



CELL MIGRATION

Exocrine gland–resident memory CD8⁺ T cells use mechanosensing for tissue surveillance

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Tissue-resident CD8⁺ T cells (T_{RM}) continuously scan peptide-MHC (pMHC) complexes in their organ of residence to intercept microbial invaders. Recent data showed that T_{RM} lodged in exocrine glands scan tissue in the absence of any chemoattractant or adhesion receptor signaling, thus bypassing the requirement for canonical migration-promoting factors. The signals eliciting this noncanonical motility and its relevance for organ surveillance have remained unknown. Using mouse models of viral infections, we report that exocrine gland T_{RM} autonomously generated front-to-back F-actin flow for locomotion, accompanied by high cortical actomyosin contractility, and leading-edge bleb formation. The distinctive mode of exocrine gland T_{RM} locomotion was triggered by sensing physical confinement and was closely correlated with nuclear deformation, which acts as a mechanosensor via an arachidonic acid and Ca²⁺ signaling pathway. By contrast, naïve CD8⁺ T cells or T_{RM} surveilling microbe-exposed epithelial barriers did not show mechanosensing capacity. Inhibition of nuclear mechanosensing disrupted exocrine gland T_{RM} scanning and impaired their ability to intercept target cells. These findings indicate that confinement is sufficient to elicit autonomous T cell surveillance in glands with restricted chemokine expression and constitutes a scanning strategy that complements chemosensing-dependent migration.

INTRODUCTION

CD8⁺ T cell–mediated protection against viral infections hinges on the rapid detection of cognate peptide–major histocompatibility complex (pMHC) displayed on antigen-presenting and infected host cells. To expedite this process, CD8⁺ T cells integrate biochemical cues from their microenvironment via chemoattractant and adhesion receptors (1). During an immune response, CD8⁺ T cells adapt their adhesion and chemoattractant receptor expression pattern to their differentiation status. These expression patterns enable clonal selection in lymphoid organs, followed by elimination of infected cells in inflamed tissue and tissue surveillance in the memory phase (2). More specifically, naïve and central memory CD8⁺ T cells (T_N and T_{CM}, respectively) express CD62L and CCR7 for homing to secondary lymphoid organs (SLOs), whereas effector and effector memory T cells (T_{EFF} and T_{EM}, respectively) display high surface levels of inflammatory chemokine and adhesion receptors to enter nonlymphoid tissues. Chemokine and adhesion receptors are also critical for interstitial scanning within target organs, the prerequisite

for antigen-specific protection afforded by T_{EFF} (3–5). Last, tissue-resident memory T cells (T_{RM}) express numerous chemokine and adhesion receptors including CXCR3, CXCR6, CD49a, and CD103, which contribute to their long-term persistence in their tissue of residence and to the surveillance of epithelial barriers exposed to microbes (6–13).

Their vigorous scanning behavior makes CD8⁺ T cells a valuable model to study amoeboid cell migration, a heterogeneous phenomenon encompassing forward and retrograde F-actin flow, as well as different types of protrusions such as lamellipodia, pseudopods, and blebs (14–19). Recent studies have dissected the precise mode of amoeboid migration of T_N. These cells generate a CCR7-driven retrograde cortical F-actin flow dependent on the guanine exchange factor (GEF) DOCK2 and its downstream targets Rac1 and Rac2 (20–23). Retrograde F-actin flow transmits weak forces via the integrin LFA-1 to its receptor intercellular adhesion molecule-1 (ICAM-1) displayed on stromal cells, providing the traction force for rapid T_N locomotion within the lymphoid parenchyma without inducing substantial adhesion (23–25).

In addition to the paradigmatic T cell migration driven by chemosensing of external cues, we have recently uncovered a distinct motility signature displayed by submandibular salivary gland (SMG) CD8⁺ T_{RM} (26). Although these cells exhibit high in vivo migration speeds of 6 to 7 μm/min, we did not find evidence for a substantial contribution for chemoattractant and adhesion receptors during homeostatic organ surveillance because neither pertussis toxin treatment (to block G_{qi}-coupled receptors) nor integrin inhibition influenced their motility. Instead, SMG CD8⁺ T_{RM} spontaneously migrate with frequent shape changes in the absence of chemoattractants and specific adhesive interactions (26), perhaps as an adaptation to the chemokine-poor milieu of noninflamed salivary glands (27). This is in contrast to epidermal CD8⁺ T_{RM}, which require external chemoattractants for motility (8, 26). In line with our

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findings, T cell lines can migrate in complete absence of chemokine and adhesion receptors using topography-dependent force transmission (28). However, the cues that trigger receptor-independent motility of CD8⁺ T_{RM} lodging in resting glands and the relevance of this noncanonical migration to intercept Ag-presenting cells remain unknown to date.

Here, we have systemically compared SMG T_{RM} motility with the canonical chemosensing-driven T_N amoeboid migration. In the absence of external biochemical cues, T_{RM} spontaneously generated retrograde F-actin flow as a force-generating module for translocation. Furthermore, T_{RM} displayed a highly contractile actomyosin cytoskeleton, leading to continuous bleb formation at the leading edge. Autonomous cell motility was also observed in lacrimal gland (LG) T_{RM} but not T_N or small intestine (SI) T_{RM}, and this was strictly dependent on their ability to sense and react to mechanical load of their microenvironment. Mechanosensing triggered an “evasion reflex,” which elicited cortical actomyosin contractility through arachidonic acid (AA) and intracellular Ca²⁺ signaling and correlated with nuclear deformation, in line with the recently uncovered function of the nucleus as mechanosensor in mammalian and nonmammalian cells (29, 30). Inhibition of mechanosensing signaling disrupted T_{RM} tissue scanning and interception of target cells. Our data suggest that memory T cells lodged in organs with low microbial exposure have co-opted a conserved mechanosensory module for immunosurveillance, suggesting an instructive role for physical confinement to elicit dynamic T cell motility. In combination with their preserved chemosensing capacity, our data identify a multitier strategy for T_{RM}-mediated tissue surveillance. Such a strategy bypasses the requirement for elevated constitutive chemokine and adhesion receptor expression in resting nonbarrier tissues, thus preventing unwarranted leukocyte influx.

RESULTS

SMG T_{RM} spontaneously generate retrograde F-actin flow in the absence of chemoattractants and specific adhesions

Our previous *in vivo* and *ex vivo* analysis uncovered high intrinsic SMG CD8⁺ T_{RM} motility (movie S1), which bypasses canonical chemoattractant sensing and adhesion receptor engagement (26). To generate SMG CD8⁺ T_{RM} for an in-depth examination of the underlying actomyosin cytoskeleton regulation, we adoptively transferred congenically marked OT-I T cell receptor (TCR) transgenic CD8⁺ T cells, which recognize the ovalbumin (OVA) peptide OVA_{257–264} in the context of H-2K^b (31), into C57BL/6 mice. One day later, recipient mice were infected with lymphocytic choriomeningitis virus–OVA (LCMV-OVA), a replication-competent, attenuated LCMV variant encoding OVA as model antigen (Fig. 1A) (32). Although rapidly cleared, LCMV-OVA infection results in OT-I expansion and formation of T_{CM} and T_{EM} populations in the spleen and lymph nodes (LNs) and bona fide CD103⁺ T_{RM} in SMGs at ≥30 days post infection (p.i.) (fig. S1A). Cell sizes were similar between T_N and endogenous or adoptively transferred CD8⁺ T_{EM} and T_{RM}, whereas T_{CM} showed a tendency toward increased cell size (fig. S1B). Similarly, levels of the F-actin regulators phospho-cofilin and DOCK2 were not elevated in T_{RM} as compared to other memory subsets, and the expression and phosphorylation of ezrin-radixin-moesin (ERM) proteins, which link cortical F-actin to the plasma membrane, remained unchanged (fig. S1, C to F). In contrast, both endogenous and adoptively transferred SMG CD8⁺ T_{RM} contained higher F-actin levels as compared with other T cell subsets (Fig. 1B).

We used the F-actin probe LifeAct-GFP (green fluorescent protein) to examine steady-state actin cytoskeleton dynamics in T_{RM} (33). We generated LifeAct-GFP⁺ SMG OT-I T_{RM} as above and compared their cortical actin cytoskeleton dynamics with LifeAct-GFP⁺ T_N as a well-characterized benchmark using total internal reflection fluorescence (TIRF) microscopy in an under-agarose system (23, 34–36). Under-agarose assays create promigratory conditions in the absence of a directional chemokine gradient, thus mimicking the random guided walk characteristic of T_N in lymphoid tissue and the homeostatic surveillance of T_{RM} in SMGs (23, 26, 37). Initially, we measured F-actin flow without cellular translocation by placing T_N and T_{RM} onto “slippery” surfaces passivated with Pluronic F-127 (pluronic), a nonionic surfactant polyol. This approach permits the quantification of cortical F-actin flow speeds during spontaneous or chemokine-induced F-actin treadmilling. In the absence of chemokines, T_N contained only sparse and short-lived cortical F-actin dots correlating with their unpolarized phenotype (Fig. 1, C and D, and movie S2). To assess the impact of biochemical stimulation on F-actin dynamics, we added CCL19 as a ligand for the T_N-expressed chemokine receptor CCR7. CCL19 rapidly induced cortical F-actin filaments that flowed from the leading to the trailing edge with speeds of 14.5 ± 8.2 μm/min (mean ± SD; Fig. 1, C and D). This resulted in a “running on the spot” phenotype on pluronic-passivated surfaces (movie S2). When CCL19-stimulated T_N were plated on ICAM-1–coated plates, retrograde F-actin flow was converted into fast chemokinetic cell movement by force-coupling of LFA-1 to ICAM-1 as described (Fig. 1, C and D, and movie S2) (23). This was accompanied by the stalling of net rearward F-actin flow, with LifeAct-GFP⁺ F-actin becoming stationary in relation to the substrate (Fig. 1D).

