept2 knockdown and over-expression of domains that sequester septins both lead to the loss of stress fibers (50; 51). The reciprocal dependence of these two cytoskeletons remains mechanistically obscure.

Septins may also interact with the actomyosin cytoskeleton via direct binding to MyoII. MyoII has been shown in CHO cells to interact directly with SEPT2, and the two proteins colocalize even when stress fibers are disrupted and septins appear in rings. The interaction has been mapped to a small portion of the MyoII coiled-coil domain (45). Over-expression of that domain blocks the interaction between SEPT2 and MyoII, and results in decreased MyoII activation. The authors suggest that one function of septin filaments is to act as scaffolds for myosin-activating proteins such as ROCK, CRIK and MLCK (45).

Some of the crosstalk between the actin and septin cytoskeletons may be regulated through Rho family GTPases. One of the first identified regulators of mammalian septins was the Cdc42 effector Borg3. Overexpression of Borg3
leads to septin mislocalization, an effect that can be abrogated by coexpression of activated Cdc42, suggesting that septins and active Cdc42 may compete for Borg3 binding (51). Similarly, Rho appears to compete with a Sept9 isoform, Sept9b for binding to septin-associated RhoGEF (SA-RhoGEF), raising the intriguing possibility that expression of certain septin isoforms could influence the rate of nucleotide exchange by Rho (52). Downstream of Rho function, the Rho effector Rhotekin also binds and effects the distribution of Sept9 (53).

**Septin-Microtubule Interactions**

The first indication that septins may interact with microtubules came when all three *Drosophila* septins were hits in a screen for proteins that interact with both actin filaments and microtubules in drosophila embryos (54). That finding was somewhat ambiguous since that study did not control for the possibility that septins co-sedimented with microtubules because of their polymerization and not due to a direct interaction with microtubules. Subsequently, however, other groups have found partial colocalization of septins with both interphase microtubules and the mitotic spindle in living animal cells, and the molecular significance of this interaction is beginning to be understood (46; 47; 55-57).

The colocalization of septins and microtubules appears to be driven by the patterning of microtubules. In HeLa and mammary epithelial cells, nocodazole treatment leads to the loss of Sept9 filaments, and a similar effect was seen for Sept5 filaments in platelets (46; 47; 55). Once associated, however, septins
appear to be important for regulating the stability of certain subsets of microtubules. Particularly, the fraction of poly-glutamated microtubules decreases with Sept2 depletion, but acetylated tubulin increases (56; 58). The net effect of this multifaceted regulation is that several microtubule-dependent processes, such as spindle positioning, polarized secretion, and axonal growth are influenced by septin depletion (47; 57; 56; 59).

An important cofactor in the microtubule-septin system is the microtuble-stabilizing protein MAP4. In the initial identification of MAP4’s role in this system, Kremer et al. showed that the SEPT2-6-7 complex competes with tubulin for MAP4 binding in HeLa cells. Depletion of septins resulted in thickening of microtubule bundles and a general increase in the number and stability of microtubules present in the cell (58). Spiliotis et al. identified a more specific role for the septin-MAP4 interaction in generating polarity in MDCK cells. Here, septins bound poly-glutamated microtubules directly, displacing MAP4 and allowing for ‘fast-tracking’ of vesicles for polarized transport (56). Interestingly, depletion of septins in this system caused the loss of the poly-glutamated subset of microtubules and the loss of epithelial polarity. Clearly, the interplay between septins, microtubules, and MAP4 is more complex or diverse than is currently appreciated, and an integrated model of how these systems interact remains elusive.

Several intriguing hypotheses have been suggested for the major importance of septin-microtubule interactions. The observation that mitotic spindle-associated septins are required for recruitment of the checkpoint protein
CENP-E and that their depletion leads to failure of chromosome segregation and cytokinesis has led some to propose that septins act as a coordinating scaffold between the actomyosin contractile ring and microtubules during mitosis (57). The evidence for septins bridging these two cytoskeletal systems under other conditions, however is scant. It has also been suggested that in interphase cells, septins work generally to maintain the continuity of microtubule networks. Specifically, since septins are less dynamic polymers than microtubules, they could align on the microtubule lattice and act as an “avidity matrix,” stabilizing both the microtubules themselves and interactions with other binding partners. (60)

**Septin-Membrane Interactions**

Purified septins from both yeast and mammals have been demonstrated to bind lipids directly, with particular affinities for PIP, PIP₂, and PIP₃, which they bind through a polybasic region just N-terminal to the GTP-binding domain (49; 61-63). It was recently shown in a cell-free system that inclusion of even small amounts (0.5%) of PIP₂ in a lipid monolayer recruits septin complexes, and increasing concentrations promote the formation of complex, organized septin superstructures such as those seen in vivo (64). Conversely, septin filaments can be disrupted in 3T3 cells by sequestration or depletion of PIP₂ and PIP₃ (62).

The functional significance of membrane-binding by septins is not completely understood. It is clear that septin complexes serve as diffusion
barriers in yeast, segregating plasma membrane, cytoplasmic, ER-membrane, and nuclear components between mother and daughter cell, and it is probable, but not formally proven, that this function relies on binding of septins to membranes (65-68). Septins have been shown or hypothesized to form similar diffusion barriers for transmembrane proteins in many settings in animal cells, including during mitosis, at the base of dendritic spines in neurons, defining the spermatozoon annulus, and at the primary cilium (69-73). The case of the primary cilium provides the best evidence for a mammalian septin diffusion barrier. There, Sept2 localizes to the base of the primary cilium and prevents lateral diffusion of a panel of cilium-resident transmembrane proteins to the cell body (73).

Septins may also play a role in membrane shaping. Addition of septins or septin-containing brain extracts to liposomes containing PIP and PIP\(_2\) changed spherical giant liposomes into tubular structures in vitro, with properties distinct from those formed by BAR proteins (63). The authors of that study suggest that despite the relatively weak affinity of individual septin molecules for PIP\(_2\), the formation of large septin arrays generates a gently curved, rigid surface with high avidity for the liposome, giving these structures the potential to shape plasma membranes in vivo. In this way, the septin cytoskeleton could shape protrusions and invaginations, and sequester excess plasma membrane. One setting in which this may be the case is at the phagocytic cup. Septins have been found localized to sites of phagocytosis of both IgG-coated beads and bacterial pathogens (74; 75). In the setting of bead endocytosis, it was shown that septins
are recruited with the same dynamics as a PIP$_2$ biosensor, suggesting that the localization of septins to this structure could be PIP$_2$ dependent, and depletion or sequestration of Sept2 reduced the rate of successful phagocytosis (74). In this system, a possible role of septins could be in membrane shaping of the phagocytic cup.

**Septins and Secretion**

One of the best–understood cellular roles for septins is in secretion in the nervous system. Hsu et al. were the first to hint at a role for septins in secretion when they immunoprecipitated the Sec6/8 exocyst complex from neurons and pulled down a filamentous complex of Sept2, Sept5, and Sept7 (76). Following this study, the molecular role of Sept5 in secretion has been investigated the most. Sept5 was found to be present on synaptic vesicles in neurons and on exocytic granules in platelets, and to directly bind syntaxins in both cells types (77; 78). Sept5 appears to play an inhibitory role in secretion. Platelets from the Sept5-knockout mouse have a hyper-responsive secretory response to collagen stimulation (77). When Sept5 is over-expressed in neuronal cell lines, secretion is modestly inhibited, and this inhibition is dependent on Sept5 GTPase activity (78; 79). Septin probably regulates secretion by competing for syntaxin binding with a-SNAP and therefore preventing vesicle fusion (80).

Sept5 is probably not the only septin involved in secretion, only the best-understood. The Sept5-knockout mouse appears to have normal
neurotransmitter release and altered expression of other septins, suggesting that those other septins, especially Sept1, are able to compensate for Sept5 function in this system (81; 82). Interestingly, it is not at all understood how septins’ role in secretion is related to the well-characterized interactions between septins and the other systems discussed above. It remains to be seen whether septins serve to integrate many systems across the of the cell, or whether they serve a number of distinct roles, a testament to the diversity of potential septin structures.

**Regulation of Septin Structures**

The factors controlling septin assembly and disassembly in mammalian cells is still a virtual black box. An array of kinases and other enzymes that control septin structures at the yeast bud neck have been established, but as yet, these enzymes have been of little use as candidates for modifiers of mammalian septins. All of the currently identified phosphorylation sites on septins are serine residues, and the majority of septin-kinase interactions were found in screens for substrates. Phosphorylation of Sept1, Sept3, Sept4, and Sept5 was identified in screens for targets of Aurora B kinase, PKG, DYRK1A, and Cdk5, respectively (83-86). Sept2 is likely phosphorylated by casein kinase 2 (CK2), and the phosphorylation site was determined by mass spectrometry and the kinase was predicted based on sequence and later validated (87; 88) (She 204, Yu 2009). Of the putative phospho-septins identified, the best demonstrated is Sept5, and it is also the only mammalian phosphorylated septin for which a function has been
identified. Cdk5 has been shown to bind and phosphorylate Sept5 in neurons, weakening its binding to the exocyst component Syntaxin. A serine to alanine mutant of Sept5 binds Syntaxin more strongly than endogenous Sept5 and results in a measurable increase in exocytosis from cells. These data suggest that phosphorylation of Sept5 is a regulator of exocytosis in neurons, but unfortunately reveal little about how Sept5 phosphorylation changes septin architecture. (79)

An important post-translational regulator of septins in *S. cerevisiae* and other fungi is SUMOylation. In G2/M arrested yeast, septins are the most abundantly SUMOylated proteins in the cell. SUMOylation appears to be a septin destabilizer, as mutating SUMOylation sites results in septin rings that failure to disassemble following cytokinesis and accumulate around the cell cortex (89). A yeast two-hybrid screen for septin-interacting proteins in *Drosophila* found two components of the SUMOylation pathway, but a direct interaction with septins could not be validated, nor was SUMOylated septin detected (90). Other evidence of septin SUMOylation in animal cells has yet to be uncovered.

**Septins and Motility**

Studies of septin expression and function in cancer cells have suggested that septins play a role in motility. Sept9 isoforms have been found to be amplified or aberrantly expressed in leukemias, and breast and ovarian tumors (91; 92). Further experiments in cell lines have identified a potential role for
Sept9 in the epithelial to mesenchymal transition. Overexpression of the Sept9_v4 isoform caused otherwise nonmotile cells to make actin-containing protrusions that resembled pseudopodia and were independent of Cdc42 activity. These cells had elevated motility in a scratch wound assay compared to control cells, but lacked directional guidance in 2D and 3D systems (93). Ectopic expression of Sept9_v1 induces a similar phenotype in breast cancer cell lines, causing them to take on a more mesenchymal morphology, and increasing their capacity for invasion and migration. Interestingly, a proteomic screen of pseudopodia isolated from six metastatic cancer cell lines identified Sept9 as a protein enriched in the pseudopodia compared to cell bodies. Subsequent knockdown of Sept9 in those cells reduced motility, invasion, and pseudopodia formation in all six cell lines (94).

Prior to the work presented in this thesis, a project was initiated in our lab with the goal of characterizing septin complexes in T cells. Septins became a candidate for study when they were identified in a microscopy screen for proteins that polarize in CD4 T cells. RT PCR and western blot studies confirmed that septins from all four groups are present in both the D10 T cell line and primary T cells, and it was demonstrated by immunoprecipitation that these septins form complexes together. All septins that were stained for are primarily distributed in a corset-like shape the T cell cortex, enriched in the mid-zone of polarized cells and largely absent from both the leading edge and the back of the uropod. (95)

A knockdown system was used to explore the function of T cell septins. Fortuitously, and has been indicated in other systems, septin stability depends on
assembly of the core septin complex, so depletion of Sept7 resulted in the loss of all other septins as well. Septin depletion resulted in a profound morphological defect. Cells had elongated uropods, around twice as long as control cells on average. Furthermore, rapid live imaging revealed that Sept7KD cells had cortical abnormalities, such as periodic rapid blebbing of the cell body, and abnormal large protrusions, especially from the “neck” region of the cell. These defects were not accompanied by abnormal phosphorylation of MyoII or Myosin Light Chain, nor did septin depletion affect actin, MyoII, or tubulin distribution.

