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Review

Mechanisms of T cell motility and arrest: Deciphering the relationship between intra- and extracellular determinants

Rachel S. Friedman, Jordan Jacobelli, Matthew F. Krummel *

Department of Pathology, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0511, USA

Abstract

T lymphocytes are capable of rapid motility in vitro and in vivo. Upon antigen recognition, they may stop crawling and form a stable cell–cell contact called the 'immunological synapse' (IS). However, it is becoming clear that this outcome may not occur with the reliability that was once presumed. T cells, particularly naïve cells, are apparently triggered partly 'on the fly' during short contacts with peptide–MHC (pMHC) bearing antigen-presenting cells (APCs) and are also influenced in both activity and synapse duration by a multitude of external cues. Underlying the emerging issues is a paucity of data concerning the cell biology of T lymphocytes. Here, we review the molecular mechanisms of crawling and adhesion versus the various potential modes of 'stopping' in T lymphocytes. Both motility and arrest involve similar processes: adhesion, actin elongation and internal tension control, but with different coordination. We will attempt to integrate this with the known and potential external cues that signal for T cell motility versus stopping to form a synapse in vivo. Finally, we discuss how this interplay may give rise to unexpectedly complex motile and morphological behavior.

Keywords: T cell; Motility; Actin; WASP; Myosin; Adhesion; Chemokines; TCR; Integrins

1. Introduction

T lymphocytes transition between motile and sedentary modes depending upon stimuli from their environment. Rapid motility permits them to scan a large number of antigen-presenting cells (APCs) and potentially infected targets, whereas stalling and stopping in response to stimuli may permit them to engage in prolonged signaling and cellular crosstalk.

Amongst the factors influencing the decision between motility and stopping, the T cell receptor (TCR) has received the greatest amount of attention. This is in part because triggering of pre-activated T cells with peptide–MHC (pMHC) complexes in vitro induces these cells to stop [1,2] and form a prolonged contact characterized by concentric arrays of TCR, adhesion receptors, and glycoproteins (termed central-, peripheral- and distal-supramolecular activating clusters (SMACs)) in the contact interface [3,4]. This ordered interface, sometimes referred to as the 'mature immunological synapse' (mature IS) forms over the first minutes of contact, following a dynamic phase termed the immature IS in which these receptors are distributed in smaller clusters. It is actually during this immature phase that tyrosine phosphorylation [5] and intracellular calcium release [6] begins. It is also clear that some effector functions such as CTL lysis of targets can clearly be initiated prior to the formation of a canonical mature IS [7].

Despite an emphasis on the 'stopped' mode as a site of antigen-engagement, there are increasing data showing that T cells can also receive antigenic stimuli during more motile encounters with APCs. For example, T cells in a collagen matrix stop very infrequently and briefly while still effectively getting activated as assessed both by surface markers and by proliferation [8]. More recent two photon scanning laser microscopy (TPSLM) imaging data [9–12] have all shown short-lived encounters with antigen-bearing APCs as a prelude to stable interactions. While it has not been conclusively established that these short-lived encounters permit signaling via the TCR, this is strongly suggested by the observed upregulation of the CD69 activation antigen in the hours preceding stable interactions [9,10].

As a general theme, the activation status of both the APC and the T cell appear to modulate the rate at which contact can trigger the formation of a mature stable synapse in vitro. For example, maturation of dendritic cells (DCs) leads to more frequent and stable T cell interactions when assayed in vitro [13]. Similarly,

* Corresponding author. Tel.: +1 415 514 3130; fax: +1 415 514 3165.
E-mail address: matthew.krummel@ucsf.edu (M.F. Krummel).

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naïve T cells were shown to take approximately 30 min to form a mature IS whereas pre-activated cells do so in less than 5 min [14]. The microenvironment may also play a role as indicated by the differences in experiments performed in vitro versus those in a collagen matrix or in an intact lymph node. While these differences have been observed, it remains unclear whether they derive from microenvironmental cues or are simply a reflection of the source and activation status of the T cells and APCs being studied.

Thus, for T cell behavior in vivo, there remains the question of how microenvironmental cues influence the T cell motility machinery and these signals integrate with TCR stimulation. We will analyze this from the perspective of their biochemical effects upon adhesion, cytoskeleton and motor proteins, and how this might integrate to produce complex interconversions between crawling and stopping.

2. T cell amoeboid movement: the interplay of cytoskeletal polymerization, adhesion and tension

Motile T cells in lymphoid organs and in peripheral tissues are most similar in morphology to neutrophils and to the amoeba *Dictyostelium discoideum*. In shape, they are said to resemble a hand-mirror or frying pan with a large bulky cell body consisting largely of the nucleus, trailed by a pinched handle-shaped tail termed a uropod containing the majority of the cytoplasm, and the microtubule organizing center (MTOC). Time-lapse analysis of T cell movement shows the protrusions called pseudopodia emanating from the leading edge and a consistent absence of protrusions and pinching at the uropod (Fig. 1). Furthermore, in contrast to many epithelial cells that move with their MTOCs closer to the leading edge relative to the nucleus, lymphocytes reverse this order and lead with the nucleus, until the onset of synapse formation. Movement in lymphocytes, as in many other cells, is modeled on three fundamental processes: (1) actin protrusion and elongation leading to expansion of a membrane border; (2) regulated adhesion along the cell surface to provide tethering spots for actin extension; and (3) regulated intracellular tension between the adhesive points, typically generated via class II myosin motor proteins. These are modeled in Fig. 2 as a means to orient the further discussion. While these can be separated in some cases, it is becoming evident that molecules involved in regulating one antagonize or promote another—thus, coordinating crawling.

One notable aspect of T cell motility is that in many settings, cells interconvert in a cyclical way between an amoeboid, motile form and a round non-polarized and relatively immobile form [15]. The basis for this is not yet clear but may represent an internal timer or possibly a weak ongoing response to an external ‘stop’ signal.