In contrast to T_N, LifeAct-GFP⁺ SMG T_{RM} plated on pluronic-passivated surfaces showed intrinsic cortical F-actin treadmilling from the front to the rear with speeds of 9.7 ± 6.8 μm/min (Fig. 1, C and D), resulting in a polarized phenotype. This was accompanied by frequent F-actin protrusion formation at the leading edge (movie S3). Thus, SMG T_{RM} not only contain more total F-actin but also exhibit a continuous front-to-back flow of F-actin filaments even in the absence of biochemical cues (i.e., chemokines). To assess the impact of chemokines on F-actin flow, we added CXCL10, the ligand for the T_{RM}-expressed chemokine receptor CXCR3, because these cells do not express CCR7. Whereas the speed of retrograde cortical F-actin flow was accelerated to 12.8 ± 8.5 μm/min upon CXCL10 exposure, the increase was lower than for chemokine-stimulated T_N (Fig. 1, C and D, and movie S3). Front-to-back F-actin flow stalled when T_{RM} were placed on lipid-free human serum albumin (HSA), which provides sufficient friction to generate traction force for cell translocation, in the absence of specific adhesive interactions (Fig. 1, C and D, and movie S3) (26). In addition, we observed occasional forward movement of F-actin filaments at the trailing edge (Fig. 1C, dashed line). This observation is indicative of a highly contractile uropod, which drags F-actin filaments toward the leading edge (38). In sum, T_{RM} use retrograde cortical F-actin flow for translocation akin to the cortical flow-force transmission model described for motile T_N (23, 39). Unlike T_N, cortical F-actin flow in T_{RM} is induced spontaneously in the absence of external chemoattractants. Nonetheless, our data confirm that SMG T_{RM} remain responsive to CXCL10. This is in line with previous observations that CXCR3^{−/−} T_{RM} do not accumulate around tissue macrophage clusters *in vivo* despite scanning salivary gland parenchyma with speeds comparable to wild-type (WT) T_{RM} (26).

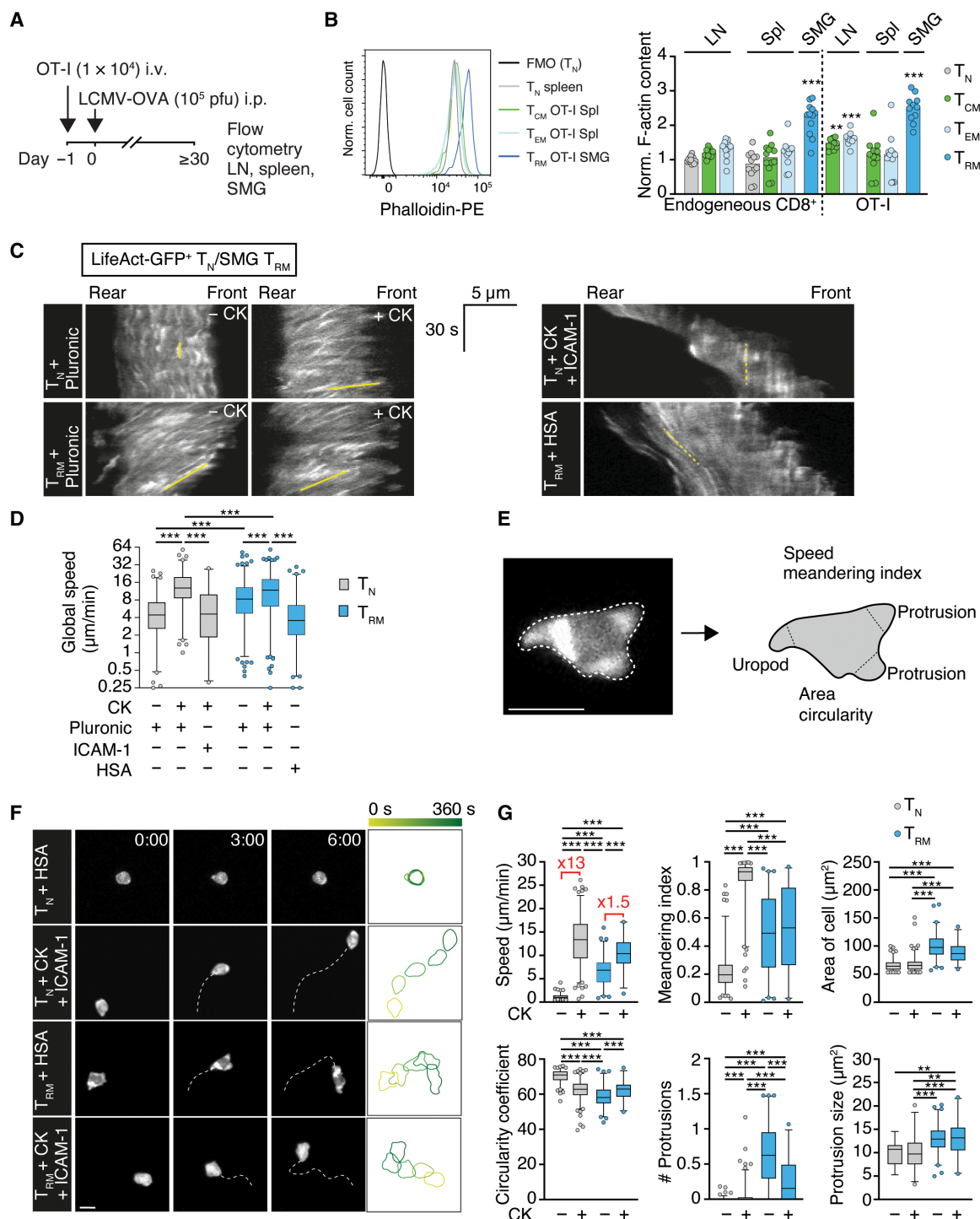


Fig. 1. SMG T_{RM} autonomously generate F-actin flow for chemokine- and specific adhesion-independent amoeboid motility. (A) Experimental layout. (B) Flow cytometry plots and quantification of F-actin content in LN and spleen (Spl) T_N , T_{CM} , T_{EM} , and SMG T_{RM} . Bars represent median. (C) TIRF images of cortical F-actin flow in LifeAct-GFP⁺ T_N and SMG T_{RM} under agarose. Note that for T_N plated in the absence of chemokine (–CK), there is no front or rear discernible because these cells are non-polarized. Yellow lines indicate rearward (continuous) and forward (dotted) F-actin flow. CK, chemokine. (D) Global F-actin flow speeds in T_N and SMG T_{RM} . (E) Schematic layout of dynamic and morphometric analysis. Scale bar, 10 μ m. (F) Wide-field fluorescence microscopy images of T_N and SMG T_{RM} under agarose migration. Time in min:s. Scale bar, 10 μ m. (G) Dynamic and morphometric analysis of T_N and SMG T_{RM} displacement. Red numbers indicate fold change. Data in (B and G) were pooled from at least two independent experiments with $n = 11$ or 12 mice (B) and $n = 60$ to 279 tracks per condition (G), and (D) is from one of two representative experiments with 21 to 24 cells per condition and $n = 411$ to 777 individual F-actin spots tracked per condition (except T_N + CK on ICAM-1: 144 F-actin spots). Statistical analysis was done by a Kruskal-Wallis test against LN T_N (B) or between all columns (D and G). ** $P < 0.01$ and *** $P < 0.001$.

Morphometric analysis uncovers high protrusion formation in spontaneously migrating SMG T_{RM}

We compared the amoeboid motility modes of T_N and T_{RM} in more detail by examining displacement parameters in combination with a morphometric (i.e., cell shape) analysis. To this end, we used wide-field fluorescence microscopy of T cells under agarose (without pluri-ionic passivation) and determined cell speeds, meandering index, cell area, and circularity, as well as numbers and sizes of leading edge protrusions using a customized analysis pipeline (Fig. 1E and fig. S2). We first compared T_N motility on HSA without CCL19 (as a nonmigratory substrate) to ICAM-1 + CCL19-coated plates to mirror conditions used for F-actin flow measurements (23). In agreement with F-actin dynamics identified by TIRF microscopy (Fig. 1C), T_N converted from a nonmotile state with rounded cell morphology in the absence of chemokines and adhesive ligands to a polarized phenotype with high speeds and directionality when ICAM-1 and CCL19 were present (from 1.2 ± 0.5 to 13.1 ± 4.8 $\mu\text{m}/\text{min}$; Fig. 1, F and G, and movie S4). T_N speeds closely matched CCL19-triggered rearward F-actin flow speeds under slippery conditions (Fig. 1G), suggesting that the F-actin flow is efficiently converted into forward movement on ICAM-1-coated substrates. Irrespective of the presence or absence of CCL19, T_N formed very few protrusions (Fig. 1G, bottom middle panel).

In line with TIRF measurements, T_{RM} acquired a spread and polarized shape with numerous protrusions and spontaneously moved on HSA-coated plates, even in the absence of chemokines and specific adhesive substrates (Fig. 1, F and G). We then examined the impact of chemokine addition in the presence of ICAM-1 coating to recreate similar promigratory conditions as used for T_N. Whereas ligands for the T_{RM}-expressed chemokine receptors CXCR3 and CXCR4 together with ICAM-1 were associated with increased SMG T_{RM} speeds (from 6.7 ± 3.0 to 10.5 ± 3.1 $\mu\text{m}/\text{min}$), the increase was less pronounced than in T_N (Fig. 1G and movie S5). Furthermore, chemokine exposure did not increase T_{RM} directionality (as assessed by the meandering index), in contrast to T_N (Fig. 1G). Instead, chemokines reduced protrusion formation in T_{RM} (Fig. 1G). Together, autonomously moving T_{RM} display a protrusion-rich amoeboid migration phenotype, which is distinct from the well-characterized T_N mode (23). Our data are in line with a model in which protrusion formation does not translate into fast leukocyte motility (38) but is instead driven by retrograde F-actin flow and force coupling to substrate (23, 39).

Constitutive DOCK2-Rac-Arp2/3 signaling is required for autonomous SMG T_{RM} motility

Retrograde F-actin flow can be generated by F-actin polymerization at the leading edge and/or by Rho-mediated contraction of the F-actin network (22, 40–42), so we used under-agarose assays to explore the roles for Rac and Cdc42 as major drivers of Arp2/3-mediated F-actin generation at the leading edge (fig. S3A) (43). Reflecting the accumulation of the Rac GEF DOCK2 at the leading edge of migrating T_{RM} (fig. S3B), the DOCK2 inhibitor CPYPP (44) and CK666, an inhibitor of the DOCK2-Rac downstream target Arp2/3 (45), decreased speed, meandering index, and protrusion formation in SMG T_{RM}, with a concomitant increase in circularity and a decrease in cell area (fig. S3, C and D, and movie S6). Similarly, inhibition of Rac1 using the W56 peptide inhibited autonomous T_{RM} motility (fig. S3, E and F). In contrast, the Cdc42 inhibitor ML141 did not

alter T_{RM} speeds and circularity, although it caused a trend to a lower meandering index and a minor decrease in cellular area and average protrusion size (fig. S3, C and D). To corroborate the roles for Rac-driven versus Cdc42-driven T_{RM} motility in vivo, we adoptively transferred WT tdTom⁺ and GFP⁺ OT-I T cells lacking DOCK2 or the Cdc42 GEF DOCK8 into C57BL/6 recipients 1 day before infection with LCMV-OVA. Although DOCK2 deficiency impairs T cell accumulation in nonlymphoid tissues (46), we were able to identify occasional DOCK2^{-/-} T_{RM} at >30 days p.i. within SMGs. Intravital imaging confirmed strongly reduced cell speeds and directionality in the absence of DOCK2 as compared with WT T_{RM} (fig. S3, G and H, and movie S7). DOCK8 has previously been shown to mediate T cell migration in skin (47). In contrast, DOCK8^{-/-} SMG T_{RM} moved with comparable speeds as WT T_{RM}, with only a minor reduction in directionality (fig. S3I). These data support a central role for leading edge DOCK2-Rac-Arp2/3-driven F-actin polymerization for SMG T_{RM}-mediated tissue surveillance.

