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Controversy and Consensus Regarding Myosin II Function at the Immunological Synapse

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Abstract

Regulated actin dynamics play a central role in modulating signaling events at the immunological synapse (IS). Polymerization of actin filaments at the periphery of the IS, coupled to depolymerization near the center, generates a centripetal flow of the actin network and associated movement of signaling molecules. A recent flurry of papers addresses the role of myosin II in facilitating these events. Investigators agree that myosin II is present at the IS, where it forms actomyosin arcs within the peripheral supramolecular activation cluster, a region corresponding to the lamellum of migrating cells. However, there is substantial disagreement about the extent to which myosin II drives IS formation and signaling events leading to T cell activation.

Introduction

Upon contact with an antigen presenting cell (APC), a T cell undergoes a series of rapid cytoskeletal rearrangements that culminate in formation of an immunological synapse (IS) [1,2]. The most notable of these is the initiation of robust actin polymerization at the outermost edges of the contact [3,4]. Given the radial symmetry of the contact, this polymerization, coupled with depolymerization near the center of the interface, creates a dramatic centripetal flow of actin just under the T cell's plasma membrane in the plane of the IS. Actin flow is widely thought to provide much of the motive force for the centripetal movement of signaling microclusters (MCs) [5–7]. This leads, over a period of several minutes, to the formation of a mature IS containing an outer ring rich in integrins (termed the peripheral supramolecular activation cluster or pSMAC [8]) and an inner region rich in TCRs and associated signaling molecules (the central supramolecular activation cluster or cSMAC [9]).

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At the leading edge of other cell types, the pushing force of actin polymerization-driven retrograde flow is coupled with a pulling force generated by myosin II-dependent actin arc contraction [10–13]. These two forces reside in structurally distinct zones, with actin polymerization in the outer lamellipodium (LP) and actomyosin II arc contraction behind the LP, in the lamellum (LM). The LP is composed largely of a branched actin network created by Arp2/3-dependent nucleation at the plasma membrane:cytoplasm interface [14,15]. The LM, on the other hand, is composed of linear actin arcs or fibers aligned roughly parallel to the leading edge. These are probably created by formin-dependent nucleation and the rearrangement of LP actin [11,16–18]. Dynamic imaging of GFP-actin in Jurkat T cells engaged on planar stimulatory surfaces reveals robust Arp2/3-dependent actin retrograde flow principally in a ring surrounding the pSMAC, termed the distal SMAC (dSMAC) [3,19–22]. Based in part on these observations, Dustin hypothesized that the IS might represent a radially symmetric version of the leading edge of a fibroblast, with the dSMAC corresponding to the LP and the pSMAC corresponding to the LM [23,24] (Figure 1). In comparison with these outer regions of the IS, the cSMAC is F-actin-poor, albeit not devoid of F-actin [25,26]. Importantly, the LM is a zone of actomyosin II arc contraction, raising the possibility that the centripetal transport of TCR and other signaling MCs is driven not only by the pushing force of actin retrograde flow, but also by the pulling force of myosin II-dependent contraction. Here, we briefly review a series of recent studies that provide evidence for and against the existence of this latter mechanism and its contribution to MC transport, IS formation and T cell signaling.

Organization of the actomyosin II network

Several labs have reported strong staining for endogenous myosin II[1] at the IS usually concentrated along with LM markers like tropomyosin in the pSMAC [27–31]. Moreover, time lapse images of Jurkat T cells expressing fluorescently-tagged myosin II reveal concentric, myosin II-rich “arcs” that first become prominent at the dSMAC/pSMAC boundary and then move inward, eventually dissipating at the pSMAC/cSMAC boundary [27,28]. While previous studies employing GFP-actin did not report corresponding actin arcs in the pSMAC [3,20–22,32,33], these structures are seen in phalloidin-stained Jurkat T cells without myosin II overexpression [27]. Moreover, they become much more obvious in live cells using an indirect reporter for F-actin like F-Tractin [27,28]. Importantly, these actin arcs exactly overlap the GFP-myosin II arcs in the pSMAC [25,26]. Together, these results support the existence of actomyosin II arcs in the pSMAC region of the maturing IS. The presence of these structures within the integrin-rich pSMAC is consistent with the fact that the pSMAC’s counterpart in fibroblasts, the actomyosin II-rich LM, is the site of pronounced, integrin-dependent attachment to the substrate [34–36].