3. Actin protrusions underlying lymphocyte motility

The fundamentals of lymphocyte movement can be thought of as beginning with actin-based protrusion. Growth of actin filaments has been highly associated with the small GTPases of the Rho/Rac/Cdc42 family. These proteins are unfolded and

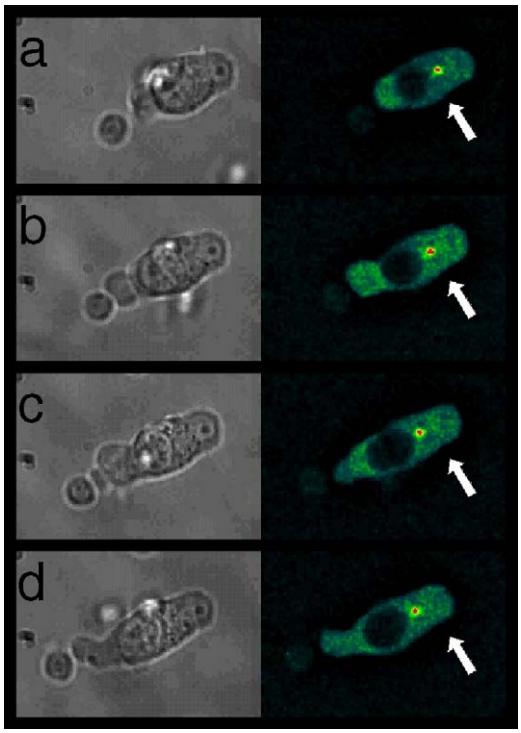

Fig. 1. Morphological dynamics, MTOC and nuclear positioning during T cell crawling. D10 T cell clones were transfected with tubulin-GFP and allowed to crawl on glass coverslips in media containing FCS. DIC and fluorescence images (a pseudocolor scale green–yellow–red) are shown at 1 min intervals. White arrow indicates starting position of the MTOC. Nucleus is round area in front of MTOC and having less fluorescence. Full movie available as Supplementary data, movie 1.

active in their GTP-bound state and assume a closed conformation when GTP is hydrolyzed to GDP. While an exposed domain in the active conformation can bind and stimulate a variety of proteins, the closed conformation typically fails to do so. In lymphocytes as well as in a majority of motile cells Rac and Cdc42, when active, tend to favor membrane protrusions while the Rho subfamily of proteins appear to oppose these.

3.1. Growth of the leading edge via Rac and Cdc42

Longstanding data on motility in *D. discoideum* demonstrates that Rac controls leading edge formations. There is clearly unregulated pseudopod extension in these cells when Rac is overactive and poor motility and an absence of the leading edge characterizes cells expressing a dominant negative form of Rac [16]. In T cells [17] as well as neutrophils [18], activated Rac also leads to increased cell spreading and pseudopodal projections. Biosensors expressed in motile fibroblasts reveal that activated Rac is more plentiful at leading, relative to trailing edges [19], consistent with this role.

One activator of Rac in T cells is the guanine nucleotide exchange factor (GEF), Tiam-1. This GEF was originally identified on the basis of a screen for gene products that promoted T lymphocyte invasion and metastasis. Tiam-1 was subsequently shown to activate Rac (reviewed in [20]). Tiam-1 overexpression

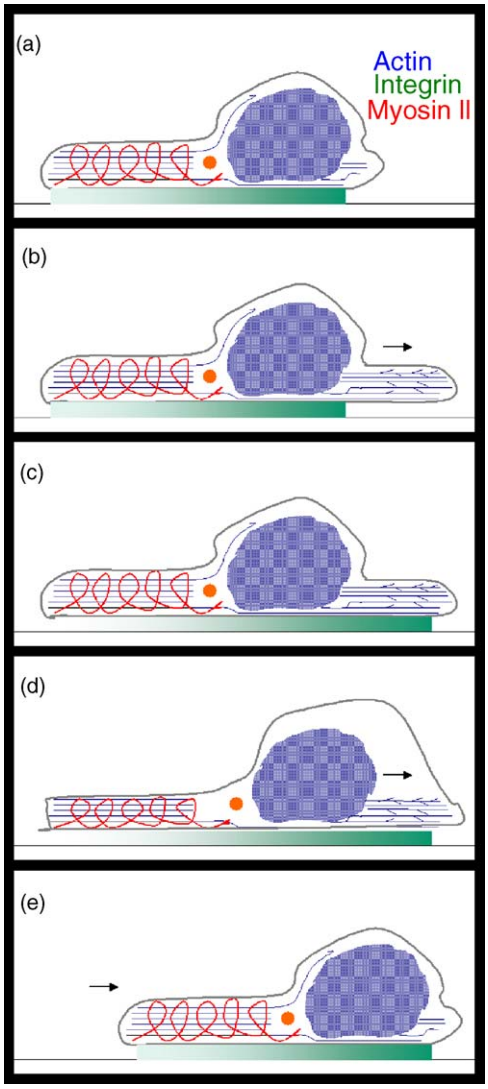

Fig. 2. Mechanistic aspects of T cell motility. A ratcheting model for motility requires: (a–b) controlled actin protrusion, (b–c) spatially restricted integrin activation, and (c–e) controlled Myosin II-mediated local tension between adhesion sites and front to rear. Arrows represents critical spatial control for each of these processes. Blue lines are actin fibers; red represents myosin, here denoted as a coil; orange dot represents the MTOC; and the density of green underneath the cell denotes the level of adhesiveness at this position. The cartoon is based on crawling on a two-dimensional planar surface but the same principle applies to amoeboid motility in 3D.

in N1E-115 neuroblastomas results in Rac dependent increases in both cell spreading and neurite outgrowth and the inability to retract neurites in response to lysophosphatidic acid [21]. Tiam-1 is primarily localized to the leading edge of normal T cells, consistent with a role in directing Rac function to the leading edge. Although, indirect evidence showing inhibition of T cell polarization by overexpressed Tiam-1 [22] would suggest a possible requirement for motility, this has not been directly assessed. Amongst the other Rac GEFs, Vav is clearly implicated in functioning during synapse establishment [23,24] but its role during motile behavior is less clear.

A related GTPase, Cdc42, shares many upstream GEFs and downstream targets with Rac and is also located at sites of protrusions [25]. However, at least as assessed in vitro, its function in motile and stationary cells may be quite different. Indeed, increasing evidence is arising to suggest Cdc42 as a compass for cellular polarity in both amoeboid and epithelial cells. In yeast, expression of a constitutively active allele of Cdc42 in unpolarized cells can lead to spontaneous polarized localization of Cdc42 and establishment of a polarized morphology. This is in part mediated by directional trafficking of Cdc42 itself using the yeast Myo5 homologue [26]. Since this property is unique to Cdc42, it has been suggested to represent a self-feeding loop in which small accumulations of Cdc42 can dictate the membrane localization of future pools.

In epithelial cells Cdc42 plays a critical, although indirect, role in establishing MTOC positioning relative to the nucleus [27]. Intriguingly, this is primarily a result of Cdc42 requirement for nuclear positioning—dominant negative Cdc42 has little effect on MTOC positioning per se, but has dramatic effects on nuclear movement, thus, swapping the position of these two structures [27]. To this extent, the observation that dominant negative Cdc42 inhibits chemokine-mediated chemotaxis [22] might well be attributed to a failure to properly orient to the gradient rather than inability to extend the cytoskeleton. During IS assembly, regulated Cdc42 function in T cells is clearly downstream of TCR and upstream of MTOC reorientation—either overactive or dominant negative forms block the reorientation [28].