The morphological defects associated with septin depletion resulted in a small retardation of crawling speed. Surprisingly, though, when Sept7KD cells were submitted to a Boyden chamber transmigration assay, it was found that they transmigrate through pores as small as 3 μm at rates 2-5 times higher than control cells (95). This unusual phenotype suggested an important and mysterious role for septins in controlling the T cell cortex. Obtaining a mechanistic understanding of how septins function at the T cell cortex has been the primary goal of this project.
CHAPTER 1: CHARACTERIZATION OF CORTICAL DEFECTS DURING AMOEBOID MOTILITY IN SEPTIN-DEFICIENT T CELLS

I. ABSTRACT

The systems that refine actomyosin forces during motility remain poorly understood. Septins assemble on the T cell cortex and are enriched at the mid-zone in filaments. Septin knockdown causes membrane blebbing, excess leading edge protrusions, and lengthening of the trailing-edge uropod. The associated loss of rigidity permits motility, but cells become uncoordinated and poorly persistent. This also relieves a previously unrecognized restriction to migration through small pores. Pharmacologically rigidifying cells counteracts this effect, and relieving cytoskeletal rigidity synergizes with septin-depletion. These data suggest that septins tune actomyosin forces during motility, and likely regulate lymphocyte trafficking in confined tissues.

II. INTRODUCTION

An important feature of T lymphocytes is their migration through tissues in search of antigen presenting cells (APC) bearing peptide-MHC complexes. Both motility rate and access to tissues by T cells is highly controlled—a mechanism that is thought to be necessary to prevent accumulation in peripheral tissues and the potential breakdown of tolerance mechanisms. Motile T cells are characterized
by an amoeboid ‘hand mirror’ shape in which the trailing edge pinches distinctly into a uropod. This morphology is required for efficient migration within lymph nodes and peripheral tissues, for crossing barriers to enter new tissues, and for their reactivity to antigen-bearing APC (96). Their migration is characterized by actin polymerization at the leading edge pseudopod (97-99), and myosin IIA-based contraction, predominantly away from the leading edge (13). Some of the polarity circuits restricting leading edge extensions to the pseudopods and retraction to the uropod are likely similar to those in neutrophils and Dictyostelium (100; 101). The mechanisms that fine-tune protrusive activity and regulate the shape of the uropod, however, remain undetermined.

Septins were first identified as cell division cycle (cdc) mutants in yeast and assemble into concentric filaments at the mother-bud neck during cell division (102-104). The Septin ring around the cytokinetic furrow in yeast functions as a diffusion barrier, maintaining cell fate determinants in the appropriate cell (65; 67). Individual septins were independently identified in mammals on the basis of their abnormal expression in a wide range of tumors, including mammary adenocarcinomas and myeloid leukemias (91; 92). Additionally, the Septin 9 locus is a site of frequent retroviral insertion leading to generation of T cell lymphomas (105). Since their identification in mammals, septins have been shown to play important roles in many cell types, including neurons, platelets, and spermatozoa (70; 72; 77).

Septins are cytoskeletal proteins that polymerize to form rings and gauzes and typically attach to the cell membrane via N-terminal phosphoinositide binding
motifs (106). In mammals, they frequently co-localize with actin stress fibers. The mammalian septins can be divided into four groups. Filaments form from combinations of these, with members of each class potentially substituting for one another and generating a great diversity of possible arrays. A recently reported crystal structure of complexed septins indicates that the essential subunit of septin superstructures is a linear hexamer of Sept2-Sept6-Sept7 with a 2:2:2 stoichiometry (43).

Septins assemble along the cell cortex, enriched in an array of fibrous strands in the mid-zone. Elimination of the septin cytoskeleton using shRNA led to marked elongation of the uropod, pronounced blebbing, and excess leading edge protrusions, suggestive of uncontrolled cytoskeletal forces (95). Here, we define features of the role of septins in T cell motility. The motion and membrane dynamics of septin-deficient cells is consistent with a loss of membrane tension. The resulting disorganized migration of septin-deficient cells strongly suggests a corset-like function for the septin cytoskeleton, providing compression and rigidity, and supporting efficient motion of motile T cells.

III. RESULTS

*Septins Assemble on the Cortex, Enriched in the T Cell Midzone*

High-resolution confocal imaging of Sept6 and Sept7 in D10 T cells revealed that septins assemble into short puncta or fibers on the T cell cortex
(Fig. 1.1a and 1.1b). In polarized cells, these structures were enriched in the midzone of the cell, forming a corset-like distribution (Fig 1.1a). In rounded cells, septins were evenly distributed across the cortex (Fig. 1.1d) Occasionally, cells with abnormal protrusions were observed, and in these cases, anti-septin antibodies tended to stain those regions very brightly, as is demonstrated with anti-Sept6 in Fig. 1.1c. Staining of primary activated T cells demonstrated a similar septin distribution at the cortex to that observed in the D10 cell line (Fig. 1.1e).

*Defective Motility in the Absence of Septins*

Previous work demonstrated severe cortical abnormalities arising from septin depletion from T cells. We therefore wondered whether cells with such cortical morphology could efficiently coordinate their motility. When control and Sept7KD cells were observed crawling on coverslips in vitro, Sept7KD T cells exhibited reduced instantaneous crawling velocities (Fig. 1.2a). Sept7KD T cells crawled $7.5 \pm 1.9$ mm/min (KD #1, n = 36) and $7.0 \pm 1.8$ mm/min (KD #2, n = 35) compared to $8.5 \pm 2.6$ mm/min for control cells (n = 30). More significantly, they had shorter net displacements than control cells (Control: $15.0 \pm 6.1$ mm/min$^{1/2}$, KD #1: $10.81 \pm 5.8$ mm/min$^{1/2}$, KD #2: $10.85 \pm 4.5$ mm/min$^{1/2}$). Interestingly, previous results indicated that the actomyosin cytoskeleton was generally normal in Sept7KD cells (Tooley). This indicates that septins perform an important function in motility that is independent of actomyosin protrusion and contraction.
Figure 1.1 High-resolution images of septin distribution in T cells. (a) D10 T cells were stained with anti-Sept7 and the membrane intercalating dye DiO. Poor colocalization indicates that Septin staining was not a result of local variations in membrane densities. (b) D10 T cells were stained with anti-Sept6 antibodies. Staining had less fibrous and more punctuate distributions. (c) A rare cell with an extra leading edge protrusion shows enrichment of Sept6 in the protrusion. (d) A round, nonmotile cell stained for Sept6 demonstrates a punctate, but evenly distributed distribution across the cell cortex. (e) A motile primary T cell stained for Sept6 shows a similar cortical staining to that of D10 cells. Scale bars represent 10 μm.
Figure 1.2 Septins support efficient motility by preventing non-productive protrusions. (a) Overall crawling velocity and (b) displacement over time were reduced by Sept7KD. KD #1, n = 31, KD #2, n = 29 and control treated cells n = 39. Similar results were obtained in three independent experiments. Statistical analysis was performed using Kruskal-Wallis tests with Dunn’s post-tests. (c) Representative frames from analysis of extra-nuclear path volume. The path of the nucleus over the entire time course is shown in blue. Measured cell body volume deviating from that path is represented in green. The red line shows the outline of the entire cell volume, for reference. (d) Analysis of multiple time points for at least 10 different cells and a Mann-Whitney U test indicates that Sept7KD cells make significantly more protrusions out of the direction of motility than control cells.
Since Sept7KD cells were more protrusive overall than control cells, we wondered whether Sept7KD cells were making non-productive protrusions outside the direction of motility that impeded their processivity. To address this, we measured the volume of motile cells that extended outside the path of motility and, therefore, did not propel the cell forward. We labeled the nuclei of GFP-transfected cells with Hoechst 33342 and acquired confocal three-dimensional time-lapses of crawling cells. We then created volumes representing the paths occupied by the nuclei during 15-minute movies (Fig. 1.2c, blue), showing the overall directions of the cells. The GFP volumes were overlaid on the nuclear path volumes at each time point and the total volumes (Fig. 1.2c, red outline) and the volumes extending outside the nuclear path were measured (Fig. 1.2c, green). The total volumes of control and Sept7KD cells were similar, but on average Sept7KD cells had about 1.5 times the extra-nuclear path volume of control cells (median volumes of 167.8 mm$^3$ and 249.5 mm$^3$ for control and Sept7KD, respectively, Fig. 1.2d), supporting the hypothesis that their excess protrusions do not contribute to processive motion and, therefore, contribute to diminished motility in Sept7KD cells.

**Septin Deficiency in Primary Cells**

To better understand the relevance of our findings in Sept7KD D10 cells, we used retrovirus to express the knockdown hairpin sequence in primary
activated DO11.10 T cells. Using this system, we were able to achieve 59% knockdown of Sept7 72 hours after transduction (Fig. 1.3a). Though this level of knockdown was not as complete as that achieved in the D10 system, we observed many phenotypic similarities with Sept7KD D10 cells. Morphologically, Sept7KD DO11.10 cells had similarly elongated uropods to those of D10 cells (Fig1.3b) though they were at times more challenging to observe, since the uropod is not as stable or pronounced in primary DO11.10 cells. Further, when DO11.10 cells were allowed to adhere to ICAM-1 coated coverslips and then subjected to shear flow, Sept7KD cells had significantly lower motility speeds than control cells, though the slight difference in their displacements over time failed to achieve statistical significance (Fig1.3c).

D10 T cells exhibit little specific chemotaxis in the presence of chemokine, so our DO11.10 system gave us the opportunity to investigate how the enhanced basal transmigration observed in Sept7KD D10 cells influenced chemokine-induced transmigration. In a chemotaxis assay using 5 µm pores, we found that septin-deficient primary cells transmigrated at a rate double that of control cells (Fig. 1.3d). This was accompanied by a small increase in their transmigration in the absence of chemokine. Together, these results demonstrate the relevance of our findings in the D10 system to primary T cell motility.
Figure 1.3 Septin regulate shape, motility, and transmigration in primary T cells. (a) Retroviral knockdown of Sept7 in KO11.10 T cell blasts resulted in a 59% depletion of Sept7 72 hours after transduction. (b) Sept7KD DO11.10 T cell blasts show a similar extended-uropod phenotype to Sept7KD D10 cells. Scale bar represents 10 μm. (c) Activated control or Sept7KD DO11 T cells were allowed to adhere on ICAM-coated glass coverslips for 3 minutes and then subjected to shear flow of 1 dyne/cm3 for 15 minutes while images were collected. Similar to non-flow conditions, Sept7KD cells traveled more slowly than control cells, though the small difference in their displacements over time did not achieve statistical significance. (d) Sept7KD DO11.10 cells transmigrate more efficiently in response to CCL21 than control cells. Transwell inserts with 5 μm pores were used in these assays. Data shown is representative of two experiments.
Transmigration Efficiency is Correlated with Cortical Rigidity

Because of the extreme compression of large cells migrating through small pores, we hypothesized that a loss of cortical rigidity in septin-deficient cells allowed them to transmigrate more efficiently. To investigate this possibility, we sought to pharmacologically mimic or counteract the septin-based loss of rigidity. We treated D10 cells with nocodazole to inhibit microtubule polymerization and relax the cell cortex or with taxol to stabilize microtubules and rigidify the cortex. Interestingly, taxol treatment has previously been shown to increase cell-permeation of collagen gels\textsuperscript{29}. After nocodazole treatment, both control and Sept7KD cells maintained their uropods, but appeared less compact and often generated multiple leading edges (Fig. 1.4a). Conversely, when treated with taxol, both groups had few protrusions, and most became rounded (Fig. 1.4a, b.)