SMG T_{RM} have high constitutive actomyosin contractility

We next examined the role of actomyosin contractility for spontaneous SMG T_{RM} motility. Non-muscle myosin IIA (MYH9)-mediated contractility of F-actin filaments supports amoeboid cell motility by pulling F-actin filaments toward the trailing edge and by generating force to push the nucleus as the biggest organelle through narrow pores (48). Using a reporter line expressing GFP-tagged myosin IIA under the endogenous *Myh9* promoter (49), we observed notably higher MYH9-GFP levels in SMG OT-I T_{RM} as compared with T_{CM} and T_{EM}, suggesting a high baseline contractility (Fig. 2A). In under-agarose assays, MYH9-GFP accumulated mostly at the trailing edge of migrating SMG T_{RM} (Fig. 2B and movie S8), consistent with local F-actin contractions observed in TIRF imaging (Fig. 1C). MYH9-GFP also accumulated in protrusions of the leading edge, followed by their subsequent retraction (Fig. 2B). This observation points to a dual role for myosin IIA activity: first, to contract the uropod and, second, to retract protrusions that are not aligned with the migratory path, as described for neutrophils (50). In line with elevated MYH9 expression, SMG T_{RM} contained two to three times higher phosphorylated myosin light chain (pMLC) levels as compared with T_{CM} and T_{EM} (Fig. 2, C and D).

We next attempted to genetically assess MYH9 function for SMG T_{RM} scanning but failed to obtain MYH9-deficient peripheral T cells in a CD4-Cre \times MYH9^{fl/fl} line (fig. S4A). Similarly, Cas9-mediated MYH9 depletion resulted in defective OT-I T cell expansion after viral infection, in line with its role in cytokinesis (fig. S4, B and C) (51). We therefore performed under-agarose assays in the presence of selected inhibitors of the MLC phosphorylation cascade (Fig. 2E). Direct inhibition of Rho using rhosin decreased SMG T_{RM} speeds and protrusion sizes (fig. S5, A and B). We next evaluated its downstream effectors, MLC kinase (MLCK) and Rho-associated coiled-coil kinase (ROCK), both of which regulate pMLC levels. MLCK inhibition slightly reduced T_{RM} speeds (from 7.1 ± 3.7 to 5.4 ± 3.0 $\mu\text{m}/\text{min}$; mean \pm SD) but did not significantly alter directionality, cell shape and area, or protrusion formation (Fig. 2, F and G, and movie S9). In contrast, the ROCK inhibitor Y-27632 caused cell rounding and a substantial decrease in speeds to 0.7 ± 2.7 $\mu\text{m}/\text{min}$, as well as low directionality and protrusion formation (Fig. 2, F and G, and movie S9). Furthermore, Y-27632 blocked topography-driven T_{RM} motility on pluri-ionic-passivated surfaces (fig. S5, C and D), as well as SMG T_{RM} surveillance in situ as assessed by intravital

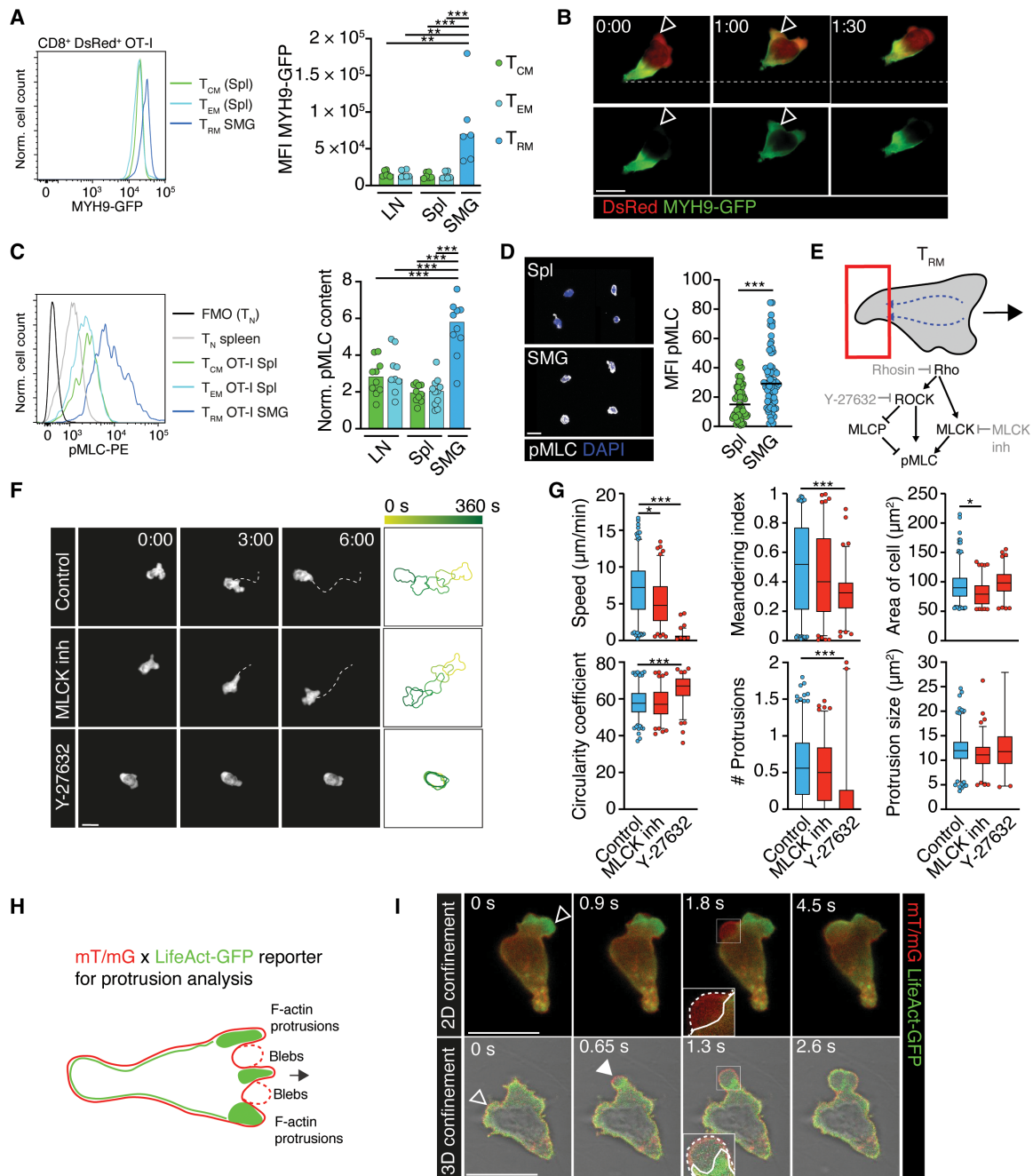


Fig. 2. SMG T_{RM} display high constitutive actomyosin contractility and bleb formation during spontaneous migration. (A) Flow cytometry analysis of MYH9-GFP-expressing T_{CM} , T_{EM} , and SMG T_{RM} DsRed⁺ OT-I T cells. (B) MYH9-GFP dynamics in DsRed⁺ OT-I T cells under agarose migration. Empty arrowheads indicate DsRed⁺ leading edge protrusions. The bottom row depicts MYH9-GFP signal only. Time in mins. Scale bar, 10 μ m. (C) Flow cytometry analysis of pMLC in endogenous T_N and OT-I T_{CM} , T_{EM} , and SMG T_{RM} . (D) Immunofluorescent analysis of pMLC in spleen and SMG memory OT-I T cells. (E) Scheme of Rho-triggered pMLC generation and selected inhibitors. (F) Wide-field fluorescence microscopy images of SMG T_{RM} under agarose migration in the presence of inhibitors. Time in mins. Scale bar, 10 μ m. (G) Dynamic and morphometric analysis of SMG T_{RM} displacement. (H) Scheme of F-actin-filled protrusion versus bleb formation in migrating LifeAct-GFP \times mT/mG SMG T_{RM} . (I) High temporal resolution protrusion analysis of migrating LifeAct-GFP \times mT/mG SMG T_{RM} OT-I under 2D and 3D confinement. Empty arrowheads indicate F-actin-filled protrusions; filled arrowheads indicate blebs. Inserts depict blebs defined by a separation of the mT/mG signal (dotted line) from the LifeAct-GFP signal (continuous line). Scale bars, 10 μ m. Bars in (A) and (C) represent medians. Data in (A) ($n = 6$ mice), (C) ($n = 9$ to 11 mice), (D) ($n = 63$ to 65 cells), and (G) ($n = 184$ to 239 tracks) were pooled from at least two independent experiments and analyzed by ANOVA (A and C), Mann-Whitney test (D), or a Kruskal-Wallis test against control (G). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

imaging (fig. S5E). Because Rho-guanosine triphosphate activates nucleation factors of the formin/mDia family, we performed under-agarose assays in the presence of the formin homology 2 domain

inhibitor SMIFH2. We observed decreased T_{RM} speeds, directionality, protrusion formation, and cell area in the presence of this inhibitor (fig. S5, F and G). As a caveat, SMIFH2 has been reported to

affect additional factors including MYH9 (52, 53). In sum, SMG T_{RM} have high intrinsic actomyosin contractility, which is required for inherent motility and organ surveillance.