Cdc42 may, thus, play a directional role in a way that Rac perhaps does not. Interestingly Rac activation can result from Cdc42 activation, making one event capable of triggering the other and suggesting that Rac effects may in some case be a subset of Cdc42 functions [29].

3.2. Downstream effectors of Cdc42 and Rac at the leading edge

Both Rac and Cdc42 are upstream activators of Wiskott–Aldrich syndrome protein (WASP) family of proteins via the interactions of GTP-bound Rac and Cdc42 with Rho-binding domains (RBD). The activation of the Wiskott–Aldrich syndrome protein and its related homologues N-WASP and the Wave/Scar family results in the de-repression of Arp2/3 binding domains and subsequent actin polymerization. This is likely the basis for Rac/Cdc42 activation/modulation of leading edge behavior.

WASP$^{-/-}$T cells do not polymerize actin [30] or cap their TCRs [31] in response to immobilized anti-CD3 and it is reported that WASP$^{-/-}$T cells fail to assemble a c-SMAC [32,33]. It is possible that this phenotype somewhat simplifies the defect in WASP$^{-/-}$T cells as the most dramatic defect in the context of APCs lies in the frequency of actual strongly attached T–APC couples (reduced from 80% to 10% in one study [32]). In RBL-2H3 leukemia cells, specific recruitment of WASP to a surface near a bead resulted in unique membrane protrusions that began to engulf the attached bead [34]. These differed from the elongated protrusions that occurred perpendicular to a recruited Cdc42 allele, suggesting that WASP may be a more precise mechanism of membrane control, suited to

generating an engulfing membrane to capture effectors. Thus, it is perhaps a failure of the T cell surface to sense and conform to the APC, perhaps just at the onset TCR engagement, that is most defective in WASP$^{-/-}$cells rather than in the molecular recruitment of proteins into the central zone of a defective contact. In this light, the ability of a leading edge to wrap around a surface may be seen as a unique form of projection and perhaps one mediated most by WASP.

Recent studies have also suggested that WASP$^{-/-}$T cells show marginally reduced homing into lymph nodes and spleen and show defective chemotaxis to CCL19 [35]. These effects are much less severe than, for example, homing of integrin deficient cells. Indeed the defect in migration to lymph nodes may follow the mold posed above as it might result from defective cell–cell adhesions normally mediated in response to chemokine or other surface molecule signaling. A small collection of chemokines might also modulate WASP independently of Rac/Cdc42, perhaps by direct phosphorylation [36]. It is believed that in non-stimulated T cells WASP is complexed together with WIP, an interaction which prevents its association with and activation by Cdc42 [37]. Ultimately WASP may play a more important part in the fine-tuning of leading edge morphologies rather than in motility-based leading edge behavior.

In contrast, the WASP-related Cdc42/Rac effectors, N-WASP and Scar/Wave, are likely to play prominent roles in leading edge behavior in T lymphocytes. Like WASP, N-WASP and Wave unfold upon Cdc42 or Rac binding to reveal an Arp2/3 binding motif. Injection of anti-N-WASP antibodies into fibroblasts prevents filopodia formation, for example [38]. However, it is unlikely that N-WASP is unique in the ability to generate filopodia, as embryonic stem-cells from N-WASP deficient cells are nevertheless capable of forming filopodia and lamellopodia [39]. Also, short hairpin RNA-mediated knockdown of the single Scar protein in *Drosophila* also results in reduced lamellopodia and filopodia formation. Similarly, the migration of neutrophils on integrins in the presence of fMLP was inhibited by blocking peptides against Scar but much less so by peptides that compete for binding with WASP [40]. At present, it is unknown which of the N-WASP and Scar/Wave players are responsible for the pseudopodial projections in T cells.

Beyond WASP, two other mechanisms arise for Rac/Cdc42 activation of leading edge behavior. First, Cdc42/Rac RBD activation of the P21 kinase Pak results in upregulation of the kinase activity and ultimately results in actin modulation. Second, Rac activation is frequently closely correlated with local production of PI(3,4,5)P3. There is no existing evidence that Rac activation directly stimulates PI3-kinase activation. However, it would appear that once locally activated PIP3 and Rac colocalization is tightly reinforced, perhaps via a feedback loop that may help polarize protrusive activities [41]. One attractive player for this synergy is the P-Rex family of Rac GEFs [42]—the GEF activity of these proteins are activated by PIP3 in combination with Ras activation and the P-Rex family is thought to act as coincidence detectors for the presence of both. In addition, Tiam-1 and Rac itself are PIP3 sensitive as a result of a PH domain that directs its localization to membranes bearing these lipids.

3.3. Inhibition of Rac/Cdc42 by Rho and the formation of the uropod

No discussion of Rac and Cdc42 in motility is complete without considering the antagonistic relationship of Rac/Cdc42 and Rho. At the biochemical level, this latter relationship can be demonstrated by introducing the active form of Rac and measuring reciprocal decreases in cellular Rho activation [43]. At the cellular level, while Rac and Cdc42 localize to the leading edge in multiple cell types, Rho localizes to the trailing edge in neutrophils [18].

Activation of Rho prevents the formation of leading edges in neuroblastomas, even those initiated by overexpression of Tiam-1 [21]. Conversely, inhibition of the major Rho effector, Rho kinase (ROCK) and in monocytes results in multiple protrusions [44]. In T lymphocytes, interfering with RhoA prevents detachment of the trailing edge and also reduces the rate of migration [45,46]. Neutrophils provide one of the most direct examples of cross inhibition by Rho and Rac—Bourne and colleagues have described these molecules functioning in concert to determine ‘frontness’ and ‘backness’. In this model, ‘frontness’ (the making of a leading edge) is enhanced by Rac and inhibited by Rho, while the making of a round non-protrusive surface (‘backness’) is enhanced by constitutively active Rho and blocked by active Rac [18]. These signals apparently feedback on one another although the molecular nature of this feedback effect is still unclear.

The net result of Rho activation (or Rac inactivation) is the suppression of membrane extension. This is ultimately a necessary feature of a uropod—a structure from which membrane protrusions are never observed. While this is partly due to an absence of actin elongation initiated by Rac/Cdc42-like effectors, it is also a result of the effects of Rho kinase on both integrin affinity and cellular tension via myosins and/or ERMs as discussed below.