Time-lapse images of nocodazole-treated cells indicated that they exhibit a similar protrusive phenotype to Sept7KD cells. While control cells generally made one or two small protrusions in a 10-minute period, Sept7KD and nocodazole-treated cells frequently made four or five (Fig. 1.4c), and the persistence of each protrusion was significantly longer for Sept7KD and nocodazole-treated than control cells (Fig. 1.4e). Like Sept7KD cells, protrusions in nocodazole-treated cells were uncoupled from the processive motion of the cell. While protrusions frequently arose at the leading edge, their growth was not restricted to that region and they extended without adhesion in many directions, sometimes reaching so far back before being retracted that the uropod of the cell
Control KD #1

No Drug

Nocodazole

Taxol

Protrusion in 10'

Protrusion Length (µm)

Protrusion Duration (s)

% of Cells Rounded

Fraction of Cells

% Transmigrated

p<0.05

p<0.01

p<0.001

p<0.001

p<0.01

n.s.
Figure 1.4 Septins and microtubules regulate rigidity and transmigration. (a) Images of control or Sept7KD D10 were taken after treatment with no drug, 5 µM nocodazole or 5 µM taxol for at least 30 minutes. (b) Taxol treatment caused both Sept7KD and control cells to become rounded implying a dependence of the Sept7KD phenotype on microtubule function. (c) Septin depletion and nocodazole treatment both caused an increase in the number of protrusions made by cells in a 10-minute observation period. Analysis represents greater than 30 cells per group. Duration of protrusions (e) but not protrusion length (d) was significantly increased in both Sept7KD and nocodazole-treated cells relative to control cells. Analysis in (d) and (e) is of the first protrusion observed in each cell from (c), and was done using the Kruskal-Wallis test with Dunn’s post-test (f) Representative time lapse images of protrusions observed in control, Sept7KD, and nocodazole-treated cells. (g) Nocodazole caused chemokine-independent transmigration of D10 cells through 3 µm pores similar to, and synergizing with, that observed in Sept7KD cells. This nonspecific transmigration was completely abolished by taxol treatment. Cells were treated with drugs for 30 min prior to the beginning of the four-hour assay. Data shown is representative of two independent experiments. Scale bars represent 10 µm.
took on a forked appearance (Fig. 1.4f). On average, these protrusions did not extended farther from the cell body than the less frequent protrusions in control cells, though occasionally, very long (>15 mm) protrusions arose from Sept7KD and nocodazole-treated cells, but not control cells (Fig. 1.4d). Additionally, the means shown in Figure 1.4d may underestimate the true population means for Sept7 KD and nocodazole-treated cells because the most severely abnormal protrusions often extended out of the imaging plane, excluding them from this analysis.

Since the cortical phenotype after nocodazole treatment was like that of SeptKD cells, we compared the migration of Sept7KD and nocodazole or taxol-treated D10 cells through 3 µm transwell pores in the absence of chemokine (Fig. 1.4g). Interestingly, nocodazole-treated control D10 cells transmigrated comparably to untreated Sept7KD cells (8.2 ± 0.7% versus 11.8 ± 0.3% and 12.5 ± 1.8% for KD #1 and KD #2, respectively) while few (1.9 ± 0.1%) control cells migrated. When Sept7KD cells were treated with nocodazole, the effect was greater than additive, with (35.1 ± 1.9% of KD #1 and 30.9 ± 7.7% of KD #2 cells). Conversely, when cells were stiffened with taxol, less than 1.5% passed through the pores, even among Sept7KDs. These data support the hypothesis that septin depletion relaxes the cell cortex, allowing highly efficient movement through spaces much smaller than the resting cell diameter.
IV. DISCUSSION

Given the many phenotypic abnormalities of septin-deficient cells, septin filaments, being cortically enriched and punctuated by occasional fibers and puncta, may function as a molecular corset. A thin and uniform septin network prevents blebs at the leading edge where actin and myosin are highly dynamic (107). We hypothesize that the dynamic turnover of actomyosin filaments and their short length may be complemented by the stays of long septin filaments which are probably stable over time (108; 109). This stability may provide background rigidity against which global and local actomyosin forces are applied.

This fine-tuning of forces is clearly evident in time-lapse microscopy of septin-deficient cells; septin loss has a profound effect on coordinated crawling and processivity of D10 cells. We propose that in highly dynamic amoeboid cells, it may be critical to contain and direct forces generated over their short lengths. At cellular lengths of 5–15 mm, compression forces would tend to propagate across the entire cytoplasm very rapidly and could generate inefficient motility if translated into bulk movements that did not reflect the required directionality (27). It is therefore interesting to note that foraging Dictyostelium lack septins and that their response to chemotactic stimuli, unlike that of T cells, is characterized by increased production of blebs and excess protrusions in multiple directions. Large numbers of protrusions extending in many directions may allow cells to better survey their environments, and Dictyostelium have evolved to select certain protrusions, rather than restrict their initial number (110).
The cortical instability of septin-deficient cells may be related to their enhanced capacity for migration across small openings. Transwell migration assays assume cells are not able to cross a barrier with dimensions smaller than the cell’s resting girth in the absence of a motive force. We found that this is not the case in the absence of septins. At present, it is unclear whether septin-dependent repression of basal migration is due to additional leading edges (septin-deficient cells find holes more easily) or the loss of cortical stability (septin-deficient cells are more deformable). Although our experiments with nocodazole treatment do not directly distinguish between those possibilities, that cortical stabilization by taxol treatment inhibits transmigration suggests that the relative fluidity of Sept7KD cells allows them to pass readily through small pores. Since T cell nuclei are typically larger than 3mm in diameter, septins may also regulate nuclear shape—indeed septins interact with anillin, which, prior to cell division is associated with the nuclear envelope (111).

These observations may have important implications for in vivo transmigration and tumor metastasis. Cells entering or exiting tissues must squeeze through or between endothelial cells; loss of a basal restriction on this may contribute to metastatic proficiency. Since septin expression is frequently altered in human and mouse tumors and Sept2 was first identified as an over-expressed gene in a tumor line selected for metastasis we speculate that destabilization of the cell cortex due to stoichiometric changes in septin complexes may facilitate tumor metastasis and progression (112-115).
CHAPTER 2: CORTICAL RETRACTION BY SEPTIN-MEDIATED MEMBRANE RECAPTURE

I. ABSTRACT

Regulation of the cortex during amoeboid motility is critical for efficient motion. It was previously shown that the septin family of GTP-binding, filament-forming proteins are required for cortical control in T cells, and that excess membrane protrusions in the absence of septins impair rapid processivity during motility. In this work, we explored the mechanism of action of septins in this system. To dissect the role of septins, we manipulated the outward hydrostatic force experienced at the cell cortex by shifting cells to hypotonic conditions. By monitoring cell size during this shift, we found that that septins are required for rapid cortical retraction following hypotonic expansion, and that they function in concert with the actomyosin cytoskeleton. Septins assembled on the cortex in rings and fibers under hypotonic conditions, and marked sites of membrane invagination. These invaginations likely represent sites of membrane retraction, where excess membrane liberated during osmotic expansion is recaptured and contorted by septin complexes. When blebs were induced in motile T cells, Sept6-GFP relocalized to the sites of those blebs. Our results suggest that septins regulate the cell cortex by assembling at actin-poor membrane extensions and mediating recapture of those membranes.
II. INTRODUCTION

Cell shape is a result of the interplay between hydrostatic forces exerting outward force on the plasma membrane, lateral tension of the membrane itself, and contractile forces exerted by the actomyosin cytoskeleton (116). Diverse structures can largely be explained by varying contributions of these forces. For instance, on the leading edges of crawling cells, protrusion is driven by the combination of densely polymerizing actin and locally elevated hydrostatic pressure that results from enriched NHE1 transporting ions into that region and driving the influx of water (25; 117). In contrast, blebs are the result of hydrostatic pressure that causes a segment of plasma membrane to rip away from the cortical actomyosin cytoskeleton, while bordering membrane remains firmly anchored by Myosin II (118). Blebs are actin-poor during their expansion phase, and this expansion is limited both by local surface area and by repolymerization of actin on the bleb surface that is associated with retraction (119). It has recently been shown that these same forces of positive hydrostatic pressure and contractile actomyosin control cell rounding during mitosis (120).

Septins are a family of GTP-binding proteins that self-assemble into hexameric or octameric quaternary structures and further into large filaments, rings, and gauzes in vitro and in vivo (42). A variety of functions have been described for septins in mammalian cells. Principle among these are contouring of phosphatidyl inositol-containing membranes in vitro, templating of actin fibers, stabilization of microtubule tracks, and interaction with exocytic machinery (44; 47; 63; 76). The relative importance of these many mechanisms of septin action
remains unclear, but in aggregate, septin deficits can lead to defects in cytokinesis, neuronal development and function, platelet activation, and spermatozoa motility (44-48; 72; 77; 121).

We have previously shown that septin depletion in T cells leads to a distinctive morphological phenotype that includes excessive blebbing and protrusion on the cell body. The observation that septins may play a role in regulating the shape of the cell cortex led us to the current study, in which we sought to understand the interplay between septins and other forces that regulate cell protrusions. To this end, we manipulated the outward hydrostatic pressure experienced by cells and investigated the roles of septins in response to that force. We found that septins are required for rapid contraction of the cell cortex, in cooperation with the actomyosin cytoskeleton. Septins assemble at sites of cortical over-extension, such as leading edge protrusions prior to retraction and inducible membrane blebs, where they mediate plasma membrane recapture.

III. RESULTS

Blebbing and protrusion in septin-deficient cells

We have previously shown that D10 T cells expressing shRNAs against Sept7 are deficient in all septins, and have profound morphological defects that include cortical instability (95). As illustrated in Fig. 2.1a, Sept7KD cells display periodic blebbing as they crawl on coverslips. The blebs are concentrated on the cell body, but generally absent from the uropod, are non-apoptotic, and are
Figure 2.1. Septins regulate cortical stability. (a.) D10 T cells crawling on ICAM-1-coated glass display periodic membrane blebs and protrusions (black arrows). (b.) Fluorescent images of D10 T cells expressing GPI-mCherry (red) and Sept6-GFP (green) indicate that leading edge protrusions (white arrow) retract into the septin collar. (c.) Control and Sept7KD cells were cell cycle-synchronized with nocodazole and then released for imaging. Time lapse images indicate profound blebbing during cytokinesis among Sept7KD cells. A blebbing index consisting of number of blebs divided by (time observed x cell perimeter) was calculated for each cell and indicates significantly elevated blebbing in Sept7KD cells. (d.) DIC and Sept6-GFP fluorescence time lapse images of cells undergoing mitosis as in (c.), demonstrating accumulations of septin complexes at the midbody late in cytokinesis. (e.) Line scan of a dividing cell indicated by the dotted line (d.) indicating enrichment of Sept6-GFP at the midbody. Scale bars represent 10 μm.
generally resolved within a few minutes of beginning, at which point the cells resume normal motility (95). This phenotype suggested an important role for septins in stabilizing the T cell cortex.

To better understand how septins function in control of the T cell cortex, we generated a Sept6-GFP fusion. In polarized cells, this fusion generally localized to the midzone of the cell in a corset-like pattern, recapitulating the major pattern of localization of endogenous septins previously observed by immunofluorescence (95). Sept6-GFP was coexpressed with GPI-linked mCherry to mark the plasma membrane, and cells were imaged while crawling on ICAM1-coated coverslips. As expected, crawling cells extended protrusions at their leading edge that propelled them forward. Strikingly, when cells changed directions in the course of undirected crawling, protrusions comprising a portion of the leading edge retracted, and in most cases (83%, n=18) those retracting protrusions dissolved into the Sept6-GFP collar (Fig. 2.1b). Though Sept6-GFP was generally absent from the cortex of the retracting protrusion, in many cases, an increase in Sept6-GFP intensity could be seen to appear at the front of the retracting protrusion, seemingly discontinuous with the bulk of the septin collar.

Amoeboid motility is a process of rapid shape change by a cell, and we wondered whether an effect of septin-depletion on cortical stability could also be observed under other conditions of shape change, such as cytokinesis. To investigate this, control and Sept7KD D10 Tcells were treated with nocodazole for 16 hours to synchronize their cell cycles, and imaged following washout. Though Sept7 is dispensable for successful mitosis in T cells, an unstable
membrane during cytokinesis was readily observed. While control cells extended and retracted a few small blebs on their surfaces, Sept7KD cells produced on average 2.5 times as many blebs per surface area and unit time (blebbing index, \textbf{Fig 2.1c.}) These blebs also tended to be more long-lived than those in control cells, often persisting from the time of their appearance until the end of the time-lapse, up to 30 minutes. We also observed Sept6-GFP distribution during cell division, and found that it was mostly concentrated at the cytokinetic furrow during the completion of mitosis (\textbf{Fig. 2.1d}), consistent with observations in other mammalian cells and in yeast (57; 122).