T_{RM} form blebs at the leading edge during two-dimensional and three-dimensional space exploration

High baseline actomyosin contractility leads to increased intracellular hydrostatic pressure that can cause blebs, short-lived F-actin-free membrane protrusions (16) (54) (55). To unequivocally assess whether SMG T_{RM} displayed bleb formation during migration, we generated LifeAct-GFP × mT/mG OT-I T cells, in which F-actin and plasma membrane are tagged with GFP and tdTom, respectively (Fig. 2H). We transferred LifeAct-GFP × mT/mG OT-I into recipient C57BL/6 mice, followed by LCMV-OVA infection and sorting of SMG T_{RM} in the memory phase. High-frame rate in vitro imaging confirmed the presence of two types of leading-edge protrusions: F-actin-filled protrusions and membrane blebs, which were rapidly (<1 s) filled with F-actin (Fig. 2I and movie S10). This correlated with quick accumulation of DOCK2 into blebs (movie S10). We did not find evidence for the formation of thin lamellipodia observed in lymphoblasts migrating on adhesive two-dimensional (2D) surfaces (56), perhaps owing to the low adhesiveness of HSA and the substantially lower amount of cytoplasm in resting versus activated T cells (57). Both F-actin-filled protrusions and blebs were short-lived and restricted to the leading edge of motile T_{RM} because they were followed either by the translocation of the cell body or by retraction. Last, we also detected F-actin-filled protrusions and rapid bleb formation that bulged out into the extracellular space at the front of T_{RM} moving in 3D collagen matrices, occasionally followed by the bulky nucleus (Fig. 2I and movie S10). Bleb-supported migration is therefore suited to rapid exploration of the available space in front of the cell's leading edge. Alternatively or in addition, blebs might contribute to motility and cell survival (17, 58). Together, motile T_{RM} and T_N differ in two key features. First, rearward F-actin flow in T_{RM} is generated even without external guidance cues. Second, T_{RM} have high intrinsic actomyosin contractility correlating with formation of blebs and F-actin-filled protrusions.

Exocrine gland T_{RM} use mechanosensing to trigger polarization and motility

We next explored the cellular and molecular mechanism underlying autonomous SMG T_{RM} motility. Previous studies have shown that activated T cells migrate rapidly on 2D surfaces without confinement (59). Furthermore, nonattached leukocytes can use rearward F-actin treadmilling or myosin II-dependent membrane flow for fast translocation (60, 61). To test whether this also applies to SMG T_{RM}, we examined their migration dynamics on 2D surfaces without agarose overlay, anticipating that these highly contractile cells would continue to display a polarized phenotype and motility (Fig. 3A). However, most T_{RM} lost their polarized cell shape and created fewer protrusions on 2D surfaces than spatially confined cells did (Fig. 3, B to D). As a result, T_{RM} displacement was essentially abolished under these conditions (Fig. 3, C and D). Thus, T_{RM} need spatial confinement to acquire a polarized phenotype and for translocation.

To further delineate the relationship between spatial confinement, cell morphometry, and autonomous motility, we exposed SMG T_{RM} to distinct mechanical loads. We adjusted the agarose concentration from 0.5% (corresponding to a Young's modulus of

2.9 kPa) to 1% (9.8 kPa) as described (23). We found that T_{RM} responded to the degree of mechanical load with changes in cell shape, protrusion formation, and motility parameters. When exposed to higher mechanical load (1% agarose), T_{RM} slowed down, moved less directionally, were less polarized, and formed fewer protrusions. These data are consistent with a model in which the forces required to lift agarose for protrusion formation and cell motility increase under high mechanical load, resulting in reduced cell migration. We then analyzed T_{RM} motility exposed to 0.125 and 0.25% agarose, assuming that reduction of the environmental resistance to deformation facilitates cell motility. We found that under conditions of reduced mechanical load, T_{RM} moved more slowly, were more spherical, and formed fewer and smaller protrusions than observed with 0.5% agarose (Fig. 3, E and F). Thus, T_{RM} react to the degree of confinement using a mechanosensing module.

Next, we examined whether mechanosensing was a property shared with T_{RM} from other internal organs. To address this point, we isolated T_{RM} from another exocrine gland, the LG, or from the epithelial barrier of the SI after LCMV-OVA infection. In under-agarose assays, LG but not SI T_{RM} displayed confinement-induced motility akin to SMG T_{RM} (Fig. 3, G and H). In combination with the observation that epidermal T_{RM} do not respond to physical confinement (26), these data suggest that exocrine gland but not skin or gut T_{RM} have acquired the ability to mechanosense their environment, thus being able to measure and respond to a mechanical load with gradual induction of polarity and cell motility.

Confinement-induced T_{RM} motility requires nuclear mechanosensing-triggered signaling pathways

We explored the mechanism underlying cellular proprioception, which translates mechanical cell deformation into adaptive cytoskeletal dynamics. We excluded classical integrin-based mechanotransduction because we observed T_{RM} polarization in the absence of specific integrin ligands, as well as on pluronic-passivated surfaces containing polystyrene (PS) beads (fig. S5C). Various mammalian and nonmammalian cell types subjected to spatial confinement use their nuclei to measure absolute environmental dimensions (29, 30). According to this “nuclear ruler” model, confinement-induced mechanical stretching of the nuclear envelope (NE) leads to activation of Ca²⁺-dependent cytosolic phospholipase A2 (PLA2) enzymes (62). The resulting production of AA leads to membrane recruitment and activation of MYH9, which, in turn, stimulates sustained actomyosin contraction, bleb formation, and motility (29, 30), which are hallmarks of SMG T_{RM} migration observed here.

To examine whether SMG T_{RM} co-opted the nucleus as mechanosensory organelle for spontaneous migration under confinement, we analyzed nuclear shape under 0.5% agarose confinement. These data confirmed that T_{RM} nuclei became stretched, as reflected by increased maximal cross-sectional area and lower heights (Fig. 4, A and B). To directly establish a link between mechanical cell deformation, cell morphometry, and motility, we used confinement chambers with defined heights to induce nuclear compression without interfering with protrusion formation (Fig. 4C). At a 7-μm chamber height, T_{RM} became trapped without substantial nuclear compression. Under these conditions, T_{RM} remained immotile and round, forming only sparse protrusions (Fig. 4D). In contrast, T_{RM} placed in 4-μm-high chambers acquired a highly polarized cell shape with multiple protrusions that correlated with

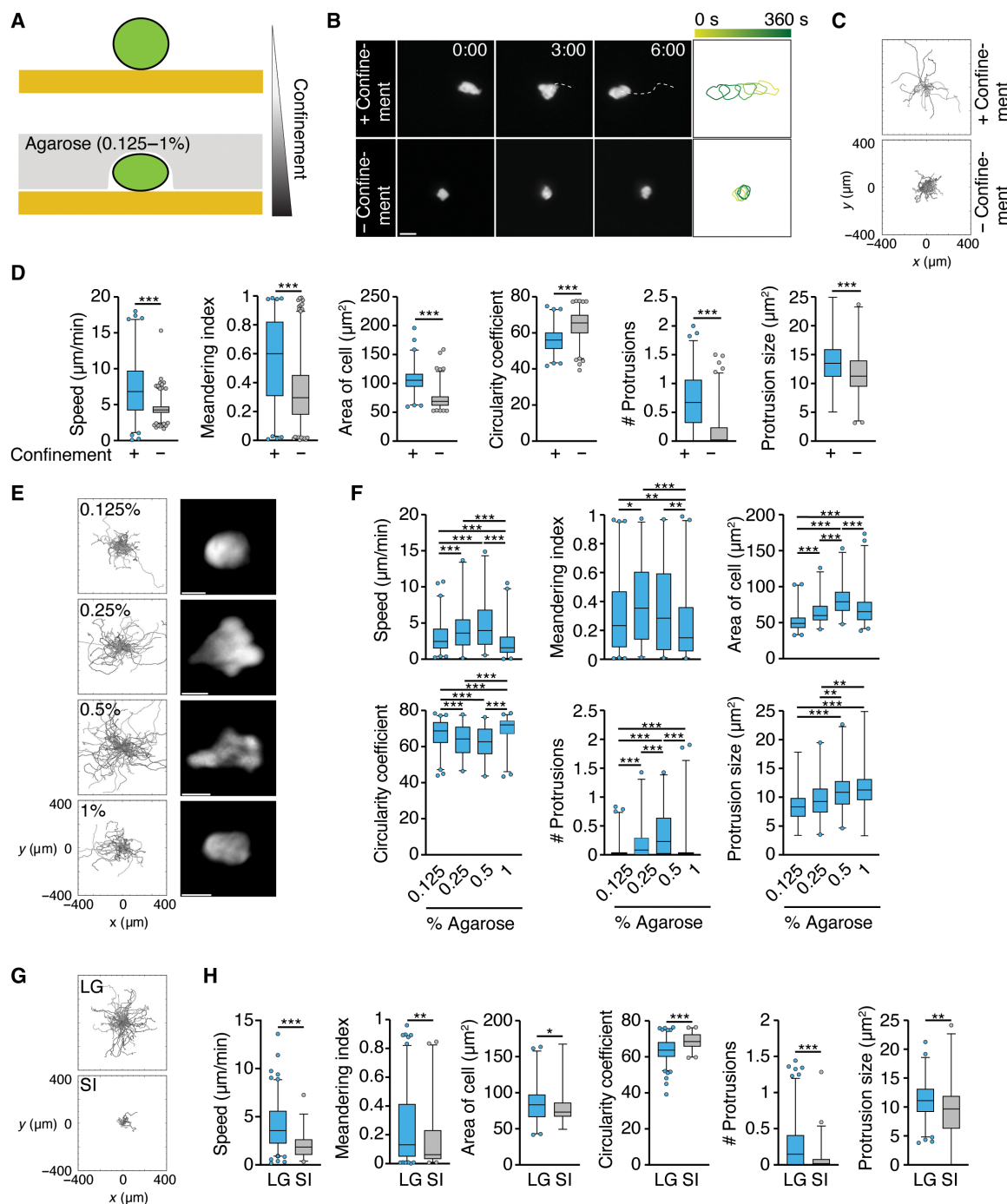


Fig. 3. Exocrine gland T_{RM} react to mechanical load of their environment with motility. (A) Experimental layout. (B) Wide-field fluorescence microscopy images of SMG T_{RM} migrating without or with confinement. (C) Exemplary tracks. (D) Dynamic and morphometric analysis of T_{RM} motility with or without confinement. Scale bar, 10 μm . (E) Exemplary tracks and wide-field fluorescence microscopy images of SMG T_{RM} migrating under distinct mechanical load. Scale bars, 5 μm . (F) Dynamic and morphometric analysis of T_{RM} motility under distinct mechanical load. (G and H) Exemplary tracks (G) and dynamic and morphometric analysis of lacrimal gland (LG) and small intestine (SI) T_{RM} motility (H) on HSA-coated plates under confinement. Data in (D) ($n = 454$ to 1000 tracks), (F) ($n = 159$ to 323 tracks), and (H) ($n = 68$ to 242 tracks) were pooled from at least two independent experiments and analyzed using a Student's t test or Mann-Whitney test (D and H) or a Kruskal-Wallis test between all columns (F). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

the induction of spontaneous motility (Fig. 4D and movie S11). To examine whether mechanosensing-triggered motility can also be induced in other T cell subsets at a distinct confinement threshold, we placed SMG T_{RM} or T_N in 2- and 4- μm -high chambers. In

contrast to T_{RM} , T_N did not display spontaneous motility at 4- μm confinement, whereas both cell populations failed to migrate under 2- μm confinement, accompanied by signs of cell death (fig. S6, A and B). Thus, in the experimental conditions used here, we