4. Regulated adhesive contacts

Localized cell adhesion and attachment of membrane proteins to the cytoskeleton is thought to allow actin extensions to be used as adhesive points for pushing/pulling, and thus, completing translation of the cell body. For this to occur, the affinity of the leading edge of lymphocytes and their trailing uropods needs to be regulated differentially. Although the adhesion receptor that takes on the differential adhesive role in vivo is unknown, it is widely speculated that integrins, particularly LFA-1 must play this role for T lymphocytes. In vitro, human T cells that normally do not migrate on glass substrates can be induced to migrate when plated on immobilized ICAM, showing that differential ICAM/LFA-1 interactions and/or LFA-1 signaling may be sufficient to permit amoeboid movement [45,47,48]. Furthermore, T cells that cannot deactivate LFA-1 due to mutations in the α-chain cytoplasmic domain are essentially unable to crawl [49]. LFA-1 ligands are widely present in the lymphoid environment including on DCs, B cells, and other neighboring T cells. Beyond LFA-1, other integrin receptors (VLA-4/α4β1) are present on selected cells and surfaces in the lymph node.

Both LFA-1 and α4β1 are known to be required for B cell entry into marginal zone in the spleen [50] and a similar multiplex requirement may be in place for the complete spectrum of T cell motile behaviors, particularly when considering non-lymphoid tissue sites.

4.1. Determinants of local integrin adhesion during dynamic movement

Integrins and LFA-1 in particular are clearly a component of the adhesive structure that constitutes the immunological synapse. This likely results from TCR activation of protein-kinase C (PKC) isoforms and PI3-kinase that signal for integrin affinity upregulation. This process is thought to highly utilize talin [4] which directly binds to and modulates integrin tails [51]. For T cell motility using LFA-1, talin mediated adhesion mechanisms are possible, however numerous other proteins bind to and modulate integrin tails. In the case of motility, the already complex regulation of global integrin affinity and avidity is increased by the need to differentially regulate affinity locally at specific sites on the cell surface. This is clearly demonstrated by the absence of crawling on ICAM-1 bilayers when LFA-1 is artificially kept in an open conformation [2].

In the case of crawling cells, a clear line of evidence connects Rho activation at the uropod to integrin deactivation. For T cells, Rho kinase at the trailing edge deactivates integrins, as blocking of ROCK results not only in extended spreading but also increased integrin activation. This is suggested to result from a requirement for ROCK to deactivate Pyk2 and Paxillin activation—both of which are otherwise associated with integrin activation [44]. In this way, de-adhesion at the trailing edge is clearly linked to ROCK and indeed, ROCK inhibition gives rise to cells with extended uropodal projections probably due to their ability to de-adhere [45].

Leading edge integrin adhesion may also occur via an additional Rac-like GTPase called Rap1. Rap1 is known to be activated by TCR engagement after which it localizes to the IS [52]. Additionally, it has recently been shown to be activated by leading edge chemokines as well [53]. Importantly, an activated form of Rap1 enhances LFA-1 clustering and adhesion and induces fast motility on ICAM/VCAM [52,53]. These effects appear to occur by Rap1 direct activation of RapL which directly binds to integrins to activate them [54]. Notably, the knockout of RapL show defects in T cell migration into secondary lymph nodes confirming its critical role in modulating this process [55].

5. Regulated intracellular tension and protrusive forces

Integrins provide the most likely source of local attachments to adjacent cells, the reticular network and extracellular matrix (ECM). A source of tension mediated by motor proteins and perhaps the Ezrin–Radixin–Moesin (ERM) proteins is then required to translate contacts into changes in cell shape and translation of the cell body. There is also feedback with actin elongation and integrin affinity here as Rho affects myosin motor function and ERM proteins in the uropod via ROCK. Additionally, this increased tension may give rise to a bias against receiving TCR signals in the uropod [1,56] despite the fact that a majority of the T cell receptor is concentrated in this relatively small patch of membrane [6].

5.1. Myosin II as a protrusive engine

As in most motile cells [57], one player mediating crawling on adhesive surfaces in T cells is active Myosin II. T cells predominantly express a single Myosin II isoform called non-muscle myosin heavy chain IIA (NMMHC-IIA) or MyH9 [58]. Interference with active Myosin IIA, either through restriction of the phosphorylation of the light chains [45], shRNA-mediated loss of the heavy chain, or drug inhibition [58] result in T cells with significantly diminished or absent motility. Notably, shRNA or drug-mediated inhibition both give rise to cells lacking a defined uropod suggesting that this structure in particular requires motor-mediated tension to exist. This is consistent with the posterior localization of Myosin II during chemotaxis in *D. discoideum* [59] and in motile T cells [58]. Inhibition of Myosin IIA, nonetheless, permits transient protrusions such as are generated suggesting that actin polymerization dependent leading edge extension does not strictly require tension but that translation of the cell body does require this function.

5.2. Spatial control of Myosin II activity to coincide with sites of binding and signaling

Myosin II motors are modulated by regulatory light chains and heavy chain phosphorylation. Myosin light chains (MLC) are activated by phosphorylation and the known kinases mediating this include two associated with the leading edge: myosin light chain kinase (MLCK) and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) [60] and two associated with the uropod: Rho kinase and the Rho-sensitive citron kinase [61]. Blockade of the calcium-sensitive MLCK using the drug ML-7 demonstrated that this is required for the initiation of adhesive contacts. Additionally, treatment of already adhered cells shows that MLCK is required for continued pseudopodal extension [45]. In contrast, Rho kinase, another kinase for myosin light chains appears to be required more for uropodal functions as treatment with ROCK inhibitors results in an inability to release the uropod and subsequently very long extensions and poor motility [45]. The functions for the remaining light chain kinases and existence of other players are as yet undiscovered.

During synapse formation, Myosin IIA/MyH9 is phosphorylated at protein-kinase C sites in the heavy chain [58]. This phosphorylation occurs as a result of increased calcium levels induced by TCR stimulation. Evidence is now clear that heavy chain phosphorylation of Myosin IIA/MyH9 at either the PKC (within ACD of coil–coil and mts binding site) or casein-kinase 2 (CK2—just outside of these) sites can significantly reduce filament formation [62]. Such a reduction leads to the loss of cortical tension provided by the actin cytoskeleton [63–65]. A similar global loss of cytoskeletal tension is likely to be responsible for the loss of the uropodal integrity as well as allowing Myosin IIA and possibly other proteins to translocate from the former uropod toward the synapse. Notably, phosphorylation on

the CK2 site in particular prevents binding of the S100 protein, mts1. This small protein is frequently overexpressed in highly motile cells [66] including lymphocytes and tumor cells where it confers metastatic potential [67,68]. At present, it is unclear whether phosphorylation of filaments at distinct cellular sites might also be a mechanism to regulate their contractile capacity during crawling. It is notable, however, that in PC12 cells, Tiam-1 overexpression or bradykinin (a Rac activator) induce phosphorylation of Myosin II isoforms [69], suggesting that these players also regulate local tension via controlling filament formation.