In many mesenchymal cell types, the speed of centripetal actin flow is faster in the LP than in the LM [10,11,37]. Consistent with this, in two studies of Jurkat T cells where flow speed was measured under conditions where cytoskeletal organization in the pSMAC was clear [27,28], the speed of actin retrograde flow in the LP/dSMAC was several fold faster than the speed of inward actomyosin II arc movement in the LM/pSMAC (although the transition in speed was reported to be rather abrupt in one study, and more gradual in the other). Yi et al. simultaneously measured the speeds of TCR MC movements and actin flow in T cells responding to activating planar lipid bilayers and found that the two rates were statistically indistinguishable [27]. In other words, centripetal TCR MC transport was fast in the LP/

[1]Mice and humans possess three type II myosin heavy chains, myosin IIA (MyH9), myosin IIB (MyH10) and myosin IIC (MyH14). Murine primary T cells express only myosin IIA [31], while human Jurkat T cells express both myosin IIA and myosin IIB [28]. Both molecules are inhibited by treatment with blebbistatin [52]. siRNA experiments performed to date target only myosin IIA; myosin IIB function has not be explicitly tested. For simplicity, we will use the general term “myosin II” throughout this review.

dSMAC and slow in the LM/pSMAC, and there appeared to be little if any uncoupling of MC movement from actin flow. This observation is at odds with the report of Kaizuka et al. [20] that TCR MCs move inward at ~half the speed of actin, a result that has been cited widely in support of the frictional coupling model of TCR MC/actin cytoskeletal interaction [38–40]. While studies employing physical barriers to TCR MC movement have provided strong support for this frictional coupling model, it may be that under unrestricted conditions the coupling between actin flow and TCR MC movement is fairly tight. Finally, it's worth noting that not all signaling MCs are coupled to actin flow; MCs containing the adapter protein SLP-76 actually move faster than actin flow in the pSMAC/LM region [28], pointing to the existence of another transport mechanism for some molecules [28,41].

Myosin II function in MC transport and IS formation

While there is consensus that myosin II is enriched in the pSMAC region of the IS, efforts to define its functional significance for MC transport and IS formation have yielded widely disparate results (Table 1). On one end of the spectrum is a study by Ilani et al. [42], who reached the conclusion that myosin II is essential for formation of a mature IS. These authors used blebbistatin (BB) to inhibit myosin II motor activity, as well as inhibitors of myosin regulatory light chain phosphorylation and siRNA-mediated myosin II knockdown to probe myosin II function in bilayer-engaged Jurkat and human primary CD4+ T cells. They presented evidence that myosin II plays major roles in the growth and centripetal transport of TCR MCs, cSMAC formation and maintenance of sustained T cell-APC contacts. Myosin II was also found to be required for key TCR signaling events, including intracellular Ca^{++} elevation and tyrosine phosphorylation downstream of Lck.

On the other end of the spectrum are two papers from the Krummel lab, both of which argue that myosin II plays no measurable role in MC transport or IS formation. Using the D10 murine T cell clone and murine primary T cell blasts, Jacobelli et al. [31] reported that while inhibition of myosin II with BB inhibited cell polarity and motility prior to APC contact (see also [43]), it had no effect on conjugate formation with antigen-specific APCs, or on segregation of TCR and LFA-1 into prototypical cSMAC and pSMAC structures. On the basis of these findings, the authors argued that myosin II activity is dispensable for formation of the mature IS. While this first study addressed IS formation using only end-point assays, Beemiller et al. [44] subsequently reported that the speed, degree of centralization, and directional persistence of TCR MC transport were all normal in bilayer-engaged mouse T cells pretreated with BB and in mouse T cells in which the myosin IIA heavy chain gene was genetically deleted *ex vivo* (of note, these latter T cells contain ~20–30% of the normal amount of myosin IIA). Based on experiments using low dose Jasplakinolide to inhibit F-actin depolymerization, the authors argued that actin retrograde flow, driven by polymerization at the IS periphery and depolymerization near the center, is what drives inward TCR MC transport and cSMAC formation. Interestingly, this study showed that while the central actin-poor zone created by actin depolymerization directly overlaps the cSMAC in stationary T cells, in migrating T cells the cSMAC trails slightly behind the actin-poor zone, and TCR MCs move toward the actin-poor zone rather than the cSMAC.