\textit{Osmotic volume change as a model for cortical dynamics}

This observation that normal cellular protrusions tend to retract into the septin collar suggested the intriguing possibility that rather than preventing protrusions and blebs, septins play a role in resolving those structures after they arise. To distinguish between those possibilities, we devised an assay in which hypotonic swelling and subsequent regulatory volume decrease (RVD) were used to model protrusions and retractions at the cell surface. We hypothesized that since blebs can arise from local hydrostatic pressure inside the cell that causes the plasma membrane to balloon away from cortical actomyosin (118), hydrostatic pressure generated by shifting cells to hypotonic media would model a cell-wide bleb. To monitor the progress of this swelling and contraction, cell volume in isotonic media was measured using the forward scatter parameter of a
flow cytometer. Cells were then shifted to isotonic media, and their volumes were monitored over time. Three features of cellular swelling and contraction could then be measured: initial volume, maximum volume, and the rate of contraction (or RVD). This experiment is schematized in Figure 2.2a and 2.2b.

As shown in Figure 2.2c, Sept7KD cells had the same initial volume and expanded to the same extent as control cells. The knockdown samples, however, had smaller slopes in the RVD period (0.320, compared to 0.490 for controls; Fig. 2.2d), indicating that Sept7KD cells are retarded in their ability to re-contract the cortex to its original size.

*Septin assembly during hypotonic volume change*

In order to better understand what role septins might be playing during cortical contraction, cells were immobilized on anti-CD44-coated coverslips and stained with antibodies to Sept7 under isotonic conditions and ten minutes after shifting to hypotonic conditions. When cells are immobilized, Sept7 distribution is less-strikingly corset-like than when cells crawl on ICAM-1 or bare glass. Nonetheless, a clear change in septin distribution could be observed when cells were moved to hypotonic media. Under isotonic conditions, most cells had Sept7 enriched at the cortex in small puncta (Fig. 2.3a). After ten minutes in hypotonic media, however, only about 20% of cells had this distribution. A greater number of cells had septin distributed in fibers, especially at the bottom surface where the cell contacted the coverslip. Other cells had 1 μm Sept7 rings on their cortexes.
Figure 2.2. Septins show slowed retraction in an osmotic swelling assay. (a.) Schematic of a flow cytometry osmotic swelling assay. D10 T cells are suspended in isotonic media and their volumes are recorded for 1 minute. Cells are then switched to 100 mOsm media and returned to the flow cytometer, where changes in their volume are recorded over time. (b.) Sample data from osmotic swelling assay indicating time periods where measurements of initial volume, maximum volume, and retraction (RVD) rate are made. (c.) Data from flow cytometry osmotic swelling assay demonstrating normal initial and maximum volumes, but slowed RVD in Sept7KD cells compared to controls. Two independent samples from each group are shown. (d.) Summary of four independent experiments showing equivalent initial and maximum volumes for Control and Sept7KD cells, but slower RVD rates for Sept7KD samples.
Figure 2.3. Septins assemble on the cell cortex during cortical retraction. (a.) DIC and fluorescence images of wild type D10 cells immobilized on anti-CD44-coated glass and stained for Sept7 before or after a shift to 100 mOsm hypotonic media. Under isotonic conditions, Sept7 is enriched at the cortex diffusely or in small puncta. Under hypotonic conditions, septins aggregate into filaments and rings (inset). Scale bar is 10 μm in main image and 1 μm in inset. (b.) Quantification of septin distributions observed in (a.). (c.) Though some Sept7 circles are observed in cells under isotonic conditions, each cell contains a greater number of rings under hypotonic conditions. (d.) Live imaging of Sept6-GFP-expressing cells shifted to hypotonic media. Within 30 seconds, Sept6-GFP begins to aggregate on the cortex, and soon assemble into cortical rings that sometimes resemble tubules. Scale bar is 10 μm. (e.) Sept6-GFP aggregations colocalize with invaginations in the plasma membrane (white arrow) as indicated by GPI-mCherry fluorescence. A dotted line overlaid on the Sept6-GFP image marks the contour of the membrane and dotted lines on the GPI-mCherry image outline this contour. Scale bar is 1 μm.
Both the frequency of cells with Sept7 rings (Fig. 2.3b) and the number of rings per cell (Fig 2.3c) was increased under hypotonic conditions.

The development of septin rings was monitored live in Sept6-GFP-expressing cells. Cells were once again immobilized on anti-CD44-coated coverslips, and after one minute of imaging, water was added to the imaging chamber to reduce the osmolarity of the media by 2/3. Within one minute after the change in osmolarity, Sept6-GFP began to aggregate on the cortex of the cells (Fig. 2.3d). Over time, these aggregates became pronounced 1 µm rings, studding the whole surface of the cell, and persisting for longer than 30 minutes after the addition of water. When Sept6-GFP rings were examined more closely in cells coexpressing GPI-mCherry to mark the plasma membrane, dimple-like membrane invaginations could be seen beneath the septin rings (Fig. 2.3e).

Pathways contributing to hypotonic volume regulation

An important step recovery from osmotic expansion is active transport of K⁺ and Cl⁻ ions that return the cytosol to normal osmolarity. We used Charybdotoxin and 5-Nitro-2-(3-phenylpropylamino)benzoic Acid (NPPB), inhibitors of Ca²⁺-gated K⁺ channels and of Cl⁻ channels, respectively, to block this ion transport during our flow cytometry volume change assay. Cells treated with the inhibitors had normal volume under isotonic conditions and normal expansion following the shift to hypotonic conditions (Fig. 2.4a). As anticipated, cells had slowed RVD in the presence of both inhibitors (Fig. 2.4b, slopes of
Figure 2.4 Septin aggregates are independent of ion transport. (a.) Treatment with ion channel inhibitors Charybdotoxin and NPPB did not influence initial or maximum volume of control or Sept7KD cells in the flow cytometry osmotic swelling assay, but both drugs significantly inhibited RVD (b.) (c.) Single-plane time lapse images of Sept6-GFP-expressing control cells or cells treated with Charybdotoxin or NPPB demonstrate that swelling-induced septin assembly on the cortex can occur in the absence of volume recovery. Bottom row of images taken 20 min after media change are 3D reconstructions of whole cells.
0.114 and 0.056 for Charybdotoxin and NPPB, compared to a slope of 0.323 for untreated control.) When Sept7KD cells were treated with the drugs, neither demonstrated an additive effect on cell shrinkage, though the effect of NPPB was so severe that an additive effect of septin depletion may have been undetectable.

When Sept6-GFP-expressing cells were shifted to hypotonic media in the presence of Charybdotoxin or NPPB, they formed Sept6-GFP rings indistinguishable from those formed by control cells (Fig. 2.4c), demonstrating that septin rings are not sufficient to mediate cell shrinkage if cells cannot recover their normal osmolarity.

The actomyosin cytoskeleton in cortical retraction

Rearrangement of the cortical actin cytoskeleton following changes in hydrostatic pressure has been described in several cell types (123-127). Since interactions of septins with both actin and MyoII have been described, we tested whether the actomyosin cytoskeleton is required for cell shrinkage. None of the inhibitors tested (blebbistatin, latrunculin B, or nocodazole) significantly influenced the volume of cells in isotonic media nor their maximum expansion (Fig. 2.5a). However, cells treated with Latrunculin B to inhibit actin polymerization shrunk with a rate of 0.289 and those treated with Blebbistatin to inhibit MyoII activity shrunk with a rate of 0.347 compared to 0.490 for vehicle-treated cells. In contrast, depolymerization of microtubules with Nocodazole had no impact on the rate of volume change (0.453) (Fig. 2.5b). Intriguingly,
Figure 2.5 Interplay between septins and the actomyosin cytoskeleton in cell shrinkage. (a.) Treatment with inhibitors of MyoII (blebbistatin), actin polymerization (latrunculin B) or microtubule polymerization (nocodazole) does not influence initial or maximum cell size in the flow cytometry osmotic swelling assay. (b.) Cortical retraction in this assay is slowed by blebbistatin or latrunculin, but unaffected by nocodazole. (c.) There is no additive or synergistic effect on cortical retraction of latrunculin B treatment of Sept7KD cells. (d.) Imaging of Sept6-GFP-expressing cells demonstrates septin aggregation into rings with latrunculin B treatment under isotonic conditions. (e.-f.) Illustration and quantification of anti-Sept7 staining of wild type cells showing that formation of septin filaments (white arrows) and rings (red arrows) is normal in cells treated with blebbistatin. Scale bars are 10 μm.
shrinkage of Sept7KD cells treated with Latrunculin B was not additionally impaired, suggesting that Septins and the actomyosin cytoskeleton may be functioning together to affect cortical contraction. (Fig. 2.5c).

Immunofluorescence for endogenous Sept7 demonstrated that cells treated with blebbistatin rearranged septin complexes similarly to control cells when hydrostatic pressure was increased, with 64% of cells assembling septin circles or filaments in control cells and 75% assembling these structures in the presence of blebbistatin (Fig. 2.5e-f) In contrast, Latrunculin B treatment induced the spontaneous formation of Sept6-GFP rings under isotonic conditions, and these rings persisted under hypotonic conditions (Fig. 2.5d). These data are consistent with septin rings observed after Latrunculin B treatment in other systems, and suggest the intriguing possibility that septin assembly following osmotic expansion might be triggered by actin depolymerization (50).

Membrane recapture by septins

Under many conditions, including swelling, cells respond to cortical stretch initially by smoothing local surface topography such that excess plasma membrane from ruffles and microvilli is recruited into the region under tension (128). Since septins have been implicated in membrane contortion in vitro (63), and since we observed topographical changes at the plasma membrane with the assembly of septin rings (Fig 2.3e), we wondered whether a role of septins in cell shrinkage might be to re-contort excess plasma membrane. To investigate this,
we took advantage of the side scatter parameter of a flow cytometer, which measures laser light scattered orthogonal to the direction of incident light, and correlates with a cell’s membrane complexity. When we tracked the Side Scatter of cells subjected to hypotonic stress, we found that upon the shift to hypotonic media, there was a precipitous drop in the average side scatter value, followed by a gradual rise over the remaining duration of the experiment (Fig. 2.6a) The kinetics of this change correlated with the changes observed in cell size (Fig. 2.2c). When we compared control and Sept7KD cells, we found that they had similar initial Side Scatter values, but following the initial drop, Sept7KD cells recovered their side scatter more slowly than control cells (Fig. 2.6a-b). This indicates that Sept7KD cells have a defect in recovering membrane complexity concurrent with their slowed shrinkage.

If septins are responsible for recapturing excess plasma membrane during RVD, they may also serve this function in more physiological structures such as blebs. We reasoned that since dynamin activity is required for many endocytic pathways, dynamin blockade would result in excess plasma membrane in need of recapture, possibly by septins. Indeed, when cells were treated with dynasore for 30 minutes prior to imaging, they displayed grossly normal morphology, but time-lapse imaging revealed that over a 10-minute imaging period, 60% of cells underwent membrane blebbing at the front half of the cell (Fig. 2.6c). Interestingly, this blebbing resembled that previously observed in Sept7KD cells in that it was stochastic and episodic, with cells crawling normally before blebbing began and resuming normal crawling after the blebbing resolved. By treating
Figure 2.6 Septins recapture excess membrane. (a.) Side scatter measurements of control and Sept7KD D10 cells in the flow cytometry osmotic swelling assay, demonstrating that scattering drops upon the shift to hypotonic media and then gradually returns to isotonic levels. Sept7KD cells are inhibited in their return to normal side scatter values. Graph shows two samples each of control and Sept7KD cells. (b.) Summary of data from 3 independent experiments showing a significant decrease in the rate of side scatter recovery in Sept7KD cells. (c.) Treatment of D10 cells with the dynamin inhibitor Dynasore caused cells to periodically bleb at their leading edges. (d.) Time-lapse imaging of Sept6-GFP-expressing cells treated with Dynasore demonstrates recruitment of septins to blebbing regions of the cell (black arrows). Scale bar is 10 μm.
Sept6-GFP-expressing cells with dynasore, we were able to directly observe the response of septins to membrane blebs. As shown in Figure 2.6d, when cells began to bleb, Sept6-GFP lost its standard midzone distribution and accumulated on the cortex at the leading edge of dynasore-treated cells. This accumulation was transient, persisting only as long as the blebbing continued, and was very rarely seen in untreated cells. The accumulation of septins at these induced blebs supports the hypothesis that septins are recruited to actin-poor membrane extensions and serve to recapture them.