5.3. ERM attachment of membrane proteins to the cytoskeleton and control of cell shape

A major integrator of cell surface morphology and cytoskeletal rearrangements is the Ezrin–Radixin–Moesin family. The canonical 4.1-ERM (FERM) domain present in these proteins (as well as in talin and in Myosin X, for example) is capable of binding highly conserved positively charged sequences on transmembrane proteins in their cytoplasmic domains while alternative domains in ERM proteins can bind to actin. Whereas c-terminal phosphorylated ERMs (cp-ERMs) are competent to bind to transmembrane proteins, the dephosphorylated forms are not. In T cells, ERMs reside primarily in the uropod during motile behavior and are described as providing rigidity to cells, primarily on the basis of studies in which ERM binding is disrupted by overexpression of FERM domains [70].

Intriguingly, ERMs and Rho form a feedback loop of their own. In L cells: maintenance of the phosphorylated cp-ERM state required active Rho [71]. In addition, ERMs oppose the actions of Rho proteins. For example a *Drosophila* epithelial line becomes highly motile and invasive when deficient in Moesin and the phenotype is reverted by halving the dose of Rho [72]. Notably, the moesin-deficient invasive phenotype of this line is mimicked by overexpression of Rho [72]. While an invasive phenotype caused by Rho may appear counterintuitive in light of evidence suggesting it prevents leading edge formation, it must be remembered that Rho is also utilized to maintain integrin adhesions and this loss of adhesiveness combined with a loss of polarity is likely to be the cause of faulty cell–cell contacts in this setting.

TCR signaling in T cells induces ERM dephosphorylation via Vav1, which is a Rac activator [70] and may, therefore, antagonize Rho function. The kinase/phosphatase pair that regulates ERM phosphorylation remains unknown, and therefore, the direct relationship between Rac and ERMs is not clear. It also remains to be determined if a similar (perhaps Tiam) induced Rac activation/Rho deactivation may reduce rigidity at leading edges during motility. In sum, Rho acting via the uropod may play a direct role not only in limiting actin protrusion via Myosin II but also in ‘tightening’ the ‘skin’ of the uropod cytoskeleton by promoting cp-ERMs and thereby permit tension to be generated for crawling. The localization of ERMs in the uropod of motile cells [22,73] is consistent with such a model.

6. Signaling for T cell motility and stopping

Crawling T cells within a lymph node are subject to perturbation of the crawling machinery as a result of multiple signaling mechanisms.

6.1. TCR and the calcium signal

TCR signaling alters the dynamics of all three of the major aspects of motility. On the one hand, TCR-induced WASP activation, presumably through leading edge activations of Vav and Rac/Cdc42 alter the dynamics of actin assembly at this site, so that rather than being purely protrusive, the leading edge now acts to engulf the triggering APC. In addition, TCR signaling induces a strong upregulation of LFA-1 activity—presumably globally through mediators such as DAG/Ca2+ and their activation of PKC, and also perhaps locally at the synapse via such players as Rap1/RapL [52]. Finally, strong calcium influx can induce phosphorylation of the Myosin II heavy chain via an unknown kinase leading to filament disassembly and the loss of the uropod.

Calcium influx is, at present, the strongest candidate to mediate the TCR-induced ‘stop’ signal. High intracellular calcium levels are strongly correlated with cell rounding and lack of motility in lymphocytes in vitro [1,74] and in thymocytes [75] in vivo. As discussed above, this calcium rise has clear effects on Myosin II phosphorylation [58] explaining cell rounding as well as lack of motility but perhaps not the tight interface formation that characterizes normal IS formation. It has also been reported that calcium-insensitive aspects of TCR-induced ‘stopping’ can be observed when integrins are artificially locked into an open position [2], giving rise to the possibility that stopping can also occur as a result of blocking integrin de-adhesion. The phenotype of integrin-only mediated stopping is not clear but may be akin to a ‘tethered contact’ (see below). The reality of the synapse is that both integrin and myosin modulation as well as Cdc42 alterations are likely to be necessary to achieve a full mature IS.

6.2. Chemokine signaling

T cells are responsive to a variety of lymphoid chemokines including lymph node CCR7 ligands (SLC/CCL21 and ELC/CCL19) and CXCR4 ligands (SDF-1α). In vitro, soluble gradients across a porous membrane can give rise to directional migration, though chemotaxis rates are rarely as good as in other systems such as bacterial chemotaxis.

Chemokine induced chemotaxis occurs through G-protein coupled receptors that function in part through Rho activation—administration of chemokine results in nucleotide exchange on Rho within seconds [76]. When Rho kinase is blocked, the resulting cells which lack uropods are unable to chemotax [73]. However, chemotaxis is not as simple as a Rho-only event as one chemokine, SDF-1α, appears to also activate Cdc42 [77]. Such Cdc42 activation may occur indirectly via integrin signaling during directional migration in other systems, for example, as has been observed in astrocyte chemotaxis [78]. A

dual requirement for Rho control as well as Cdc42 activation is consistent with requirements for both leading edge protrusions and uropodal tension to produce bona fide migration.

Chemokines also trigger upregulation of LFA-1 affinity, which requires the activation of Rho as well as an atypical PKC isoform [79]. Such atypical PKC isoforms rely on membrane targeting for their function and are also associated with the 'partitioning' (Par) proteins in a number of species [80]. This raises the possibility that similar molecules are required for effective chemokine signaling in T cells. Additionally, chemokine-mediated adhesion to low-density but not high-density ICAM-bearing surfaces appears to require PI3-kinase function, suggesting that multiple pathways are involved in chemokine-mediated adhesions [81]. Notably, chemokine induced adhesion on its own is transient, on the order of 5 min [82], perhaps allowing for the cell to rapidly redirect its attention to other stimuli after responding briefly to the first.

One of the most timely developments in the study of chemokine regulated adhesion is the demonstration by Shamri et al. [83] that surface immobilized chemokines have more profound effects as compared with the same chemokines in soluble form. In a recent study, this group demonstrated that immobilized chemokine triggers localized LFA-1 extension and suggest that ICAM ligands bound to the same surface are uniquely capable of capturing and stabilizing the upregulated integrin. This is particularly intriguing for the T cell chemokines with respect to motility in the lymph node as it suggests that surface-bound chemokines might function via highly localized 'spot' adhesions to direct pulling. In such a model, depicted in Fig. 3, leading edge protrusions in T lymphocytes would encounter chemokine signals, inducing activation of localized integrin patches on the triggering cell. Over time, this adhesion would end up in the uropod as new protrusions are formed on adjacent membrane as myosin tension in the uropod ratchets the cell body forward. Engagement of even higher chemokine levels on another cell might then induce a new leading edge adhesion and allow the cell to pull away from the first patch (Fig. 3b–e), whereas contact with a surface bearing lower levels of chemokine may not induce a strong contact and subsequent movement in that direction (Fig. 3f–i). The appealing aspects of this model are that it generally induces movement up a chemokine gradient as is observed in B cell movement toward the T–B border in vivo [84]. In addition, such a mechanism explains why T cell movement in the T cell zone may appear random over shorter times [11]. Since chemokine-bearing cells may diffuse away from the source of the chemokine in diverse ways, the chemokine gradient is always being 'stirred' by the orthogonal movement of the very chemokine-bearing cells that are being used to for T cell motion.