Finally, bracketed by these studies are four reports that can be loosely grouped as arguing for a significant albeit non-essential role for myosin II in MC transport and IS formation. We say loosely because the magnitude of myosin II's contribution, as well as the specific function attributed to it, varies widely within this group of papers. On the "high" end of the spectrum is work by Yi et al. [27]. In addition to showing the existence of actomyosin II arcs within the pSMAC, these authors showed using BB that myosin II contributes significantly to the centripetal flow of actin and TCR MCs in bilayer-engaged Jurkat T cells. Moreover,

simultaneous inhibition of both myosin II and actin assembly resulted in a complete cessation of actin flow and centripetal MC transport. Based on these and other observations, Yi and colleagues concluded that the relatively fast pushing force of polymerization-driven actin retrograde flow in the dSMAC and the slower pulling force of actomyosin II arc contraction in the pSMAC cooperate in an interdependent fashion to drive centripetal TCR MC transport at the IS at two distinct rates. While this work indicates that myosin II is not absolutely required for TCR MC transport (MCs still move inward in the presence of BB, albeit slowly and haphazardly due to the disorganization of actin arcs in the pSMAC), it argues that the myosin II contributes significantly to the kinetics of MC transport and SMAC coalescence.

On the “low” end of the spectrum is work by Babich et al. [28]. Like Yi et al., this group found clear evidence for the existence of actomyosin II arcs in the pSMAC and quantitated actin flow rates across the IS. However, using coverslip-engaged Jurkat and human primary T cells, Babich and colleagues concluded that actin polymerization is the primary driver of actin retrograde flow, since flow rate was not affected by various myosin II inhibitors or by suppression of myosin II. While the authors did not measure TCR MC mobility, they showed that inhibition of myosin II contraction using a Rho kinase inhibitor had no effect on the centripetal transport of SLP-76 MCs (although further addition of Jasplakinolide led to a complete cessation of both actin flow and SLP-76 MC transport). This raises the important point that individual MC components may utilize distinct mechanisms for centripetal movement. Although this study points to a minimal role for myosin II function, these authors did present evidence that myosin II contributes to the maintenance of radial symmetry at the IS.

In the third paper in this middle group, Kumari et al. [45] showed that suppressing myosin IIA in bilayer-engaged primary mouse CD4+ T cells had a profound inhibitory effect on the formation of the typical bull’s eye-patterned IS. Somewhat surprisingly, however, this effect was not due to a defect in centripetal TCR MC transport, which slowed only slightly, but rather to enhanced cell spreading (resulting in larger distances to traverse) and reduced directionality of movement (formation of an organized pSMAC was also inhibited, albeit to a lesser extent). Finally, Yu et al. [29] showed that inhibition of myosin II in bilayer-engaged mouse primary T cells completely suppressed a transient (0–2 min) acceleration of in inward actin flow and TCR MC movement that occurred immediately after contact with the activating bilayer. After this early phase, however, myosin II played no obvious role in retrograde actin flow or centripetal TCR MC movement. While myosin II inhibition caused a slight delay in coalescence of the cSMAC, the authors concluded that polymerization-driven actin retrograde flow acting across the entire IS provides the long-lasting driving force for the centripetal transport of TCR MCs to the cSMAC.

We (and we assume the reader as well!) are struck by the wide variation in results regarding the functional significance of myosin II in TCR MC transport and IS formation (Table 1). Some of the discrepancies could be due to significant differences in experimental parameters, which include differences in cell type (Jurkat versus primary mouse T cells), method of engagement (glass, bilayer, conjugate, anti-CD3 versus antigen-specific stimulation), methods of analysis (end-stage assays versus dynamic data), type and degree of myosin II inhibition (small amounts of myosin II in knockdown and knockout cells may be functionally sufficient), off-target effects of chemical inhibitors, and differences in visualization probes (GFP-actin versus F-Tractin).