IV. DISCUSSION

In this study we investigated the mechanistic role of the septin cytoskeleton in controlling the T cell cortex. We observed abnormally frequent and persistent membrane blebs during motility and during cell division in septin-deficient cells and found that retracting protrusions in crawling cells usually resolve into the septin-rich midzone of the cell. These findings led us to develop an experimental system in which local blebs were modeled by global hypotonic expansion of the whole cell and bleb retraction was modeled by compensatory regulatory volume decrease. This system allowed us to easily test the influence of various molecular pathways on cortical dynamics by using flow cytometry to monitor cell volume. Using this technique, we found that hypotonic expansion is normal in septin-depleted T cells, but that RVD was significantly slowed. Combined with the finding that protrusions generally retract into a septin-rich
region, these data indicate that in T cells, septins may play a general role in the retraction of plasma membrane.

During RVD, septins assemble on the cortex in rings similar to those observed during Latrunculin B-induced actin depolymerization in our system and others (50). A feature of the response to hypotonic conditions in several cell types is the loss of actin stress fibers and subsequent repolymerization of actin in peripheral patches (123; 124). We therefore postulate that septins are recruited to the cortex downstream of the loss of cortical F-actin. Septin rings were persistent on the cortex, and represented sites of membrane invagination. These structures may be analogous to tubular structures formed by purified septins applied to giant unilamellar vesicles in vitro (63). Formation of those tubules is enhanced by the presence of phosphatidyl inositides, especially PIP and PIP$_2$ in the membrane, and it is possible that these signaling lipids also play a role in septin recruitment during osmotic swelling and shrinkage. This would be consistent with a study of Erlich ascites cells that demonstrated a drop in total cellular PIP$_2$ during initial hypotonic swelling and increased PIP$_2$ production during regulatory volume decrease (129).

One cellular response to cortical tension is local redistribution of a plasma membrane reservoir that, in the steady state, is ordinarily sequestered in membrane contours such as ruffles and microvilli (130; 131). Side scatter measurements over the course of our osmotic swelling assay support the hypothesis that in this system, those membrane contours are rapidly smoothened and then gradually re-contorted as the cell shrinks. The correlation between
slowed cell shrinkage and the slowed increase in side scatter in Sept7KD cells suggests that septins may participate in cell shrinkage by recapturing and folding membranes. Our model predicts that septins should also control blebs via membrane recapture. In support of this, we found that septins redistributed to the sites of pharmacologically-induced blebs, consistent with the idea that they were recruited by loose, actin-poor cortical regions and served a function in retracting those regions.

Together, our findings provide mechanistic insight into earlier observations of the phenotypes of septin-deficient T and demonstrate the first in vivo evidence of the in vitro phenomenon of membrane contortion of by septin complexes. Our data suggest that interactions with membranes may be the dominant mechanism by which septins regulate the cortex of amoeboid cells. These observations also add an important new molecular player to our understanding of how cellular protrusions are controlled and retracted.
CHAPTER 3: SEPTINS IN PRIMARY T CELLS: PROGRESS AND FUTURE DIRECTIONS

I. ABSTRACT

The physiological roles of septins are extremely diverse, and efforts to connect knowledge of their cell biology to in vivo functions have been sparse. In this chapter, I describe our progress in characterizing T cells from the Sept7 conditional knockout mouse. Thymic development proceeds normally in these mice, and they contain normal numbers of T cells in their secondary lymphoid organs, with excellent depletion of septins from all four groups. T cells from these mice recapitulate several features of a septin-deficient T cell line, such as elongation and cortical instabilities. Activation and trafficking of Sept7-deficient primary cells is grossly normal. The availability of these mice and the viability of their cells make this model an excellent tool for the study of septins in amoeboid cells in vivo.

II. INTRODUCTION

Though a number of important discoveries have been made in the mammalian septin field in the last fifteen years, there has been little progress in connecting the biochemistry and cell biology of septins to their role in physiology in vivo. Some major challenges in this endeavor include the ubiquity of septin expression and the redundancy within septin groups. A number of septin knockout mice have been made, and their phenotypes range from undetectable
(Sept3<sup>−/−</sup>) to failure to gastrulate (Sept7<sup>−/−</sup>) ((132) and personal communication from M. Kinoshita). In between, several interesting phenotypes have emerged. For instance, the Sept4<sup>−/−</sup> mouse has defects in multiple tissues. These mice have structural defects in their spermatazoa leading to asthenospermia that mimics a human disease, a propensity towards liver fibrosis, abnormal dopamine transport in the brain, and elevated numbers of hematopoietic stem cells (72; 121; 133; 134). Mice lacking Sept5 have hyper-reactive platelets, but apparently normal brains, a surprising finding given that Sept5’s role in neuronal secretion in vitro is well-known (77; 132).

Because Sept7 is part of the core complex of mammalian septins and is the sole member of Group IV in mice, its depletion leads to depletion of the majority of septin complexes. For this reason, a Sept7-null mouse would be ideal for the gross study of the requirements for septins in vivo. Complete knockout of Sept7 results in mice that fail to gastrulate, which, though an impressive illustration of the importance of septins in mouse development, render them useless for studies of lymphocytes. To combat this problem, mice carrying a floxed Sept7 allele have been generated by our collaborators, and we have crossed those mice to CD4-Cre transgenic mice. This chapter will present our progress to date in characterizing T cells from those mice, as well as suggest some future lines of inquiry.
III. RESULTS

*Normal T cell Development in Sept7cKO Mice*

We began our study of CD4-Cre x Sept7^lox/lox^ conditional knockout (Sept7cKO) T cells by examining primary and secondary lymphoid organs from these mice. Thymi were analyzed from 6-week old Sept7cKO and littermate control mice. The organs had equivalent cellularity and proportions of CD4^+, CD8^+, and double positive thymocytes (**Fig. 3.1a**). When these thymocytes were permeabilized and stained with anti-Sept7 antibodies, it was revealed that, as expected, deletion of the Sept7 gene occurred between the double negative and double positive stage of T cell development. While over 60% of cells expressed Sept7 at the double-negative stage, by the double positive stage, 66% of cells were clearly negative for Sept7, and the whole population appeared to be expressing less Sept7 than double positive thymocytes from littermate controls. In both single positive populations from Sept7cKO mice, around 90% of cells were negative for Sept7 expression. (**Fig. 3.1b**)

Mature lymph node T cells showed near-complete deletion of Sept7; peripheral CD4^+^ cells were 87% Sept7-negative, whereas CD8^+^ cells were 98% Sept7-negative by FACS (**Fig. 3.1c**). Western blot analysis revealed that purified CD8^+^ T cells from Sept7cKO mice were depleted for septins from all four groups, recapitulating our previous results in D10 T cells and validating Sept7cKO T cells as a model for studying gross septin function (**Fig. 3.1d**). Despite having virtually
a. 

![Control vs. Sept7cKO](image)

b. 

![Flow cytometry graphs](image)

c. 

![Peripheral CD4+ vs. CD8+](image)

d. 

![Western blot analysis](image)

e. 

![Cellularity of Lymphoid Organs](image)
Figure 3.1. Efficient deletion and normal development of Septin-deficient T cells. (a.) and (b.) Thymi were dissected from 6 week-old Sept7cKO and littermate control mice. A single cell suspension was fixed and permeabilized and stained for CD4, CD8, and Sept7. (a.) Cellularity of the thymus was equivalent between the two mice. (b.) Sub-gating of developmental subsets demonstrates that Sept7 is efficiently deleted between the double positive and double negative stages, and that most T cells lack Sept7 by the time they reach the single-positive stage of development. (c.) FACS analysis of Sept7 staining in lymph node cells demonstrates nearly complete knockout of Sept7 in both CD4 and CD8 subsets. (d.) western blots of lysates from purified CD8 T cells show depletion of septins from all four groups in Sept7cKO cells. Numbers indicate the percentage of protein remaining in the Sept7cKO sample. (e.) Spleens and lymph nodes were analyzed from 7 week-old Sept7cKO and control mice. The groups had equivalent distributions of CD4 and CD8 T cells in those organs, as well as equivalent total cellularity.
no septin complexes, CD4 and CD8 T cells were present in equivalent numbers to littermate controls in both spleen an lymph nodes (**Fig. 3.1e**).

*Morphological Features of Sept7cKO T Cells*

Knockdown of Sept7 in constitutively motile D10 T cells results in cells with unstable cortexes, typified by periodic blebbing and excess membrane protrusions from the cell body and neck region of the cell. Since naïve T cells are generally unpolarized and non-motile in 2D culture, we used in vitro activated Sept7cKO-OT1 blasts and littermate control OT1 blasts to investigate the morphology of primary T cells in the absence of septins. Similarly to the D10 system, we found that Sept7cKO OT1 blasts crawling in 2D had a greater frequency of both membrane blebs and excess protrusions than control cells (**Fig. 3.2b-c**). Though not as dramatic as the morphology seen in D10 cells, we were also able to measure significant elongation of Sept7cKO cells compared to control cells (**Fig. 3.2 a and c**). This elongation appeared to be somewhat bimodal, as the distribution of cell lengths among Sept7cKO cells mostly overlapped with that of control cells, but there was also a population of very long Sept7cKO cells (**Fig. 3.2d**).
Figure 3.2 Morphological changes in septin-deficient T cells. Activated Sept7cKO and control OT1 cells were plated on ICAM-1-coated coverslips and imaged for morphological measurements. (a.) Images of control and Sept7cKO cells illustrating the elongated uropod of Sept7cKO cells. Crawling Sept7cKo cells demonstrate more frequent membrane blebs (b.) and excess membrane protrusions (c.) than control cells. (d.) Cell length measurements indicate that Sept7cKo cells are longer than control cells. (e.) histogram showing the distribution of cell lengths of control and Sept7cKO cells.
**T Cell Activation**

T cell septins have been observed to localize to the immunological synapse (IS) (A. Tooley, unpublished observation), but their role at that site is presently unknown. The well-characterized function of septins as diffusion barriers in other systems, as well as our results indicating septin-membrane interactions in T cells, raise the possibility that they could play a similar role in T cells, perhaps serving to confine the T cell receptor or other components of the signaling apparatus to the IS. Septins could also serve as a placeholder for T cell polarity during activation. In yeast, septins maintain cell polarity by marking the bud site between cell divisions (135). Some evidence suggests that T cells derive their effector functions by undergoing asymmetric cell divisions, with that asymmetry maintained by some kind of persistent structure marked by the IS (136). Stable septin complexes accumulating at the IS could serve as this placeholder structure during T cell asymmetric cell division.

To begin an investigation of what role septins play in T cell activation and differentiation, we assessed in vivo proliferation of Sept7cKO OT1 and littermate OT1 control T cells in response to immunization. Purified CD8 T cells from these mice were transferred into CD45.1 congenic recipient mice, which were then immunized using the DEC205-OVA/anti-CD40 system, and their draining lymph nodes were analyzed 72 hours later. As shown in Figure 3.3, proliferation, as indicated by CFSE dilution, was very similar between control and Sept7cKO OT1 cells. These results support our previous finding that, unlike in some other mammalian cell types, septins are not required for cell division in T cells (95).
Figure 3.3 Normal activation of Septin-deficient cytotoxic T cells. 1 million CD8 T cells from Sept7cKO-OT1 or littermate control OT1 mice were CFSE-labeled and transferred into B6-CD45.1 recipients. Recipients were immunized subcutaneously with DEC-205-conjugated ovalbumin protein and anti-CD40 in the inguinal area. 72 hours later, draining lymph nodes were harvested and cells were restimulated with PMA and ionomycin for subsequent intracellular cytokine staining. Transferred cells were identified by CD45.2 staining. Equivalent activation of Sept7cKO and Control cells demonstrated equivalent proliferation (CFSE-dilution, top panels) and effector cytokine production (IFN-γ intracellular stain, bottom panels.)
Further, when these cells were restimulated in vitro and stained for intracellular IFN-γ, similar proportions were positive, indicating that by this measure, Sept7cKO cells underwent normal activation and differentiation into effector cells.