6.3. Competitive actions of chemotactic and antigen-receptor signaling

As both chemokines and TCR ligands are presented in the lymph node, Bromley et al. [85] have investigated whether the two signals might compete at the level of T cell motility. These ligands were competed with one another in a cell-free setting by using porous membranes coated with ICAM and TCR ligands, and a chemokine gradient across the membrane. Notably, a majority of chemokines tested did not prevent cells from stopping and adhering on the membrane as a result of TCR stimulation. However, a select group including both CCR7 ligands (SLC/CCL21 and ELC/CCL19) as well as one CXCR3 ligand (IP-10/CXCL10) continued to induce transmigration even through a TCR presenting surface showing a potential dominance of these signals over TCR-induced 'stopping'. In an alternative experimental system, TCR stimulation was shown to inhibit chemotaxis to SDF-1 (CXCL12) gradients [86] suggesting that TCR-induced stopping could supercede chemotaxis in this case. In sum, depending on the system under study, chemokine-mediated motility does indeed compete with stop signals in some contexts.

The result of Alon and co-workers comparing immobilized and soluble chemokine signals raises the possibility that the 'competition' might be altered by surface presentation of chemokines by APCs and that perhaps chemokine-mediated adhesion might facilitate TCR-induced activation. Comparisons of IS formed on supported lipid bilayers containing peptide–MHC complexes and ICAM in the presence or absence of immobilized chemokines have shown that, while the integrin density at the contacts are unaltered and the ability of TCR to stimulate a mature immunological synapse is unchanged, the presence of chemokines permits more contacts to form [87]. Emerging evidence is demonstrating that CCR5, CXCR4 and CCR7 ligands costimulate T cell activation [88,89]. Together, this raises the issue of how these signals may interact during normal cell encounters to alter motility and adhesive behavior in response to the APC.

One unique area in which chemokines might influence T cell reactivity lies in the formation of small microvilli on the surface of T cells. These small protrusions are the first point of contact between T cells and other surfaces and their membranes are composed of unique contents relative to the planar membrane of the cell (the planar body). For example, CD4 and CD8 are apparently uniquely localized to microvilli whereas CD3 is on microvilli but also along the planar body [90]. Such localization matters, for example, selective exclusion of L-selectin from microvilli by fusion of its ectodomain to the CD44 transmembrane/intracellular domains considerably inhibits its ability to bind and mediate contact [91]. Notably, cp-ERMs localize to microvilli (perhaps mediating their existence) and chemokine signaling can induce resorbtion of the microvilli through a mechanism that involved ERM dephosphorylation [92].

6.4. Oxygen and energy sensing?

In vivo imaging of T cells in lymph nodes under a cover slip has suggested that basal motility is sensitive to oxygen levels [15]. In Fig. 4, we present data obtained using TPSLM on a lymph node either in the absence or presence of perfused oxygen. Perfusion and temperature were maintained identically in this experiment and the data clearly shows dramatically increased track lengths (Fig. 4a versus b) as well as accelerated cellular velocities (Fig. 4c versus d) in the presence of oxygen.