Fortunately, there are several important points of consensus. First, it is now clear that the dSMAC and pSMAC regions of the IS are analogous to LP and LM regions of other cells, a useful insight since knowledge about other cell types can be brought to bear on T cell

biology. Second, researchers agree that myosin II is recruited to the IS, and that it is organized into arcs within the LM/pSMAC region. Finally, there is wide agreement that actin polymerization in the LP region is a major driver of MC assembly and centripetal movement. The debate is really over the degree to which myosin II contractility augments this process, and the role that myosin II plays in TCR signaling (discussed below). The preponderance of evidence indicates that while myosin II is not essential for IS formation, it plays a supporting role. Most studies show that perturbation of myosin II leads to slowed mobility and increased meandering of TCR MCs, and to delayed or defective formation of compact cSMAC and/or pSMAC regions within the IS. The one notable exception to this is the study by Beemiller et al., which showed no effects of BB treatment or partial myosin IIA depletion on TCR MC speed, track length or straightness. Interestingly, this is the only study to use CD8+ T cells, raising the interesting possibility that differences in actomyosin II regulatory mechanisms contribute to the known differences in CD4+ and CD8+ T cell triggering.

Myosin II function in T cell signaling

Importantly, four of the seven papers reviewed in detail above addressed not only the role of myosin II in the dynamic architecture of the IS, but also its role in promoting TCR signaling. Here again, results are divergent. Ilani et al. showed that treatment with BB led to an immediate drop in intracellular Ca^{++} and reduced tyrosine phosphorylation of LAT and Zap70 downstream of Lck. Yu et al. showed similar effects using ML-7 to inhibit MLCK activity. Using myosin IIA siRNA, Kumari et al. showed inhibition of Src kinase phosphorylation, but reported only blunting of Ca^{++} mobilization. Finally, Babich et al. showed no effect of a Rho-kinase inhibitor on either Ca^{++} mobilization or tyrosine phosphorylation, although addition of both Rho kinase inhibitor and Jasplakinolide, which immobilizes the actin cytoskeleton, inhibited Ca^{++} signaling at the level of PLCγ1 phosphorylation.

What sense can be made of these disparate findings? With the exception of the work by Babich et al, there is agreement that myosin II function is needed for optimal phosphorylation of early signaling intermediates, beginning at least with ZAP-70. Discrepancies about the involvement of Lck may be due to the stimulatory conditions used, since Lck phosphorylation is affected by myosin II perturbation when T cells are stimulated with pMHC [29,45], but not with anti-TCR [42], and pMHC engages CD4, which signals strongly through Lck. In the case of Ca^{++} mobilization, it is important to consider possible off-target effects of the inhibitors used. Of note, the Ca^{++} data in the Ilani paper are based on treatment of cells with BB, which has known cytotoxic effects at the wavelengths used for Ca^{++} measurements [46]. Similar concerns apply for the Yu et al. paper, since ML-7 is structurally similar to ML-9, an inhibitor that was recently shown to inhibit Ca^{++} influx in a myosin-independent manner [47]. Thus, if we consider only studies where siRNA or other inhibitors are used, it seems that Ca^{++} responses are either unaffected or blunted. Given that Ca^{++} signaling depends on tyrosine phosphorylation events, it makes sense that the response should be blunted under conditions where phosphorylation is diminished. Finally, the lack of signaling defects in the Babich et al. paper may be attributable to the use of stimulatory ligands immobilized on glass surfaces vs. the mobile ligands used in all other studies. Evidently, the requirement for myosin II activity is context dependent – an effect that could reflect either differences in tension-based signaling mechanisms or the ability to continuously form new contacts with TCR ligands.

Conclusions and future directions

Recent studies clearly show that actomyosin II arc-like structures exist in the pSMAC, but controversy remains about their functional significance. While it seems reasonable to assume that the disruption of these structures would have some measurable effect on IS formation and T cell signaling, this effect could be relatively small and context dependent. Perhaps the field needs to look for additional readouts. One obvious area would be effects on integrin dynamics and function, given the tight structural and functional association between myosin II and integrin-based adhesions in other cell types [48,49]. Indeed, several groups have already shown a reduction in the phosphorylation of the mechanosensing protein CasL in myosin II-inhibited cells [29,45], consistent with significant myosin II-dependent force generation at the IS. Interestingly, Yi et al. [27] showed that an accumulation of integrin clusters at the inner aspect of the LM/pSMAC depends on actomyosin II contraction. Moreover, loss of myosin II has been linked to defects in de-adhesion from ICAM1 [50], and treatment with BB was recently shown to decrease mechanosensitivity in mouse T cells [51]. Importantly, any myosin II-dependent mechanotransduction in T cells would involve the ability of actomyosin II structures to generate tension through adhesive contacts between T cell and APC. Therefore, future efforts to define the role of myosin II in mechanotransduction will require detailed studies in cell conjugates where, unlike in bilayer-engaged T cells, there is a significant resistance to the movement of integrin clusters in the plane of the membrane. Moreover, the force generated by the physical coupling of myosin II contractile arrays to integrin clusters may be highly variable as the T cell adapts to differences in substrate stiffness, ligand mobility and other parameters. If so, understanding such adaptation may ultimately resolve current controversies regarding the role of myosin II at the IS [52].