_Trafficking of Sept7cKO Cells to Tissues_

Our previously published work demonstrated a role for septins in restricting transmigration through small pores in vitro (95). We wanted to determine how this observation translates to an in vivo setting where T cells must frequently pass through restrictive environments, such as endothelial cell layers, to access tissues, so we assessed homing of naïve T cells to several tissues. Sept7cKO and control CD8 T cells were labeled with different dyes and transferred in equal numbers to the same CD45.1 congenic recipient mice. Organs were analyzed 2 and 18 hours later, and the ratio of Sept7cKO to WT cells in each organ was determined. Our hypothesis, based on their elevated transmigration in vitro, was that Sept7cKO cells would traffic more rapidly into the spleen and lymph nodes of recipient mice, but as Figure 3.4 demonstrates, trafficking was equivalent to that of control cells at both time points.

Cells transferred intravenously transit through the lung before reaching other tissues. We wondered whether Sept7cKO cells might be aberrantly retained in the lung due to dysregulated migration through endothelial cells of the lung vasculature. Alternatively, Sept7cKO cells with their excessive protrusions and unusual shapes could become trapped in the narrow capillaries of the lung.
Figure 3.4. Normal trafficking of Sept7cKO T cells. (a.) CD8 T cells were selected from Sept7cKO and littermate control mice, labeled with CFSE and CMTMR, respectively, and co-transferred at a 1:1 ratio into CD45.1 recipients. Organs were harvested at the times indicated and the percent of cells of each color in the organs were analyzed. Ratios are normalized to the ratio in the blood at each time point to compensate for variation in injection. No significant differences were observed between control and Sept7cKO cells in any organ analyzed. (b.) Analysis of in vivo T cell motility analyzed by 2-photon microscopy, demonstrating that control and Sept7cKO cells have equivalent speeds and track straightness in lymph nodes.
However, when we isolated lymphocytes from lungs of recipient mice and compared the numbers of control and Sept7cKO cells, we found them to be equivalent, demonstrating no unusual trapping of cells in the lung capillaries or migration of cells into the lung parenchyma (Fig 3.4). Together, these results suggest that the requirements for transmigration in a Boyden chamber are different enough from the requirements for transmigration in vivo that the effect of septin-depletion on squeezing through small spaces cannot be detected in vivo.

To further assess role of septins in T cell movement in a mouse, we used 2-photon microscopy to image control and Sept7cKO T cells in lymph nodes. The two cell types were labeled with different dyes, cotransferred into a recipient mouse, and explanted lymph nodes were imaged the next day. We found that both control and Sept7cKO T cells exhibited normal motility in the lymph node environment, with both equivalent average track speeds and average turning angles. This brings up the possibility that the role of septins in motility is most relevant in a 2D setting.

IV. DISCUSSION AND FUTURE DIRECTIONS

The data presented here represent the very beginning of the potential study of septins in T cells in vivo. Our finding that septin complexes can be nearly completely eliminated from naïve CD4 and CD8 T cells using the Sept7\(^{\text{lox/lox}}\) x CD4-Cre system, and that the resulting cells develop and remain in normal numbers demonstrates that these mice will be a powerful resource for asking a
range of questions about T cell septins. Principally, our goals will be to understand the roles septins play in T cell activation and differentiation and in motility in vivo. Both of those issues remain incompletely explored.

We have shown that in the presence of a strong stimulus, Sept7cKO T cells can be equivalently activated and differentiated to cytokine-producing effectors. It is not yet known, however, whether more rigorous activation conditions, such as infection with a pathogen or immunization with a weaker antigen dose would yield the same result. If septins are required for stabilizing immunological synapses, an abundance of antigen-bearing APCs could obviate this role, since establishing long-lived contacts could be more important when antigen is scarce. Further, we have not tested whether Sept7cKO cells are capable of becoming memory cells, a more stringent test of differentiation than IFN-γ production. One model of memory cell development relies on a stable polarization of T cells with respect to the immunological synapse, and a diffusion barrier may be required to maintain that polarity (136). It is possible that septins serve this function, and in vivo studies of septin-dependent T cell activation or differentiation could have fascinating correlates to TIRF studies of Sept7cKO or Sept6-GFP-expressing cells interacting with lipid bilayers.

Our finding that elevated transmigration in septin-deficient T cells is not recapitulated in vivo brings up several interesting questions. First, the Boyden chamber assay may represent a particular step of transendothelial migration in vivo that is not rate-limiting, and it would be helpful to understand what that step is, so that better connections could be drawn between trafficking in vitro and in
vivo. Second, it could be that septins are regulated to disassemble during transendothelial migration in vivo, so the trafficking phenotype of septin-deficient cells is not as informative as some sort of constitutively active septin would be. As more is learned about mechanisms of mammalian septin regulation, we hope to be able to address that possibility. Finally, it is interesting to ponder how the model presented in Chapter 2, which identifies the primary role of septins as capturers and contorters of expanded membranes rather than maintainers of cortical rigidity, relates to the ability of septins to pass through small pores.

Perhaps constitutively protruding membranes are more able to find permissive sites for passage through a transwell membrane.

Finally, other aspects of motility in vivo remain to be investigated. Though Sept7cKO T cell motility in lymph nodes appears quite normal by 2-photon microscopy, it is unclear whether this would also be the case in a confined interstitial environment, such as skin, or under other conditions where excess protrusion in the Sept7cKO could cause cells to get caught. We have begun to model this using a combination of in vivo imaging of T cells in capillary beds of the lungs and in vitro imaging of cells crawling in confined branching PDMS microchannels (137; 138). We are hopeful that these systems used together can begin to elucidate the real effects of septins on primary T cell motility in vivo.

In conclusion, my work has demonstrated a clear role for septins in controlling the T cell cortex. While early evidence suggested that septins served to rigidify T cells, a closer examination of septin-deficient cells both in vitro and in vivo does not support that conclusion. In vitro, I have demonstrated that septins
function in a retractive phase of controlling membrane protrusions, rather than rigidifying the cortex to prevent protrusions to begin with. In vivo, the hypothesis that septin-deficient cells are less rigid than control cells predicted elevated migration through restricted environments, which was not borne out experimentally. It is difficult to predict what effect un-retracted protrusions have on T cell functions in vivo. To my knowledge, no similar phenotype of unrestricted protrusions in cells that are still globally healthy and normally polarized has been described. It is likely, therefore, that the further study of Sept7cKO T cells in vivo will reveal much about not only septin biology, but about the importance of cortical control in lymphocytes in general.
REFERENCES


34. Lämmermann T, Bader BL, Monkley SJ, Worbs T, Wedlich-Söldner R,


42. Kinoshita M. Assembly of mammalian septins. J. Biochem 2003


50. Kinoshita M, Field CM, Coughlin ML, Straight AF, Mitchison TJ. Self-


60. Spiliotis ET. Regulation of microtubule organization and functions by septin GTPases. Cytoskeleton 2010;:NA-NA.


67. Takizawa PA, DeRisi JL, Wilhelm JE, Vale RD. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin


125. Pedersen SF, Mills JW, Hoffmann EK. Role of the F-actin cytoskeleton in


150. Etienne-Manneville S, Manneville J, Nicholls S, Ferenczi MA, Hall A. Cdc42
and Par6-PKCaζeta regulate the spatially localized association of Dlg1 and APC to control cell polarization. J. Cell Biol 2005 Sep;170(6):895-901.


158. Giagulli C, Scarpini E, Ottoboni L, Narumiya S, Butcher EC, Constantin G, Laudanna C. RhoA and zeta PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in


APPENDIX I: CONSERVED POLARITY PROTEINS IN T CELL BIOLOGY

I. ABSTRACT

Polarity is essential for nearly all T cell functions, including migration, activation, and effector activity. Still, it is unknown how T cells maintain this polarity while making dramatic transitions in cell shape, such as transitioning from an amoeboid crawling morphology to stable coupling with antigen presenting cells. The Lgl/Par6/PKCζ system has been shown to be essential for polarity establishment and maintenance in model invertebrates and mammalian epithelial cells. We sought to investigate whether this conserved system also regulates polarity in T cells. We found that PKCζ kinase activity was required for immunological synapse morphology and stability, but failed to find evidence of roles for other members of this pathway in T cells.

II. INTRODUCTION

Transitions in shape, motion, and polarity are required for T cells to enter lymphoid and inflammatory environments and to interact with antigen-presenting cells (APCs) and targets. Upon encountering its cognate antigen on the surface of an APC, a T cell undergoes a transition from a motile “hand-mirror” morphology to a stopped, rounded morphology with a tight, organized synapse at the interface with the APC. During this transition, the T cell remains polarized, but dramatically reorganizes cytoskeletal components, cell-surface proteins, and intracellular signaling molecules to face the APC. While some effectors of the T
cell stop signal have begun to be elucidated, many players in this pathway remain to be determined.

Much of our knowledge about the regulation of cell polarity has come from developing invertebrates and mammalian epithelial cell lines. Though the cell types studied in these systems are relatively static compared to a T cell, they may provide clues as to the protein players that allow T cells to crawl and stop while maintaining, but adjusting, their polarity. A screen for genes affecting asymmetrical cell division in *C. elegans* revealed a set of polarity-determining genes numbered Par1-6 (for Partitioning defective), and Par3 and Par6 have been shown to work in an important polarity-regulating system in several models (139). Par3 and Par6 proteins contain multiple PDZ protein-protein interaction domains and use them to form regulated, competitive complexes in which atypical PKCζ and Par6 form a core cassette that can either interact with Lgl or Par3 and Cdc42 (140). Variations in the composition of these complexes give them a number of different effects in different organisms and tissues. A complex containing Par6, PKCζ, and Par3 is required in several epithelial systems for the establishment of tight junctions and this complex may be antagonized by competition of Lgl for Par6 and PKCζ binding (141-144). In invertebrate systems, there seems to be a role for this system separate from junctional adhesions. Various parts of the complex have been shown to be required for asymmetrical cell division in both *Drosophila* and *C. elegans* (145-149). Questions remain as to what signals control the composition and localization of Par complexes, as well as the mechanisms by which they mediate their effects on cell polarity. Some
evidence suggests that Cdc42 in its active conformation may recruit Par6 and PKCζ, and the kinase activity of PKCζ may be involved in shifting the balance of the composition toward Par3 and Cdc42 (145; 150; 151).

Data from *Drosophila* and mammalian epithelial and neuronal systems suggest that Lgl may play a particularly important role in establishing and maintaining cellular polarity. Lgl was first identified as a neoplastic tumor suppressor in *Drosophila* and homozygous mutations in the Lgl gene result in loss of polarity and overproliferation of cells in multiple tissues in the *Drosophila* embryo (152). The protein has multiple WD-40 motifs, as well as a characteristic C-terminal Lgl domain, well-conserved in its homologs from *C. elegans* to mammals (153). In the *Drosophila* neuroblast, Lgl interacts with Myosin II to restrict its distribution on the cell cortex and this interaction is required for the proper segregation of basal cell fate determinants such as Miranda and Prospero (148). This function of Lgl is regulated by the *Drosophila* PKCζ homolog (145). A role for Lgl in development is conserved in mammals. In mice lacking Lgl1, one of two mammalian homologs, neural progenitors do not differentiate, due to an inability to polarize Numb, and pups die neonatally with hydrocephalus and neuroepithelial rosettes in the brain (154). Whether Lgl1’s segregation of cell fate determinants in mammals occurs through Myosin II is unclear, and some evidence suggests that Lgl may exert its polarizing effects in mammalian epithelia by interacting with the basolateral exocytosis machinery (155). Homologues of Lgl1 in yeast interact with both homologs of Class II Myosin and
the secretory apparatus, suggesting that Lgl could function both ways in the same cells (156).

In this study, we sought to address the role of Lgl and other proteins with which it interacts in T cell biology, including immunological synapse morphology and immune effector function.