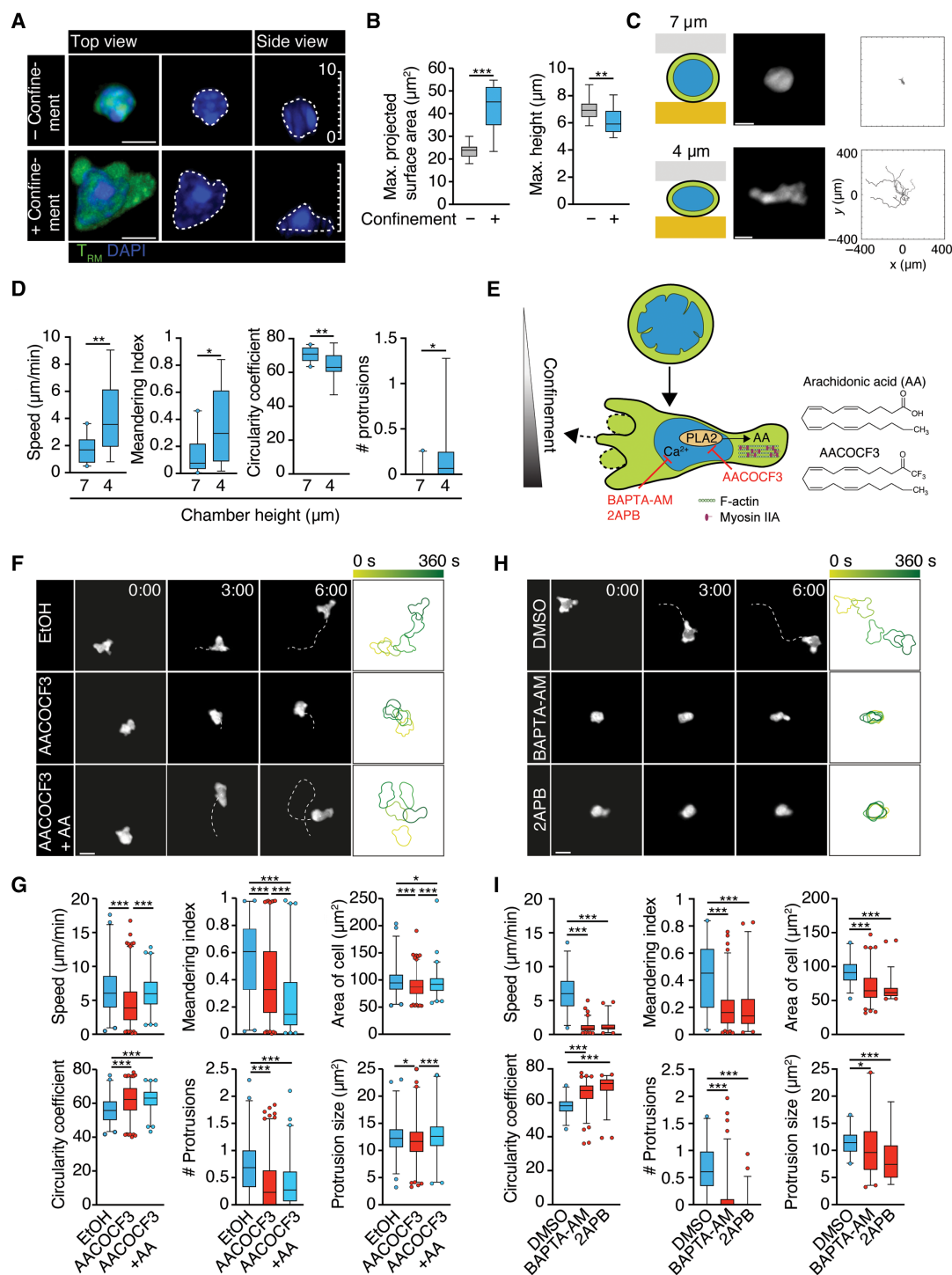


Fig. 4. Confinement-induced nuclear deformation correlates with Ca^{2+} and AA-dependent autonomous SMG T_{RM} motility. (A) Confocal image of nuclear deformation in under-agarose confinement. Scale bars, 5 μm. (B) Projected maximum cross-sectional area and maximum nuclear height with and without confinement ($n = 20$ to 23 nuclei). (C) Schematic layout, micrograph, and exemplary tracks of SMG T_{RM} in the confinement chamber. Scale bars, 5 μm. (D) Dynamic and morphometric analysis of T_{RM} motility in confinement chambers. (E) Scheme of confinement-induced nuclear stretching and selected inhibitors of evasive reflex mediated by Ca^{2+} flux from intracellular stores, induction of PLA2 activity, and release of arachidonic acid (AA). 2APB, BAPTA-AM, and the AA analog AACOCF3 are inhibitors of intracellular Ca^{2+} channels, intracellular Ca^{2+} , and PLA2 activity, respectively. (F) Wide-field fluorescence microscopy images of SMG T_{RM} under agarose migration in the presence of AACOCF3 and AA. Time in min:s. Scale bar, 10 μm. (G) Dynamic and morphometric analysis of T_{RM} motility in the presence of AACOCF3 and AA. Scale bar, 10 μm. (H) Wide-field fluorescence microscopy images of SMG T_{RM} under agarose migration in the presence of BAPTA-AM and 2APB. Time in min:s. Scale bar, 10 μm. (I) Dynamic and morphometric analysis of T_{RM} motility under agarose in the presence of BAPTA-AM and 2APB. Data in (D) ($n = 35$ to 43 tracks), (G) ($n = 296$ to 708 tracks), and (I) ($n = 63$ to 199 tracks) were pooled from at least two independent experiments and analyzed using Student's t test and Mann-Whitney test (D) or Kruskal-Wallis against control (G and I). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

could not induce T_{RM} -like motility in T_N isolated from lymphoid tissue.

To further corroborate the link between nuclear deformation and confinement-induced T_{RM} motility, we used the AA analog AACOCF3 as an inhibitor of PLA2 and other AA-producing enzymes (Fig. 4E) (29). In under-agarose assays, AACOCF3 treatment led to decreased T_{RM} speeds, as well as reduced directionality and number and size of leading edge protrusions (Fig. 4, F and G, and movie S12). Addition of excess AA to AACOCF3-treated T_{RM} rescued SMG T_{RM} speeds, cell spreading, and protrusion sizes. AA-treated T_{RM} cells frequently changed direction, resulting in a low meandering index (Fig. 4, F and G, and movie S12). These data suggest that although SMG T_{RM} are able to respond to external AA, this treatment affects the preservation of stable polarity. We examined whether other T cell populations responded similarly to AA stimulation. Whereas the addition of AA slightly increased chemokinetic T_N speeds (from 0.6 to 1.5 $\mu\text{m}/\text{min}$), this effect was much lower compared with the impact of CCL21, suggesting that AA is not sufficient to induce robust motility in these cells. Along the same line, AACOCF3 treatment did not impair CCL21-induced T_N motility, whereas it partially reduced CXCL10-induced T_{RM} speeds and directionality (fig. S6, C and D).

In addition to AA production, nuclear mechanosensing requires generation of an intracellular Ca^{2+} flux, whereas extracellular Ca^{2+} is not required (29, 30). To address the role for intracellular Ca^{2+} sensing, we blocked intracellular Ca^{2+} by 1,2-bis(2-aminophenoxy) ethane- N,N,N',N' -tetraacetic acid-acetoxymethyl (BAPTA-AM). In parallel, we added 2-aminoethoxydiphenylborane (2APB) to inhibit stretch-sensitive channels, such as inositol triphosphate receptors (InsP3R), which liberate Ca^{2+} from NE and perinuclear endoplasmic reticulum membranes. Both inhibitors resulted in strongly decreased T_{RM} speeds, directionality, cell polarization, and protrusion formation (Fig. 4H and I, and movie S13). Together, confinement-induced SMG T_{RM} migration is susceptible to inhibitors of the nuclear mechanosensing module (29, 30).

Next, we analyzed whether T_{RM} expressed candidate factors involved in nuclear mechanosensing (Fig. 4E). The ubiquitously expressed family member cPLA2a (encoded by *Pla2g4a*) has been implicated in regulating the mechanosensitive response in dendritic cells (DCs) and other cell types (29, 30, 62). However, mining the ImmGen database (www.immgen.org/) showed low to absent

Pla2g4a expression in T cells except for double-negative thymocytes, which we confirmed by quantitative polymerase chain reaction analysis (fig. S7, A and B). For increased sensitivity, we performed a “NanoString” multiplex gene expression analysis of factors potentially involved in the mechanosensitive response by comparing sorted T_N , T_{CM} , T_{EM} , and SMG T_{RM} OT-I T cells. First, we validated the sorting strategy by confirming subset-specific marker detection (fig. S7C). Although this sensitive approach corroborated low to absent *Pla2g4a* expression in T cells, we detected the expression of *Pla2g4b*, *Pla2g10*, *Pla2g12a*, and *Pla2g15* in T_{RM} (fig. S7D). SMG T_{RM} also expressed stretch-activated InsP3Rs, which are involved in intracellular Ca^{2+} release upon nuclear compression (fig. S7E) (29, 63). We did not detect noticeable differences between memory T cell subset expression of factors regulating NE structure, including lamin A/C, lamin B receptor, and emerin, as well as members of the linker of nucleoskeleton and cytoskeleton complex Sun1/2 and nesprins 1 to 4 (fig. S7, F and G). Together, T_{RM} express several Ca^{2+} channels and PLA2 isoforms that might contribute to mechanosensing.