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Highlights

- F-actin and myosin II form arcs at the immunological synapse.
- IS domains correspond to the lamellipodium and lamellum of other cells.
- Researchers disagree about the magnitude of myosin II's role in IS formation and T cell signaling.
- The magnitude of myosin II's contribution may be context dependent.

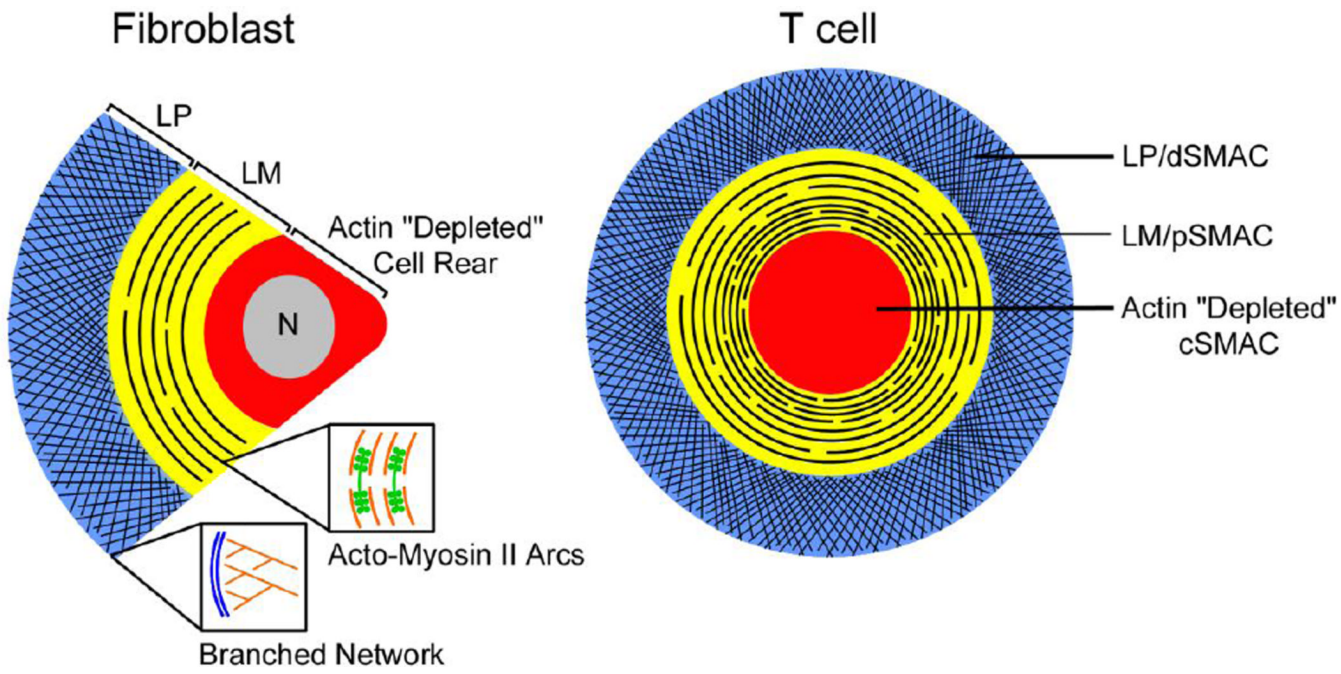

Figure 1. Organization of the actomyosin II network at the IS

The cartoon highlights the close relationship between the organization of the actin cytoskeleton in migrating mesenchymal cells (right) and in the radially symmetric IS of T cells (left). Specifically, the T cell's dSMAC and pSMAC correspond to lamellipodial (LP) and lamellar (LM) actin structures seen in mesenchymal cells, respectively. The insets show the distinct organization of F-actin in these two zones, with branched filament arrays dominating the dSMAC/LP and actomyosin II arcs/fibers dominating the pSMAC/LM (red, F-actin; green, myosin II filaments). These latter structures may be difficult to discern using GFP-actin because GFP-actin is a poor substrate for formin-dependent actin nucleation [53], which probably plays an important role in the assembly of LM (i.e. pSMAC) actin [13]. While F-actin reporters like F-Tractin that highlight these actin arcs could in principle augment their formation (although see [27]), the fact that these structures are seen in untransfected, phalloidin-stained cells supports their existence.

Summary of Findings Regarding Myosin II Function

Paper	Cells	Stimulation	Myosin II perturbation	molecules Signaling at the IS	cSMAC formation	pSMAC formation	TCR MC translocation	Actin dynamics	Ca^{++} signaling	Kinase signaling	Additional Relevant Findings
Jacobelli 2004 [31]	D10 (murine CD4+ T cell clone)	Ag pulsed B cells	Blebbistatin	Yes	Yes – fixed at late time point	Yes – fixed at late time point	–	–	–	–	T cell migration is inhibited by BB. TCR signals induce phosphorylation of myosin II HC.