III. RESULTS

**Localization of Polarity Proteins at the Immunological Synapse (IS)**

We started by staining T cell-APC couples for members of conserved polarity complexes. Others have observed polarization of polarity proteins, such as Scribble, Dlg, and Lgl, which all localize to the uropod of crawling cells and many of these uropodal proteins are redistributed to the IS upon coupling to an antigen presenting cell (157). We found that in our hands, as well, anti-Lgl1 staining was brightest at the T-APC interface, as was anti-Par6. 3-D rotations of cells stained with each antibody revealed a pSMAC-like distribution for both. Staining for PKCζ revealed a quite different distribution. PKCζ was difficult to precisely localize, since it stained very brightly in APCs as well, but T cell PKCζ also appeared to be concentrated at the T-APC interface. (Fig A.1)
Figure A.1 Patterning of Par complex members during T:APC coupling. Staining of Par6, Lgl1, and PKCζ in couples between D10 T cells and CH27 APCs, 5C.C7 T cell blasts and CH27 APCs, and DO11.10 T cell blasts and A20 APCs, respectively. Pseudocolor images are reconstructions of the boxed regions, generated by rotating z-stacks 90 degrees such that the viewer is looking down the axis of the T cell.
PKCζ Activity is Required for IS Stability and Morphology

A myristoylated peptide corresponding to the pseudosubstrate sequence of PKCζ (called PKCζ-PS hereafter) can be used to specifically inhibit its kinase activity in living cells. To investigate the role of PKCζ activity in T-APC synapses, we pre-treated activated DO11.10 T PKCζ-PS and used these cells in a flow cytometry-based coupling assay. The results of these studies strongly suggest a role for PKCζ in mediating adhesion at the T:APC interface. Figure A.2a. shows the frequency of coupling between DO11.10 T cells and a professional APC line, A20, or CHO cells that have been transfected with the necessary MHC allele and B7. While PKCζ inhibition has relatively little effect on coupling to A20, there is a clear defect in antigen-specific coupling to CHO, which lack normal mouse LFA-1-ligands. When those same T cells are allowed to couple to A20 with a greater degree of agitation during fixing, a far lower percentage of T cells couple to APCs after treatment with PKCζ-PS (Fig A.2b). The inability of couples containing PKCζ-PS-treated cells to withstand agitation suggests that adhesive contacts in these couples are not being properly assembled. Others have reported, using the same drug, that PKCζ activity is required for T cells to adhere to ICAM spotted at low density on a slide, but not high-density ICAM, and that this difference may be accounted for by the reduced ability of those cells to cluster LFA in the absence of PKCζ activity (158).
Figure A.2 PKCζ activity is required for normal adhesion and morphology during coupling. (a.) Percent of T cells coupled to professional or artificial APCs after 30 minutes without agitation. (b.) Percent of T cells coupled to professional APCs after 30 minutes when cells were briefly vortexed during fixation. (c.) Illustration of MTOC orientation phenotypes, demonstrating properly (top panels) and improperly (bottom panels) polarized MTOCs, marked by white arrows. (d.) Quantification of the frequencies of MTOC orientation phenotypes in control cells and cells treated with 10 μM PKCζ-PS. (e.) Illustrations of common morphologies of control and PKCζ-PS treated T:APC couples, with orange marks indicating how contact angle measurements were made. (f.) Histogram showing the distribution of the sums of two contact angles for each T:APC couple.
Several aspects of T:APC couple morphology are also disrupted when T cells are pre-treated with PKCζ-PS. As shown in Figure A.2c, normal T cells orient their MTOC toward the APC rapidly after coupling, but pericentrin staining indicated that a large proportion of PKCζ-PS-treated T cells failed to do this. Instead, their MTOCs were either undetectable or located askew from the T-APC interface (Figure A.2d). Furthermore, in untreated couples, T cell membranes become tightly flattened against APCs, but the T cells treated with PKCζ-PS maintained a rounded membrane (Fig. A.2e.) This was quantified by measuring the contact angles on both sides of the T cell in a DIC image. Figure A.2f shows the distribution of the sums of those angles for a set of treated and untreated cells.

Other Polarity Proteins in IS Formation

Because of the dramatic effects of PKCζ inhibition on the IS, we investigated whether other players in this pathway might phenocopy PKCζ-PS treatment. First, we used a retroviral vector to over-express wild type Lgl1 (WT Lgl1) or a constitutively active Lgl-1 mutant, which has its PKCζ serine phosphorylation sites mutated to alanines (Lgl1-SA), in activated DO11.10 T cells. We reasoned that if PKCζ’s function in IS formation is carried out through its phosphorylation and inactivation of Lgl1, Lgl1-SA-expressing cells might have similar defects at the IS. This was not the case, however. Cells expressing Lgl1-SA coupled to APCs with the same frequency as cells expressing WT-Lgl1 or
transduced with empty vector (Fig. A.3a). When we examined these cells more closely, we found that they had normal synapse morphology, including nearly perfect MTOC polarization to the IS, and equivalent cSMAC formation to WT-Lgl cells (Fig. A.3c-d). These results suggest that Lgl1 regulation is dispensible for IS assembly, and that the importance of PKCζ activity in IS formation is probably independent Lgl1 as a substrate.

Since Par6 and PKCζ form an essential complex for the recruitment of other polarity proteins, we sought to disrupt this interaction to better understand the mechanism by which PKCζ controls IS assembly. To this end, we over-expressed a Par6B mutant that lacks the N Terminus and therefore cannot bind PKCζ (Par6B-DN) and acts as a dominant negative. When cells expressing this construct were used in a coupling assay, we found that at multiple time points and antigen concentrations, they coupled to APCs equivalently to cells transduced with empty vector (Fig. A.3b) Together, our data indicate that neither phosphorylation of Lgl1 by PKCζ, nor assembly of a conserved polarity complex of PKCζ and Par6 is required for IS formation.

Normal T Cell Activation and Migration in the Absence of Lgl1

The Lgl1 knockout mouse dies within a few days of birth, so to generate Lgl1 knockout T cells for use, we used mice with a floxed Lgl1 allele crossed to the Lck-Cre mouse, in which Cre is turned on late in thymocyte development. Cells from these mice (Lgl1cKO) were compared to littermate controls in a variety
**Figure A.3 Polarity complexes are dispensable for IS formation.** (a.) Percent of DO11.10 T cells transduced with the indicated viral vectors coupled to A20 APCs 10 and 40 minutes after the two cell types were combined. (b.) Coupling between peptide-pulsed A20 APCs and DO11.10 T cells expressing empty pMIG vector or Par6b-DN at multiple antigen concentrations and time points indicates no effect of Par6 inhibition. (c.) Quantification of immunofluorescence staining of T:APC couples for MTOC pericentrin, indicating that the MTOC orients normally in couples where the T cell expresses constitutively active Lgl1-SA. (d.) Quantification of immunofluorescence staining of T:APC couples for cSMAC marker CD3, demonstrating that normal cSMACs are formed in T cells expressing Lgl-SA.
of standard assays. First, we addressed general polarity of the cells by assaying their chemotaxis. We found that both control and Lgl1cKO cells transmigrated robustly in response to SLC in a Transwell assay (Fig. A.4a.)

Next, we assessed the ability of naïve cells to interact with APCs. A coupling assay was performed using Lgl1cKO OT1 cells and ova peptide-pulsed bone marrow-derived dendritic cells (BMDCs). Over 60 minutes, around 70% of both control and Lgl1cKO cells coupled to dendritic cells, demonstrating once again, that the effects of PKCz inhibition of T-APC couples are probably not due to PKCz interactions with Lgl1 (Fig. A.4b). Further, T-APC couples formed by Lgl1cKO OT1 cells seem to lead to normal T cell activation. Control and Lgl1cKO OT1 cells were transferred into wild type hosts which were then immunized with peptide-pulsed BMDCs. When draining lymph nodes were analyzed 72 hours later, we found that regardless of peptide dose, Lgl1cKO and control cells proliferated equivalently, as assessed by CFSE dilution (Fig. A.4c).

Finally, we used several methods to evaluate whether differentiation into effector cells was influenced by Lgl1. First DO11.10 cells were activated in vitro and transduced with an empty vector, WT-Lgl1, or Lgl1-SA. This activation was carried out under unskewed, Th1-skewing, Th2-skewing, or Th17-skewing conditions. After 96 hours, differentiation to T-helper subsets was determined by intracellular cytokine staining. As demonstrated in Figure A.4d, expression of Lgl1-SA did not influence the percentage of cells that produced IFN-γ under any polarizing condition, indicating that Lgl1 regulation is not required for T helper cell differentiation. Lgl1 is also dispensable for cytotoxic T cell differentiation, as Lgl-
Figure A.4 Normal T cell function in Lgl1cKO mice. (a.) Chemotaxis of naïve CD8 T cells from Lgl1cKO or littermate control mice. (b.) Coupling between OVA peptide-pulsed BMDCs and naïve Lgl1cKO OT1 or control OT1 cells. Cells were combined 60 min prior to analysis. (c.) In vivo activation of Lgl1cKO and control OT1 cells in mice immunized with subcutaneous DCs pulsed with ova peptide at the indicated concentrations. (d.) IFN-γ production by DO11.10 T cells transduced with the indicated viral vectors under multiple T helper-polarizing conditions. (e.) Specific lysis of 51Cr-loaded EL4 target cells by in vitro activated Lgl1cKO OT1 or control OT1 cells. (f.) real time-PCR for Lgl1 and Lgl2 in naïve and in vitro activated CD4 and CD8 T cells and in the D10 T cell line demonstrating that Lgl1 is the dominantly expressed Lgl gene at the transcript level in all populations.
cKO and control OT1 cells lysed EL4 targets with similar efficiency (Figure A.4e).

IV. DISCUSSION

Our results using the PKCζ -PS inhibitor were an initially promising suggestion of a role for conserved polarity complexes in T cell biology. Unfortunately, our attempts to otherwise perturb these complexes have yielded exclusively negative results. There may be several explanations for this. First PKCζ -PS may have undescribed off-target effects that account for the phenotypes we've observed. We did find that concentrations even slightly greater than those used here led to an unacceptable level of cytotoxicity (data not show), indicating that PKCζ -PS may be disturbing multiple systems. A second possibility is that redundancy of other proteins in this pathway makes it difficult to perturb. For instance, a second Lgl gene, Lgl2, may compensate for Lgl1 function in some cases. We have found Lgl1 to be much more highly expressed than Lgl2, but our data does not rule out the possibility that small amounts of Lgl2 are sufficient to compensate for deleted Lgl1.

Finally, the most likely possibility PKCζ has functions independent of polarity complexes that are most important for T cell biology. Giagulli et al have shown that upon chemokine stimulation, PKCζ activation promotes lateral mobility of LFA-1, allowing for higher avidity interactions with integrins, and adhesion to substrates sparsely populated with ICAM1 (158). This function of
PKCζ does not appear to rely on interactions with any other conserved polarity proteins, and could therefore explain why our results are specific to PKCζ.

Surface-bound chemokine, or perhaps by other costimulatory molecules on the surface of an APC, could activate PKCζ very early upon T cell-APC contact, promoting upregulation of integrin avidity that allows for maturation of that contact into an IS. The aberrant membrane morphology observed could be due to a requirement for very strong adhesion in order to “stick” the edges of a rounded T cell to the APC. MTOC placement may also depend on LFA-1 activation, as microtubules have been observed to run from the MTOC to the pSMAC, and LFA-1 ligation is known to lead to ERK signaling, which promotes MTOC reorientation (159; 160).
APPENDIX II: METHODS

Cells lines

The A20, CH27, and D10.G4 cell lines were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, β-mercaptoethanol. D10.G4 media was supplemented with 10 U/ml human interleukin-2 (IL-2). As D10.G4 cells were used only for studies of motility and not activation, they were not periodically restimulated as has been previously described. Cells were maintained at a density less than $10^6$ cells/mL. A subclone of the D10.G4 line was generated by electroporating cells with the Sept6-GFP plasmid described below, sorting cells using a MoFlo cell sorter (DakoCytomation) and maintaining in the media described above supplemented with 50 µg/mL geneticin for selection. Phoenix cells used for generating retrovirus and CHO cells were grown in DMEM with 10% FCS, L-glutamine, penicillin, streptomycin, β-mercaptoethanol, and Heps. Both were maintained at or below 80% confluency.