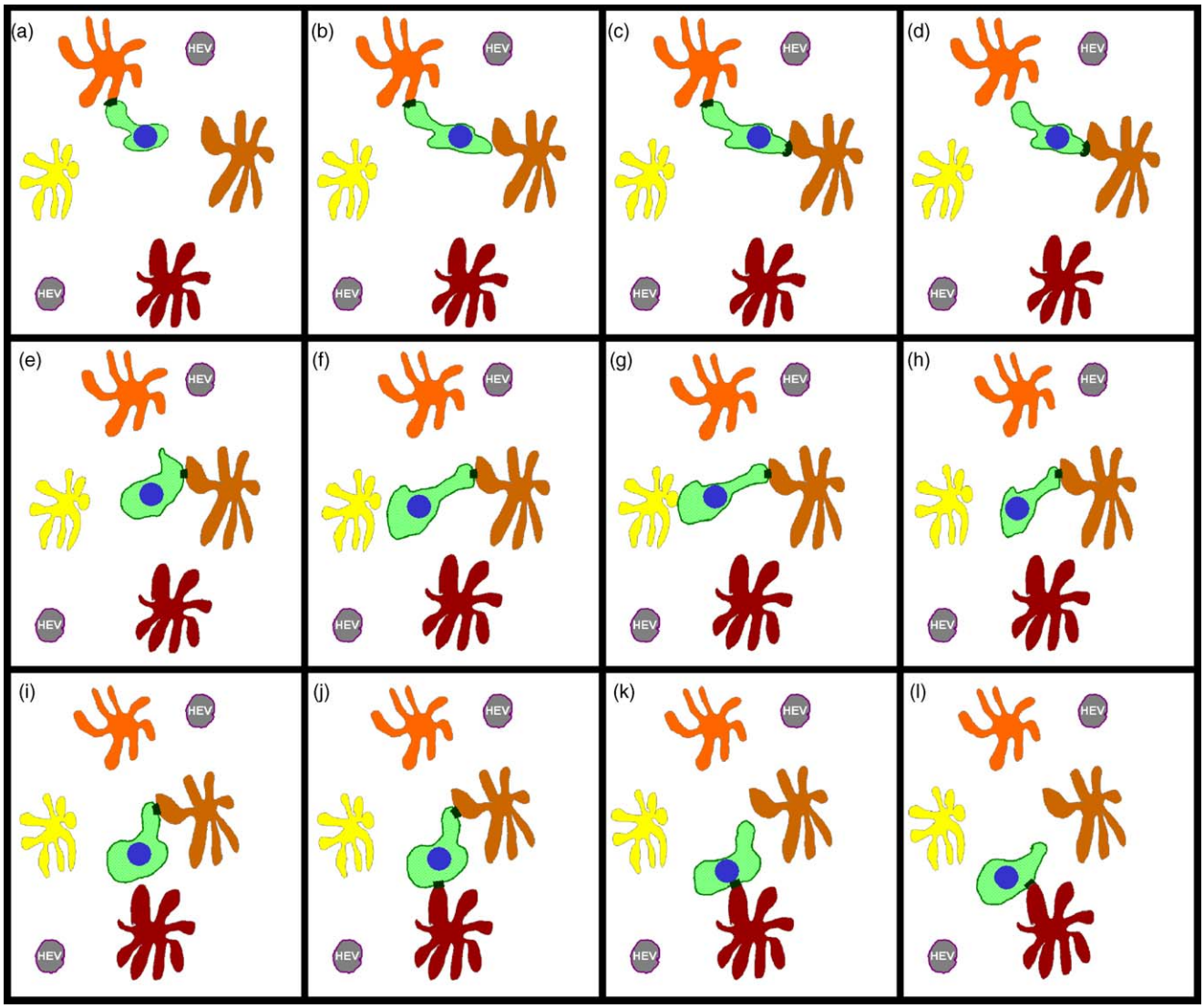

Fig. 3. Motility on immobilized chemokine-bearing surfaces via competitive cellular adhesions. Immobilized chemokines with direct effects on integrin adhesiveness may permit competitive cellular adhesions by presentation of these chemokines. This is shown in schematic for a T cell scanning a series of dendritic cells. The surface loading of chemokine is indicated by the DC coloration, with darker DCs bearing more chemokine. Integrin adhesive contacts are indicated by a dark green patch at the T cell-DC contact. In (a–d) and (i–l), contact with an APC bearing more chemokine gives rise to a transfer of the cell body from one cell contact to another. In (e–h), a DC bearing lower levels of chemokine is encountered and no transfer of adhesive site occurs. Supplementary data, animation available as movie 2.

This raises the possibility of distinct oxygen sensation in lymphocytes. A haem-containing guanylate cyclase homologue has been found in *C. elegans* and this permits worms to sense and chemotax towards distinct oxygen levels, for example, in soil. The guanylate cyclase and an associated ion channel are thereby responsible for social behavior as well as perhaps the depth of localization of the worm in soil [93]. Homologues of this gene are not clearly present in the mammalian genomes but the observed oxygen dependence of general motility in lymph nodes as well as evidence of HEV binding of T cells in lymph nodes [9] might suggest a related mechanism to guide T cells to and around vasculature. Notably, the cGMP pathway in worms detects an optimum level of oxygen—both higher and lower oxygen levels produce suboptimal migration. Such carefully tuned oxygen sensing mechanisms might also help T cells to know whether they were in the blood stream (higher oxygen tension—at which point they also round up) or isolated in lymphoid compartments (intermediate levels at which they crawl) or perhaps deep in target tissue (where rapid crawling might once again be less favored).

7. Complex interactions in the milieu

The signals from antigen receptors, chemokines, integrin activation events and perhaps gas/energy sensing mechanism obviously do not work in isolation and this raises the interesting possibility of crosstalk between these mechanisms that alter the fundamentals of ‘stopping’. In some cases, crawling cells might thereby be restrained (by stable integrin activation patches at the site of a contact) though still attempting to crawl (a ‘tether’)

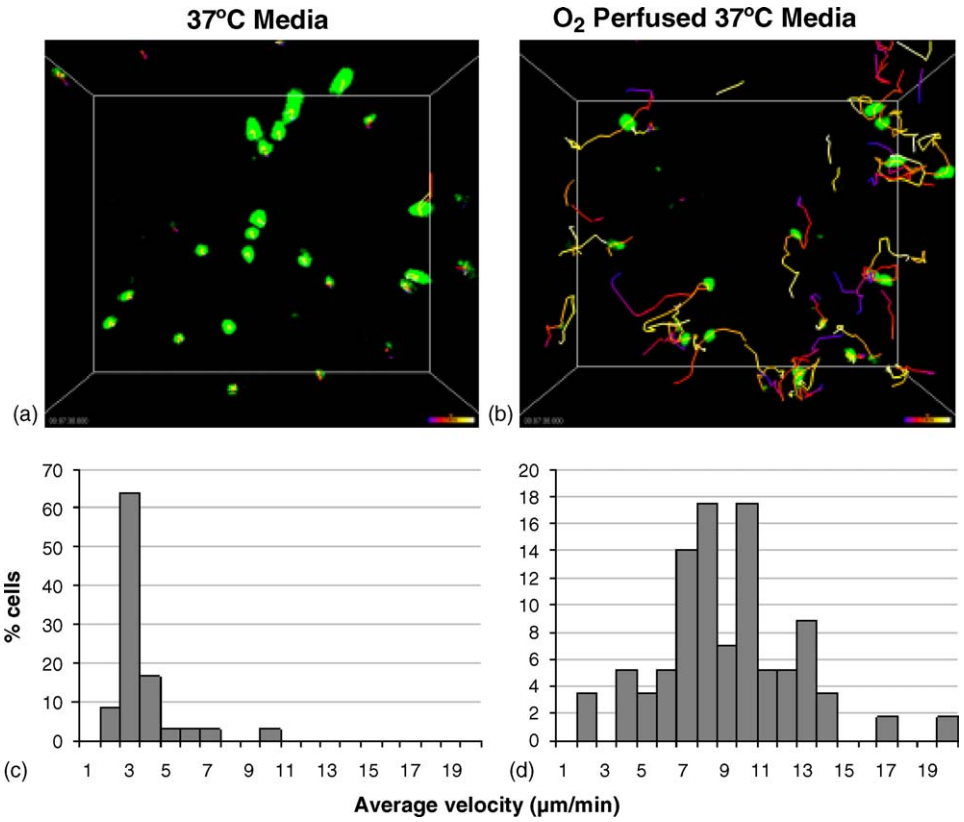

Fig. 4. Oxygen-dependent motility of T cells in isolated lymph nodes. T cells were labeled with CFSE and transferred to recipient mice. Inguinal lymph nodes were isolated and perfused with RPMI alone (a) or with RPMI saturated with bubbling 95% O2 (b) and subjected to TPSLM analysis. In regions imaged at identical depths within the lymph node, T cells were tracked in three-dimensions over time. Tracks (colored lines in (a) and (b)) were used to extract velocity information as shown in (c–d) and show dramatically increased velocities in the presence of high oxygen concentrations. Supplementary data, movie 3 shows the dynamics of cells in these differing environments.

or might round up due to calcium influx despite highly adhesive membrane patches that might otherwise guide chemotaxis. Examples of some of these behaviors for T cells in lymph nodes have previously been described [11] and those discussed below are shown in Supplementary data, movie 4. Here we explore these behaviors in some greater detail and speculate as to their possible biochemical causes.

7.1. Canonical "immunological synapse" stopping

As discussed previously and presented in Fig. 5a, conventional TCR engagement in the absence of chemokine signaling gives rise to an immunological synapse through a continuous series of stopping events. This starts when TCR signaling induces high calcium signaling, localized integrin activation at the IS and a loss of uropodal integrity and motility. Ultimately, receptors are recruited to the flattened contact site where they assume central-, peripheral-, distal-, or mixed-SMAC patterns.