Ilani 2009 [42]	Jurkat (human CD4+ T lymphoma)	OKT3/ICAM1 on bilayers	Blebbistatin	Yes	Inhibited	–	inhibited	–	Inhibited	pSrc OK, pLAT and pZAP70 reduced	BB destabilizes T cell-APC contacts. TCR signals induce phosphorylation of myosin II light chain.
	Human primary CD4+ T cell blasts	OKT3/ICAM1 on bilayers	siRNA	Yes	Inhibited	–	inhibited	–	–	pSrc OK, pLAT and pZAP70 reduced	
Yi 2012 [27]	Jurkat	OKT3/ICAM1 on bilayers	Blebbistatin	Yes	Delayed	Poorly focused	slowed, meandering increased	Slowed, arcs buckle	–	–	Rates of centripetal actin flow and TCR MC transport match in the dSMAC/LP and pSMAC/LM.
Yu 2012 [29]	Murine CD4+ AND T cell blasts	pMHC/ICAM1 on bilayers	ML-7	Yes	Slightly delayed	Unaffected	Eliminates early fast phase; later phase unaffected	Eliminates early fast phase; later phase unaffected	Inhibited	pZAP70 reduced	BB and ML-7 inhibit phosphorylation of the mechano-sensing protein CasL.
			Blebbistatin	Yes	Slightly delayed	Unaffected	Eliminates early fast phase; later phase unaffected	–	–	pZAP70 reduced	
Babich 2012 [28]	Jurkat	OKT3 on glass	Y-27632	Yes	–	–	TCR MCs not measured, but SLP76 MCs unaffected	Unaffected	Unaffected	pZAP70 and pPLCγ1 OK	Actin dynamics are required for sustained signaling through PLCγ1.
			Blebbistatin	–	–	–	–	Unaffected	–	–	
			siRNA	–	–	–	–	Unaffected	–	–	
	Human primary CD4+ T cell blasts	OKT3 on glass	Y-27632	–	–	–	–	Unaffected	–	–	
Kumari 2012 [45]	Murine CD4+ AND T cell blasts	pMHC/ICAM1 on bilayers	siRNA	Yes	Inhibited	Poorly focused	TCR slowed slightly, path length unaffected, meandering increased	–	Blunted	pSrc inhibited	Myosin II suppression inhibits TCR-induced phosphorylation of CasL.
Beemiller 2012 [44]	Murine CD8+ OTI T cell blasts	pMHC/ICAM1 on bilayers	Blebbistatin	Yes	Unaffected	–	Unaffected (track length, speed, straightness)	–	–	–	The central actin depleted zone and the
			Ex vivo gene deletion	Yes	Unaffected	–	Unaffected (track length, speed, straightness)	–	–	–	

Paper	Cells	Stimulation	Myosin II perturbation	molecules Signaling at the IS	cSMAC formation	pSMAC formation	TCR MC translocation	Actin dynamics	Ca^{++} signaling	Kinase signaling	Additional Relevant Findings
											cSMAC are offset in migrating T cells.