Mechanosensing-induced T_{RM} motility enhances target cell interception

We designed an in vitro system to examine the impact of T_{RM} mechanosensing on target cell encounter as a simplified tissue surveillance test. We placed SMG T_{RM} with a majority of unpulsed B cells spiked with rare cognate pMHC-pulsed, fluorescently labeled B cells in under-agarose confinement (Fig. 5A). In this system, T_{RM} need to actively migrate to identify dispersed target cells among an excess of bystander cells. T_{RM} were actively scanning B cells for the presence of cognate pMHC (movie S14). Within the observation period, almost half of the control T_{RM} (47%) were able to identify and engage with at least one target cell during the observation period, with 8% of T_{RM} interacting with four or more targets (Fig. 5, B and C). Many interactions were short-lived and resulted in cell death as assessed by dye leakage (movie S14). Addition of the PLA2 inhibitor AACOCF3 disrupted the “search and destroy” pattern (Fig. 5B), with only 32 and 2% of T_{RM} engaged with more than one and a maximum of four targets, respectively (Fig. 5C). In sum, confinement-induced random motility enhances the ability of T_{RM} to efficiently track and eliminate dispersed target cells.

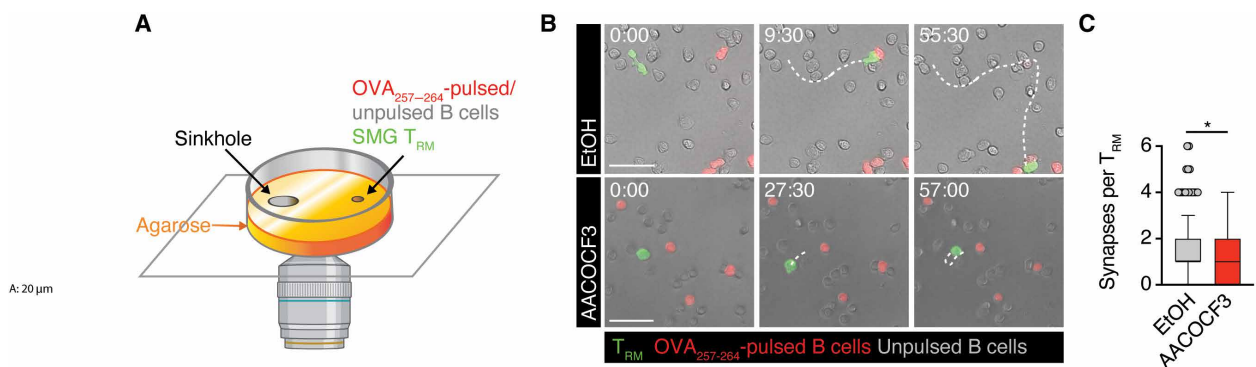


Fig. 5. Blocking mechanosensing impairs T_{RM} target cell identification. (A) Experimental layout. (B) Wide-field fluorescence microscopy images of confined SMG T_{RM} tracking of dispersed target cells with and without AACOCF3. Time in min:s. Scale bars, 30 μm . (C) Quantification of T_{RM} – target B cell synapse formation with or without AACOCF3. Data in (C) ($n = 139$ to 341 synapses) were pooled from two independent experiments and analyzed by Mann-Whitney test. * $P < 0.05$.

In vivo interference with mechanosensing impairs SMG**T_{RM} surveillance**

We set out to expand our *in vitro* observations to tissue surveillance *in vivo*. Confocal analysis confirmed nuclear deformation occurring in SMG T_{RM} within dense secretory epithelium-rich tissue (Fig. 6A). To examine nuclear mechanosensing-triggered pathways during homeostatic SMG surveillance, we used two-photon intravital microscopy (2PM) to quantify baseline OT-I T_{RM} motility parameters in LCMV-OVA-immunized SMGs before overlaying 20 or 50 μ M BAPTA-AM on the SMG preparation for 20 min and recording T_{RM} motility for additional 1 to 4 hours (Fig. 6B and movie S15). BAPTA-AM has been reported to accumulate efficiently in cells *in vivo* (64). Accordingly, the short BAPTA-AM superfusion caused a dose-dependent decrease in SMG T_{RM} speeds and directionality (Fig. 6, C to F), with a concomitant increase in arrest coefficients and sphericity (Fig. 6, F and G). These observations are consistent with the pronounced inhibition of T_{RM} migration by intracellular Ca²⁺ chelation in under-agarose assays (Fig. 4, H and I).

Last, we examined whether mechanosensing contributes to SMG T_{RM} surveillance in response to a local virus rechallenge. We administered an attenuated murine cytomegalovirus (MCMV)-3D- Δ vRAP strain expressing mCherry and the OVA_{257–264} peptide epitope into SMGs via the Wharton's duct of LCMV-OVA-immunized mice in the memory phase, as described (26). Recipient mice were subsequently treated with dimethyl sulfoxide (DMSO) or AACOCF3 for 68 hours after MCMV infection (Fig. 6H). We chose AACOCF3 over BAPTA-AM to avoid an impact on T_{RM} function resulting from impaired intracellular Ca²⁺ flux. Intravital imaging confirmed that immediately after AACOCF3 treatment of LCMV-OVA-memory mice, the SMG T_{RM} scanning behavior was disrupted as measured by a drop in directionality, although speeds were not significantly affected in the conditions used here (fig. S8, A and B). This may reflect the weaker *in vitro* inhibition by AACOCF3 as compared with Ca²⁺ chelators (Fig. 4F). After MCMV infection via the Wharton's duct, we administered anti- α 4 and α _L (LFA-1)-blocking monoclonal antibodies (mAbs) to prevent recruitment of circulating T cells without affecting interstitial T_{RM} motility (26). Neither administration of AACOCF3 over 3 days alone or in combination with anti- α 4 and LFA-1 blocking mAbs had a notable effect on total T_{RM} numbers or their CD69 and CD103 expression (fig. S8, C and D). In control experiments, MCMV-3D- Δ vRAP infection into one SMG lobe did not yield a significant increase in T_{RM} numbers as compared to uninfected contralateral lobes (fig. S8, E and F), irrespective of treatment with anti- α 4 and LFA-1 integrin mAbs or the lymphocyte-sequestering drug FTY720 (fig. S8, F and G). These data suggested no or only limited local proliferation of SMG T_{RM} or T cell recruitment during rechallenge with the attenuated MCMV strain used here. In accordance, we observed only few mCherry⁺ MCMV-infected cells in control- and AACOCF3-treated SMG sections at 68 hours p.i. (fig. S8H), in line with the key role for tissue-resident macrophages for clearance of infected cells (26). Consistent with these observations, a histological analysis of MCMV-challenged SMG sections on day 3 p.i. showed comparable numbers of SMG T_{RM} in control- and AACOCF3-treated recipients (Fig. 6, J and I). In contrast, when we examined T_{RM} distribution as a readout for inflammation-induced T cell accumulation, we observed an average of 10.5 ± 4.6 T_{RM} clusters/section (mean \pm SEM; median, 4.8) in control-treated SMGs, with T_{RM} accumulating both in EpCAM^{high} epithelial tubes and EpCAM^{low} acini (Fig. 6, I and K).

In contrast, T_{RM} clustering in infected SMG was substantially reduced to 1.9 ± 1.9 clusters (median 1.3) in AACOCF3-treated recipients (Fig. 6, I and K). Together, these data support a role for mechanosensing for T_{RM} tissue surveillance of exocrine glands as a complementary mechanism to canonical motility driven by biochemical cues (Fig. 6L) (29, 30, 65, 66).

DISCUSSION

CD8⁺ T_{RM} surveil organs with diverse tissue architectures, cellular and extracellular matrix compositions, and varying degrees of microbial exposure. Conceivably, organ surveillance is accomplished by local adaptations of their patrolling modus to enable efficient cell scanning and interception of viral infections. In epithelial barriers that are constitutively exposed to microbes, chemokines and integrin ligands contribute to T_{RM} surveillance (6–12). Here, we report that exocrine gland T_{RM} have the capacity of spontaneous F-actin treadmilling, which endows them with an internal force generation module that bypasses the requirement for chemoattractant and adhesion receptor sensing. Instead, a mechanosensing module allows these cells to measure the degree of confinement-induced mechanical load. This module is coupled to a promigratory response and is susceptible to inhibitors of the nuclear mechanogauge, implying a nongenetic function of the nucleus in this process. Thus, in these nonbarrier organs with low constitutive chemokine expression (27), memory T cell subsets are equipped to sense and react to the physical properties of their tissue of residence, extracting promigratory cues from spatial confinement for baseline surveillance. At the same time, they retain their chemosensing capacity to complement their protective function during infection, suggesting a multitiered surveillance strategy.

Amoeboid cell migration is based on continuous polymerization of F-actin at the leading edge and contraction of F-actin filaments by myosin motors at the trailing edge of the cell (48). Depending on the cell type examined, amoeboid migration spans a continuum from mostly Rho-driven actomyosin contractility-driven (as in some DCs) to mostly Rac-driven motility lacking protrusions (as in T_N) (23, 42). Our data suggest that SMG T_{RM} acquire a blend of these migration modes, presumably as an adaptation to the confined microenvironment of exocrine glands (26). Our data further indicate that mechanical load is sufficient to trigger exocrine gland T_{RM} polarization and motility, accompanied by increased cortical actomyosin contractility. Mechanosensing is increasingly acknowledged to play a central role in the immune system (67–70). On a molecular level, T cells use their TCR as a mechanosensitive receptor to gauge pMHC affinity and for target cell elimination, in a process called mechanosurveillance (71, 72). In the innate immune system, mechanical cues detected by Piezo1 ion channels in macrophages contribute to lung inflammation (73), and subsets of spleen DCs use adhesion G protein-coupled receptors as cues for proper positioning close to venous sinusoids (74). In motile T_{RM}, mechanosensing is susceptible to pharmacological inhibitors of signaling pathways induced by nuclear compression. Until recently, the nucleus has been mainly considered as a passive storage for genetic information. Furthermore, as the cell's biggest organelle, the nucleus constitutes a major obstacle to cellular passage through constricted spaces (75). However, recent findings in DC and other leukocytes have uncovered a central role for the nucleus in selecting the migratory path by determining