Primary cells

When purified naïve T cells were used, they were selected using the StemSep CD4 or CD8 T cell negative selection kit (STEMCELL Technologies Inc.). Bone marrow dendritic cells were generated by culturing bone marrow cells from wild type B6 mice at a concentration of $2 \times 10^6$ per mL in IMDM.
with 10% FCS, penicillin, streptomycin, and L-glutamine, and supplemented with GM-CSF for 7 days, then supplemented additionally with IL-4 for 2 more days. DO11.10 T cell blasts were made by making a single-cell suspension from spleens and lymph nodes from DO11.10+/− mice. This suspension was cultured in RPMI (10% FCS, penicillin, streptomycin, and L-glutamine) with 1 µg/mL ova 323-339 peptide. On day 2 following stimulation, cultures were expanded four-fold and supplemented with 10 U/mL human IL-2. To make OT1 T cell blasts, splenocytes from wild type B6 mice, were pulsed for 30 minutes with ova 257-264 peptide, extensively washed, and then combined with lymph node cells from OT1 mice. After two days of culture, cells were expanded and supplemented with 10 U/mL human IL-2. Both DO11.10 and OT1 blasts were used for experiments between 4 and 7 days after activation.

**Plasmids and transfections**

All transfections were performed by electroporation using a BioRad Gene Pulser. shRNA against Septin7 and control shRNA plasmids were used as described (95). Cells transfected with knockdown plasmids for Sept7 were cotransfected with Clontech eGFP-N1 as a marker. For retroviral expression of shRNAs, pSIREN-Retro-Q-ZsGreen1 was used (Clontech). Sept6-GFP consists of the mouse Sept6 sequence with a 22-amino acid linker sequence added by PCR and
cloned into Clontech eGFP-C1. mCherry-GPI was a gift from G.S. Baron (161). Par6b-DN consists of amino acids 102-371 and was subcloned into pMIG-W. The original sequence was a gift from I. Macara (141). Lgl-SA is the mouse Mgl-1 sequence with S to A mutations at positions 650, 654, 658, 662, and 673 (155). It was a gift from P. Brennwald and was subcloned into pMIG-W for expression.

**Mice**

Sept7 floxed mice have loxp sites flanking exon 3 generated directly on the C57/Bl6 background by M. Kinoshita. They were subsequently crossed onto OT1 and CD4-Cre backgrounds (162). Lgl1 floxed mice were made on the 129 background and have loxp sites flanking exon 2. They were a gift from V. Vasioukin (154). These mice were backcrossed onto the C57/Bl6 background and crossed to Lck-Cre and OT1 mice (162). DO11.10 mice (Jackson) used for retroviral transduction are on the Balb/c background.

**Immunofluorescence**

For studies of crawling cells, cells were allowed to adhere to poly-L-lysine or 0.2 µg/mL ICAM-1-coated coverslips for 30 minutes prior to fixation. For IS experiments, T cells and APCs were combined on coverslips and allowed to interact for 30 minutes prior to fixation. For volume change experiments, chambered coverslips (Nunc) were coated with 0.5 ug/mL anti-CD44 antibody
(clone IM7) for 60 minutes and then washed 3 times. Next, cells were suspended in 150 μL serum-deficient RPMI 1640 and allowed to adhere to coated coverslips for 30 minutes. 300 μL volume of either prewarmed deionized water or prewarmed RPMI 1640 was added to each chamber (for hypotonic and isotonic conditions, respectively). After these preparations, prewarmed 16% paraformaldehyde (Electron Microscopy Sciences) was added to each chamber for fixation at a final concentration of 4% for 10 minutes. Following thorough washing with PBS, fixed cells were blocked with 2% donkey serum and 2% fetal calf serum and permeabilized with 0.2% saponin (Sigma) in PBS for 30 min. Cells were incubated with primary antibodies for 120 min in permeabilization buffer, washed, then stained with secondary antibodies in permeabilization buffer for 60 min and washed again. Samples were imaged immediately or stored at 4 degrees overnight for subsequent imaging. Primary antibodies used were: rabbit α-Sept7 (IBL), rabbit α-Lgl1 (raised against full-length mouse Lgl1-GST fusion, Affinity BioReagents), rabbit α-Par6, rabbit α-PKCζ (Santa Cruz), hamster α-CD3 clone 500A2, rabbit α-Pericentrin (Covance). Secondary antibodies were from Jackson Immunoresearch.

Microscopy

Widefield images were acquired on a modified ZeissAxiovert 200M microscope with a plan-neofluor 63X objective (Carl Zeiss). The microscope was fitted with dual excitation and emission filter wheels and a Coolsnap HQ camera (Roper
Scientific). Image acquisition was performed using Metamorph imaging software (Molecular Devices). For motility and mitosis imaging, 0.25% low-melting point agarose was included in the media to minimize drift. Time-lapse images were acquired every 30 seconds for mitosis and hypotonic stress experiments and every 1 or 2.5 seconds for motility experiments.

**Inhibitors**

Nocodazole (Sigma-Aldrich) was used at 5 µM for flow cytometry and imaging experiments and 13 µM for cell cycle synchronization. Taxol was used at 5 µM. Latrunculin B (Sigma-Aldrich) was used at 10 µM. Blebbistatin (racemic, SOURCE) was used at 100 µM. Dynasore (Sigma-Aldrich) was used at 80 µM. Charybdotoxin (EMD) was used at 1 µM and 5-Nitro-2-(3-phenylpropylamino)benzoic Acid (NPPB, from EMD) was used at 100 µM. PKCζ-PS (Biosource) was used at 10 or 25 µM. Nocodazole, Taxol, Latrunculin B, Blebbistatin, and Dynasore stock solutions were in DMSO. Stock solutions of Charybdotoxin, NPPB, and PKCζ-PS were in water.

**Flow cytometry volume change assay**

Cells were suspended in serum-free RPMI 1640 at a concentration of 2e6/mL containing 10 nM SYTOX Red stain (Invitrogen) for dead cell exclusion and incubated at 37 degrees with or without inhibitors at the concentrations indicated
for at least 60 minutes prior to the assay. To monitor the response to osmotic stress, Forward Scatter values were recorded over time, and after 1 minute of recording, cells were transferred to a hypotonic solution of 100 mOsm sucrose, which contained SYTOX Red and inhibitors, as necessary. Samples were kept warm with a water jacket for the length of the 6 minute time course. For analysis, no Forward- or Side-Scatter gates were used. Rather, gating was on SYTOX Red-negative and, when necessary, GFP-positive cells. All experiments were performed on a BD FACSCalibur flow cytometer.

**Cell cycle synchronization**

D10 cells were incubated with 13 µM nocodazole for 16-20 hours, then washed in 15 mL media 4 times to remove the drug. They were then resuspended in a small volume and incubated at 37°C in chambered coverslips for 30 minutes prior to imaging.

**Flow cytometry analysis of organs**

Organs were isolated from mice and suspended in FACS buffer (PBS, 2% FCS, 0.01% Sodium Azide). For surface stains, cells were blocked with 2.4G2 (α-CD16) and stained on ice with α-CD4-FITC and α-CD8-PE (eBioscience). For intracellular staining of Sept7, cells were fixed with 4% PFA, and permeabilized with 0.5% saponin. Cells were then incubated with rabbit α-Sept7, washed 3
times, and stained with goat α-rabbit-Dylight649 (Jackson Immunoresearch), and washed 3 more times prior to FACS analysis.

**In vivo activation**

$10^6$ CFSE-labeled T cells were transferred into B6 CD45.1 recipients. Recipients were immunized with one of two regimens. For BMDC immunized mice, BMDCs were pulsed with the indicated concentration of ova peptide for 30 minutes, then extensively washed. BMDCs were then resuspended in PBS and $5 \times 10^5$ cells were transferred with 5 ng of LPS subcutaneously into each footpad. For DEC205-ova immunized mice, α-DEC205-conjugated ovalbumin protein was. 72 hours after either immunization, draining lymph nodes were harvested and restimulated with 50 µg/mL PMA and 500 µg/mL ionomycin for 4 hours, with 10 µg/mL Brefeldin A (Epicenter) for the final 2 hours. Cells were then fixed, permeabilized, and stained with α-CD45.2-PE to identify transferred cells and α-IFNγ.

**Coupling assay**

T cells and peptide-pulsed APCs were combined in equal numbers in prewarmed RPMI, briefly centrifuged, and incubated for the indicated time. 0.5% PFA was then added and cells were lightly vortexed to break up nonspecific couples. After
washing, samples were stained for T cell and APC markers and analyzed on a FACSCalibur flow cytometer.

**Transmigration assay**

10^6 T cells in serum-free media were placed in the top of transwell chambers (Corning) with 3-µm, 5-µm or 8-µm pores in the presence or absence of chemokine. Transmigration was allowed to proceed at 37 °C for 4 h before cells were collected and counted for a fixed period of time on a BD FACSCalibur flow cytometer.

**In vivo trafficking assay**

Naïve CD8 T cells from Sept7cKO and littermate control mice were labeled with 2 µM CFSE and 10 µM CMTMR (each dye was used on each genotype in at least one experiment.) Cells were washed three times, combined at a 1:1 ratio and 2x10^6 of each cell type was transferred intravenously into B6 CD45.1 recipients. At the indicated time points, mice were sacrificed, organs were stained with α-CD45.1-Alexa647 (BD Pharmingen), and analyzed by flow cytometry.

**Retroviral infections.**

Retroviral plasmids were used in calcium phosphate transfection of Phoenix cells. Virus-containing supernatant from these cells was used on two consecutive
days (days 2 and 3 after activation) to spin-infect DO11.10 T cell blasts. Cells were sorted according to their ZsGreen or GFP expression on day 4 and rested for 24 h before experiments were performed.

**Western blotting**

D10 T cells were lysed in PBS containing 1% Triton X-100 in the presence of a cocktail of protease and phosphatase inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 mM PMSF, 10 mM sodium fluoride, 10 mM iodoacetamide and 1 mM sodium orthovanadate). Equal amounts of cell lysates, as determined with the BioRad detergent-compatible protein assay, were then resolved by SDS-PAGE, and immunoblotting analysis was performed using the antibodies indicated.

**Cytokine skewing**

Cells were activated with 1 µg/mL plate-bound α-CD3(clone 500A2) and 1 µg/mL α-CD28 in the presence of the following cytokines and antibodies for cytokine skewing: for Th1 conditions, 5 ng/mL IL-12 (Immunotools) and 10 µg/mL α-IL4; for Th2 conditions, 50 ng/mL IL-4 (eBioscience) and 10 µg/mL α-IFNγ; for Th17 conditions, 10 ng/mL IL-6 (eBioscience), 5 ng/mL TGF-β (R&D Systems), 10 µg/mL α-IFNγ, and 10 µg/mL α-IL-4. On day 5 following initial activation, cells were restimulated with PMA and ionomycin for 4 hours with brefeldin A present for the final 2 hours. Cells were then fixed with 4% PFA, permeabilized with 0.5% saponin, and stained for FACS analysis.
**Cytotoxicity assay**

Five days after activation Lgl1cKO and control T cells were used in a standard 4-hour $^{51}$Cr release cytotoxicity assay using $^{51}$Cr (PerkinElmer)-labeled EL-4 cells as targets either pulsed with 100 ng/ml ova peptide or left unpulsed. Maximum lysis was determined by 1% SDS lysis of target cells.

**Cell sorting**

Cell sorting was performed on a MoFlow Cell Sorter (DakoCytomation) or a BD FACSAria2 SORP using 70 µm nozzles.

**Analysis and statistics**

All flow cytometry analysis was performed in FlowJo (Treestar Inc.) and the Flowjo kinetics platform was used for osmotic volume change measurements. Images were analyzed in Metamorph (Molecular Devices) or Imaris (Bitplane) depending on the type of analysis. Metamorph was used for 3D reconstructions. Imaris was used for velocity and displacement measurements. Image processing and measurements for extra-nuclear path volume was done in Matlab using Image Processing Toolbox functions (The Mathworks). Mitotic blebbing index consists of the number of blebs observed divided by the product of the time followed (in seconds) and the circumference of the cell (in µm). All statistical analyses were performed in Graphpad Prism and individual tests are named in the figure legends.
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