7.2. T cell "drive by" behavior

TCR triggering, particularly suboptimal and especially in naïve cells, may be insufficient to turn off motility. Thus, although mild adhesion may be generated and a slight increase in dwell time on the APC may result, the cell ultimately continues to form leading edge protrusions capable of grabbing new partners while the uropodal-based myosin network continues to pump the cell forward (Fig. 5b). These short contacts may, nonetheless, stimulate the cell to some degree and ultimately second messengers may accumulate during each engagement such that full-synapses can ultimately be formed—particularly on 'sticky', chemokine rich or otherwise highly costimulatory activated APCs. Examples of TCR mediated encounters that fail to induce rounding up and stopping consistent with such a mechanism have clearly been observed in collagen matrixes in vitro [8] and probably also during the first hours of lymph node priming in vivo [9–12].

7.3. T cell "stalling"

Crawling requires a precisely tuned level of a number of second messengers, in particular calcium. In the presence of too little calcium, the MLCK requirement for calcium/calmodulin may not be met, and in the absence of MLCK T cells are poorly adhesive to ICAM and show poor motility [45]. In contrast, when intracellular calcium levels are high, Myosin IIA/MyH9 heavy chain phosphorylation results in loss of myosin filamentation and contractile function. Thus, cyclical stopping and rounding may result from imbalances, either up or down, in calcium levels, perhaps as a result of protein turnover or ER functioning

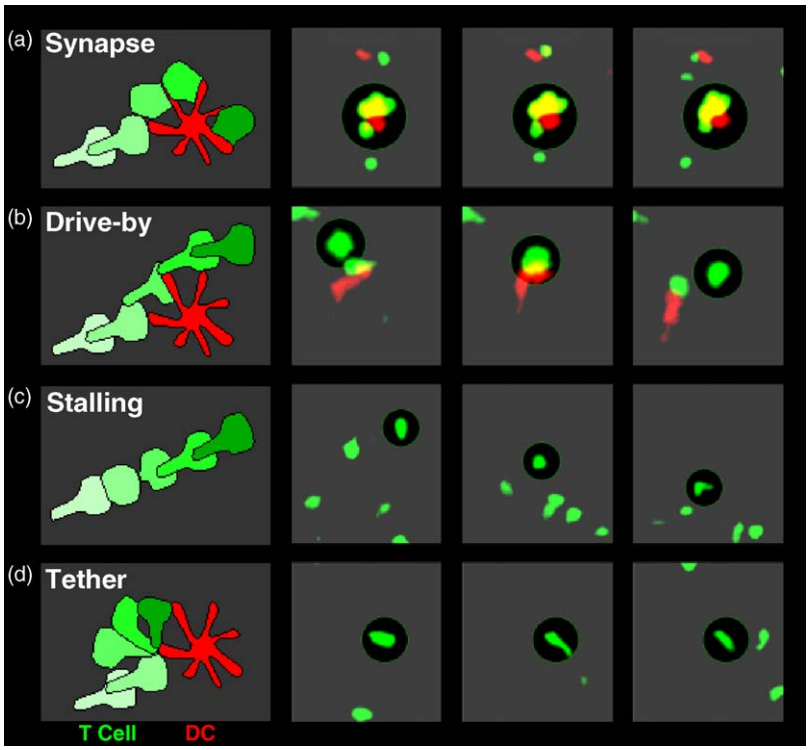

Fig. 5. Alternative synapses and cellular behaviors. T cells are shown at time intervals during deviations from crawling. Sequential times are indicated as degrees of green in cartoon models. Examples depicting these behaviors in a popliteal lymph node are shown in adjacent panels. Labeled T cells (green) and bone marrow derived dendritic cells (red) were imaged through TPSLM in excised lymph nodes maintained in O_2 perfused media. Full movies, playing at 300× real-time available as Supplementary data, movie 4a–d. (a) Canonical IS formation involves loss of uropodal structures, tight adhesion to an APC via a flattened leading edge and receptor reorganization to form a c-SMAC. (b–d) Selected interactions lacking the canonical c-SMAC behavior are depicted. (b) "Drive by" interaction in which insufficient calcium signal/Myosin IIA depolymerization is achieved during engagement with an APC. (c) T cell "stalling" in which intracellular calcium imbalance results in either MLC global dephosphorylation due to insufficient calcium levels to activate MLCK and/or debundling of motor proteins due to non-TCR-induced calcium signaling leading to Myosin IIA filament disassembly. Note that TCR may nevertheless remain polarized in the domain formerly comprising the uropod, however, no flattened leading edge is formed with adjacent cells. (d) "Tethered contact" in which a high-affinity integrin adhesion patch prevents progress but distal leading edge behavior continues or is enhanced as a result of polarized Rho activation at the site of integrin tethering.

associated with calcium stores. It should be noted that such 'stopped' cells are distinguished from prototypical immunological synapses on the basis of their morphology—they would have no flattened leading edges (Fig. 5c).

7.4. "Tethered contacts"

An imbalance of adhesive forces may arise in T lymphocytes as a result of the numerous pathways mediating integrin adhesion. Overactive adhesion contacts localized to a leading edge that are not associated with a downregulation of actin polymerization and/or myosin motor repression prevent the cell from detaching. Such adhesions ultimately reside in the uropod as the cell continues to attempt actin- and myosin-mediated translation resulting in a 'tethered' cell (Fig. 5d). Such 'tethered' cells with uropodal adhesions have been observed in intact lymph nodes [11] although the details surrounding/initiating the behavior have note been well characterized. Also, T cells in vitro appear to bind to one another via contacts on the uropod in the presence of chemokines [94]. It has been argued that integrin activation in the uropod resulting in a tether may be a mechanism for recruitment of lymphocytes into tissues [94] although their function in the context of dendritic cells and a total lymph node environment [11] has not been determined.

8. Concluding remarks

There are numerous questions remaining to be answered about how motility is internally regulated. Most notably, it remains unclear how actin elongation and myosin tension crosstalk to regulate the morphology. The evidence of ROCK inhibition giving rise to hyperextended cells would suggest that the Rho/Rac system plays one such role. However, the details of this crosstalk have yet to be worked out.

More dramatically, it seems clear that regulation of each aspect of motility independently can give rise to highly complex motile behaviors as well as a select variety of 'stopped' behaviors in T lymphocytes. Transient, stalled or tethered contacts, for example, may both influence T cell fate and also serve as an indicators of the balance of specific signaling pathways in T lymphocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.smim.2005.09.006.

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