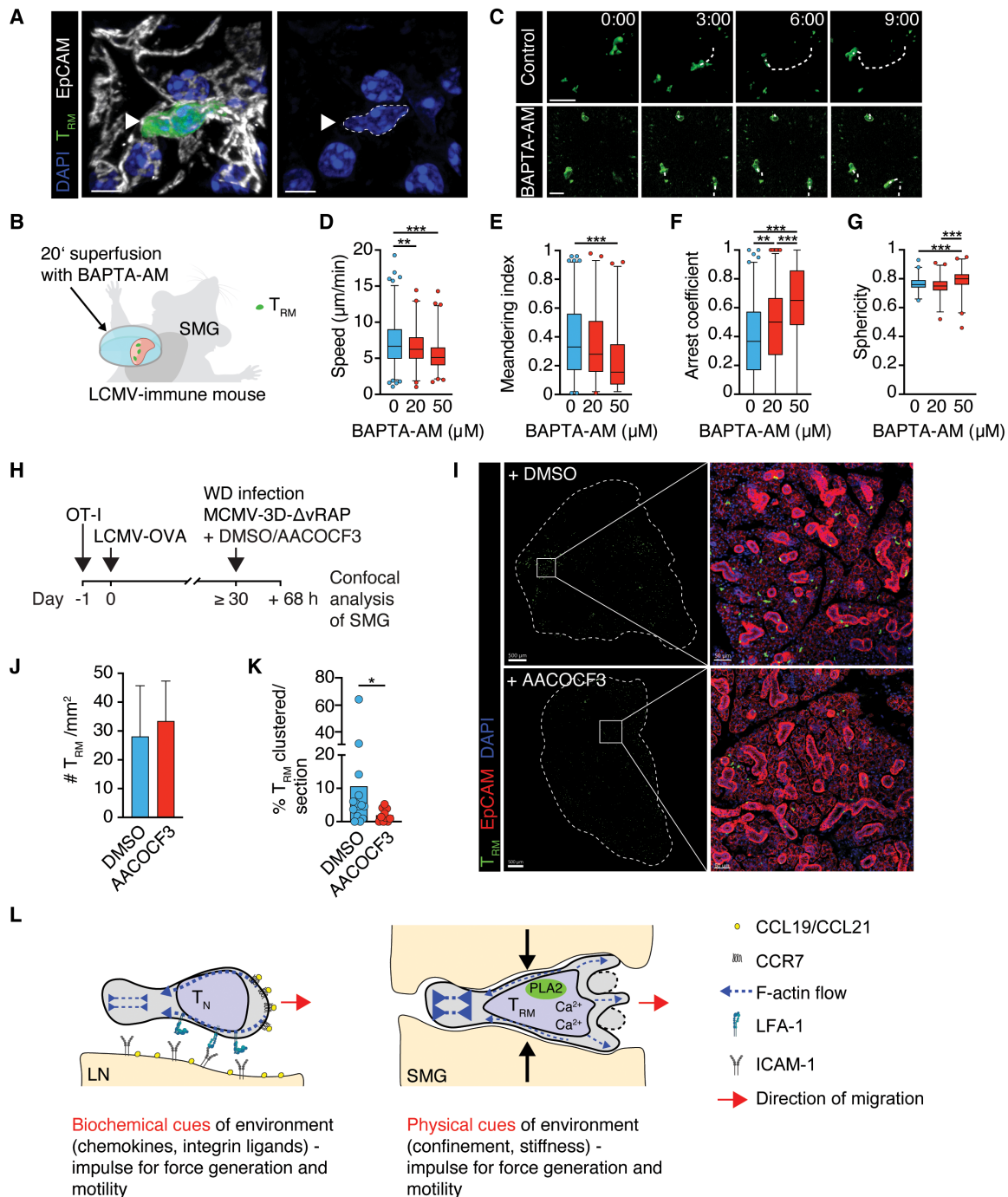


Fig. 6. Blocking mechanosensing disrupts in vivo SMG T_{RM} migration. (A) Confocal image of SMG T_{RM} (arrowhead) in situ. Scale bars, 5 μm . (B) Scheme of BAPTA-AM superfusion. (C) 2PM images of SMG T_{RM} migration before (baseline) and after 50 μM BAPTA-AM superfusion. Scale bars, 10 μm . (D to G) Speeds (D), meandering index (E), arrest coefficient (F), and sphericity (G) of T_{RM} before and 1 to 4 hours after superfusion with BAPTA-AM. (H) Experimental layout of MCMV-3D- $\Delta vRAP$ rechallenge. (I) Confocal sections of MCMV-challenged SMGs in control (DMSO)- and AACOCF3-treated recipients. The dotted line marks the outline of SMG section; the square depicts insert. Scale bar, 500 and 50 (insert) μm . (J) Number of T_{RM} per area in control- and AACOCF3-treated SMGs. (K) Percent of clustered T_{RM} in control- and AACOCF3-treated recipients in 10 to 14 sections from three independent experiments ($n = 24$ control- and AACOCF3-treated mice in total). (L) Graphical summary. Data in (D to G) ($n = 207$ to 323 tracks) and (J and K) are pooled from at least two experiments and analyzed using Kruskal-Wallis (D to G), Student's t test (J), or Mann-Whitney test (K). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

permissive pore sizes (76). In line with these findings, the nucleus also serves as a central element for cellular proprioception. Much like humans sense and react to their environment through sight,

hearing, touch, smell, and taste, recent studies have provided compelling evidence for nuclear deformation and an autonomous contractile response as the cellular correlate of space and pressure

sensing (65, 77). To accomplish this feat, the nucleus measures absolute dimensions through the stretching of its NE, beyond which a cell-specific set point triggers an “evasive reflex” via increased cortical contractility and spontaneous cell translocation (29, 30). Given its low constitutive membrane trafficking, the quiescent NE is particularly suited as low noise detector to convert mechanical perturbations into chemical signals as compared with plasma membranes, which display rapid turnover (78).

To date, the precise factors generating AA and other signals involved in nuclear mechanosensing are not well characterized owing to the complex cell type-specific regulation of lipid metabolism and Ca^{2+} channel expression (79–81). As an example, the cPLA2a isoform, which has been implicated in regulating the contractile response in DCs and zebrafish progenitor stem cells (29, 30, 82), is absent or only expressed at very low levels in mature T cells. Together with the fact that compensatory mechanisms between different isoforms might occur, genetic approaches to disrupt mechanosensing in T_{RM} constitute a formidable challenge. Although many cell types display a Ca^{2+} and PLA2-mediated response upon nuclear disturbance (29, 30, 62), our data suggest that mechanosensing-driven motility is not a universal feature of all T cell subsets, at least not in the experimental conditions applied here. Thus, T_{N} , T_{CM} , and T_{RM} lodged in gut and skin epithelium do not spontaneously move when placed under confinement (26). For the latter population, this may reflect their greater dependence on $\text{G}\alpha_i$ -coupled signaling as compared with exocrine T_{RM} (8). Local chemokines induced by constant microbial exposure might help avoid accidental egress of epithelial T_{RM} outside the host by restricting them in close vicinity to the basement membrane. In turn, mechanosensing-triggered motility for baseline surveillance might be preferentially induced in tissues with limited microbial exposure, where steady-state chemokine levels are low (27). This surveillance strategy equips T_{RM} with flexibility to surveil multiple organs with diverse cellular and matrix composition, while retaining responsiveness to inflammation-triggered local chemokine gradients.

As a limitation, despite providing solid evidence for mechanosensing capacities in T_{RM} subsets, the molecular pathways involved in this process remain undefined. Furthermore, the use of pharmacological inhibitors to interfere with confinement-induced T_{RM} motility might affect additional signaling pathways in these cells or influence bystander cells in *in vivo* experiments. Along the same line, the *in vivo* pharmacokinetics of the PLA2 and Ca^{2+} flux inhibitors are not well defined, rendering an assessment of the degree of inhibition after administration difficult. Last, our study does not address to which extent mechanosensing is a property shared with other leukocytes, such as natural killer cells lodged in exocrine glands.

In sum, although it is well established that the transition of T_{N} to T_{EFF} and memory T cell subsets is accompanied by changes in the chemoattractant and adhesion receptor repertoire, our data suggest that these changes do not fully explain the scanning behavior of these cells in distinct organs. Thus, physical properties of the tissue of residency are integrated by adaptive immune cells with local biochemical cues for a specific multitier immune surveillance strategy. It is conceivable that mechanosensing elicits site-specific signaling and epigenetic and metabolic adaptations in local immune cell populations, encouraging further investigation into the underlying molecular regulation.

MATERIALS AND METHODS

Study design

The aim of this study was to examine the mechanisms underlying intrinsic motility of SMG T_{RM} . To this end, we systemically administered LCMV-OVA and isolated Ag-specific CD8^+ T cells from SMG and other organs. We applied *ex vivo* migration assays, immunofluorescence, intravital imaging, gene expression analysis, and flow cytometry to compare their migration requirements with CD8^+ T_{N} . Furthermore, we administered pharmacological inhibitors of small guanosine triphosphatase and nuclear mechanosensing signaling pathways to functional assays. Last, we assessed the role of the T_{RM} mechanosensing module for target finding and cell clustering after viral rechallenge. Mice or isolated T cells were randomly assigned to experimental groups and analyzed without excluding outliers, and experimenters were not blinded. The numbers of independent experiments are indicated in the figure legends.

Mice

Tg(TcraTcrb)1100Mjb (“OT-I”) TCR transgenic mice (31) were backcrossed to C57BL/6-Tg(CAG-EGFP)10sb/J (“GFP⁺”) (83), hCD2-dsRed (“dsRed⁺”) (84), tdTomato-expressing Ai14 \times ZP3 (“tdTom⁺”) (85, 86), mT/mG (87), and LifeAct-GFP lines (33). GFP⁺ OT-I were further crossed to CD4-Cre \times MYH9^{flox/flox} (88), DOCK2^{-/-} (40) and DOCK8^{-/-} (89) lines, and dsRed⁺ OT-I to the MYH9-GFP reporter line (49). DOCK2-GFP was described before (90). All mice were bred at the animal facility of the University of Bern and the University of Fribourg, Switzerland and were used as lymphocyte donor mice. Six- to 10-week-old male and female sex-matched C57BL/6J mice (Janvier, Le Genest-Saint-Isle, France) were used as recipient mice. All experiments were performed in accordance to federal animal experimentation regulations and approved by the cantonal committee.

T cell transfer and viral infections

CD8^+ T cells were negatively isolated from spleen and peripheral LNs using the EasySep Mouse CD8^+ T cell Isolation Kit (STEM-CELL Technologies) or the MojoSort Mouse CD8^+ T cell Isolation Kit (BioLegend) according to the manufacturer’s instructions. OT-I T cells (5×10^4) were intravenously transferred into recipient mice 24 hours before intraperitoneal infection with 10^5 plaque-forming units (PFU) LCMV-OVA (32).

Reagents

Sodium pyruvate (P04-43100), Hepes buffer (P05-01100), minimum essential medium nonessential amino acids (P08-32100), L-glutamine (200 mM; P04-80100), and PenStrep (P06-07100) were purchased from PAN-Biotech, and RPMI 1640 (#21875-034) and fetal bovine serum (10270-106) were from Gibco. CPYPP (Tocris) was used at 50 μM ; CK666 (R&D), at 100 μM ; ML141 (Tocris) and MLCK inhibitor peptide 18 (Calbiochem), at 20 μM ; Y27632 (Sigma-Aldrich or Hello Bio), at 20 μM or 200 μg per mouse *in vivo*; AACOCF3 (Tocris or Enzo Life Sciences), at 20 μM or 300 μg per mouse *in vivo*; BAPTA-AM (Cayman Chemical), at 10 μM ; AA (Cayman Chemical), at 70 μM ; 2APB (Tocris), at 100 μM ; W56/F56 (R&D), at 100 μM ; and Rhosin (Sigma-Aldrich), at 10 μM .

Flow cytometry analysis

At indicated time points, single-cell suspensions of spleens, LNs, and SMGs were prepared for flow cytometry as described in the

Supplementary Materials and acquired on Attune NxT Flow Cytometer (Thermo Fisher Scientific) or LSRFortessa (BD). Data were analyzed with FlowJo.

Immunofluorescence

OT-I memory cells were isolated from SMGs and spleens of C57BL/6 mice infected with LCMV-OVA at >30 days p.i. Single-cell suspensions of SMGs and spleens were generated as above and sorted for tdT^+ T cells. Ibidi sticky slides (untreated side) with a 12-well removable chamber were coated with fibronectin (10 $\mu\text{g}/\text{ml}$; Sigma-Aldrich). Cells were added and incubated for 2 hours at 37°C , fixed with 4% paraformaldehyde for 20 min at room temperature (RT), washed, and permeabilized using BD Fixation/Permeabilization solution 20 min at 4°C . Cells were blocked with BD Perm/Wash 2% goat serum for 1 hour at RT, stained with rabbit anti-pMLC (Cell Signaling Technology, 3674) overnight at 4°C , washed, stained with a goat anti-rabbit AF647 (Invitrogen, A32733) for 1 hour at RT, and washed again. The 12-well removable chamber was removed, and the sticky slide was mounted on a glass slide with Prolong Gold Antifade with DAPI (Thermo Fisher Scientific) and let dry at RT for 24 hours. Images were taken with a Leica SP5 confocal microscope using a $63\times$ glycerin objective [APO CS, numerical aperture (NA) 1.3]. Data were analyzed with Imaris (Bitplane). For SMG sections, SMGs were frozen in optimal cutting temperature (OCT) and cryosectioned for immunofluorescence (6 or 10 μm), followed by labeling with goat anti-GFP (Rockland, 600-101-215) and anti-EpCAM AF647 (BioLegend, 118212) for epithelium.

2PM image acquisition and analysis

2PM intravital imaging of SMGs was performed as described in (91) and in the Supplementary Materials. ImSpector software was used to control the 2PM system and to acquire images with an automated system providing real-time drift correction (92). Data were analyzed with Imaris (Bitplane) and a customized script for arrest coefficient analysis with a threshold of 4 $\mu\text{m}/\text{min}$ as described (26).

Under-agarose and confinement chamber assays

T_N were isolated from spleen and PLN of a naive mouse using the EasySep Mouse CD8^+ T cell Isolation Kit (STEMCELL Technologies) or the MojoSort Mouse CD8^+ T cell Isolation Kit (BioLegend) according to the manufacturer's instructions. T_RM were isolated from SMGs, LGs, or SIs of C57BL/6 mice infected by LCMV-OVA for >30 days by flow cytometry sorting. Under-agarose assays were performed as described in (26) and in the Supplementary Materials. For target cell engagement experiments, 2×10^4 SMG T_RM were mixed with 0.2×10^6 1 nM OVA_{257–264}-pulsed B cells isolated using the EasySep Mouse B cell Isolation Kit (STEMCELL Technologies) and labeled with 2.5 μM (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (CMTMR) together with 1×10^6 unpulsed, nonlabeled B cells in the presence of B cell-activating factor of the tumour-necrosis-factor family (BAFF) (100 ng/ml; R&D Systems, 8876-BF-010). Time-lapse images were taken from the center of the dish using a Zeiss fluorescent microscope (Axio Observer, Zeiss) or a GE DeltaVision Elite wide-field fluorescent microscope (U plan, S Apo, NA 0.75). Images were taken every 20 s for 15 to 20 min. For the TIRF images, the GE DeltaVision microscope was used with a laser module and a $60\times$ TIRF objective.

A Dynamic Cell Confiner device (4Dcell, France) was used to study T_RM cell migration under defined chamber heights. Polydimethylsiloxane suction cups with 4- or 7- μm -high micropillars were prepared as described in the manufacturer's instructions. Plates were coated with 2% HSA. Sorted SMG T_RM (10^5 cells) were transferred into each plate. Before imaging, the suction cup was plugged to an adjustable vacuum source. Once confined at a pressure from -50 to -100 mbar, cells were imaged with a GE DeltaVision microscope as above.

Dynamic and morphometric cell analysis

Cortical F-actin flow in TIRF image sequences was measured using the Imaris (Bitplane) particle tracking function to generate tracks of GFP^+ F-actin speckles, as previously described (35, 36, 93). The global speed depicts displacement over tracking time for each track. Morphometric data were analyzed with Imaris or a custom-made FIJI plugin (source code is available at https://github.com/Matthieu-Palayret/DynMorpho_Analysis; fig. S2). For dynamic and morphometric cell analysis, cell contours were determined using the ADAPT plugin (94). For each trajectory, the dynamic and morphometric analysis consisted of calculating six outputs: the average speed of the cell; the meandering index of the trajectory, which is a measure of its linearity, calculated as the ratio of the distance separating the initial and final points of the trajectory over the distance traveled by the cell (the ratio equals 1 if the trajectory is exactly linear); the average area of the cell; its average circularity coefficient, calculated as the average over the trajectory of the ratio of the true area of the cell over the area of a circular cell that would have the same perimeter ($=4\pi \times \text{area}/\text{perimeter}^2$ – the coefficient equals 100 when the cell is perfectly circular); and both the average number and size of the protrusions of the cell (the uropod being excluded) over its whole trajectory.

3D collagen matrix migration

T_RM migration through a confined 3D collagen type I matrix was performed in μ -Slide Chemotaxis ibiTreat chambers (ibidi, Switzerland) as described (95). Briefly, sorted LifeAct-GFP \times mT/mG T_RM were resuspended at 5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. PureCol collagen I (30 μl ; CellSystems, Troisdorf, Germany), 4 μl of $10\times$ DMEM, and 2 μl of 7.5% NaHCO_3 were premixed, carefully mixed with 18 μl of cell suspension, and applied to μ -Slide Chemotaxis chambers. Collagen was allowed to polymerize for 40 min at 37°C in a humidified incubator. Live cell imaging was performed on a laser scanning microscope (Leica TCS SP5; Leica, Switzerland) using an HCX PL APO CS $63.0\times$ (NA 1.40) oil ultraviolet objective at 0.65-s intervals on a microscope stage fitted with a Tokai Hit Thermo Plate at 37°C .

MCMV rechallenge and cluster analysis

MCMV rechallenge experiments were performed as described (26). Briefly, GFP^+ OT-I cells (5×10^4 per mouse) were transferred into C57BL/6 mice and intraperitoneally infected on the following day with 10^5 PFU of LCMV-OVA. At >30 days p.i., mice were treated with vehicle (DMSO) or 300 μg of AACOCF₃, together with anti- $\alpha 4$ (clone PS/2) and anti- αL (LFA-1; clone FD441.8; each 100 μg per mouse) mAbs (26). After 2 hours, 10^6 PFU of the attenuated MCMV-3D- Δ vRAP strain expressing the SIINFEKL peptide epitope (96) were locally administered to the right SMG via Wharton's duct injection. Recipient mice were treated every 12 hours with 300 μg of AACOCF₃.

or vehicle (final volume, 100 μ l in saline) and at 48 hours p.i. again with PS/2 and FD441.8 as above. Alternatively, we administered FTY720 (2 μ g/g body weight) daily. On day 3 (68 hours after MCMV injection), mice were perfused with 4% paraformaldehyde (PFA), and the right SMG was harvested, fixed overnight at 4°C in 5 ml of 4% PFA/phosphate-buffered saline, transferred into 30% sucrose, and kept at 4°C for 24 hours. SMGs were frozen in OCT and cryosectioned for immunofluorescence (6 or 10 μ m), followed by labeling with goat anti-GFP (Rockland, 600-101-215) and donkey anti-goat AF488 (Thermo Fisher Scientific, A11055), and rabbit anti-red fluorescent protein (Abcam, ab62341), and donkey anti-rabbit AF555 (Thermo Fisher Scientific, A32794) for viral foci. Tile scans were obtained by wide-field (DeltaVision) or confocal (Leica STELLARIS Falcon 8) fluorescence microscopy, and T_{RM} were identified using the Imaris “spots” function. Nine neighbor clusters were empirically defined by choosing a maximum distance of 62 μ m between spots. The percentage of clustered cells was obtained by dividing the number of clustered over total number of spots.

Statistical analysis

Two-tailed, unpaired Student's *t* test, Mann-Whitney *U* test, one-way analysis of variance (ANOVA) with Dunn's multiple comparisons test, Kruskal-Wallis test, or a Wilcoxon rank test was used to determine statistical significance (Prism, GraphPad). Whiskers in box-and-whisker plots depict a range of 90 to 100% of individual values (nonincluded values are shown as individual dots), whereas the box comprises 50% of all data points and the line within the box displaying the median. Significance was set at *P* < 0.05.

Supplementary Materials

This PDF file includes:

Material and Methods

Figs. S1 to S8

Other Supplementary Material for this manuscript includes the following:

Movies S1 to S15

Data file S1

MDAR Reproducibility Checklist

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Exocrine gland–resident memory CD8⁺ T cells use mechanosensing for tissue surveillance

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Editor's summary

Tissue-resident CD8⁺ T cells (T_{RM}) are constantly surveilling organs and tissues for the presence of uninvited microbes. Previous studies have shown that T_{RM} migration is triggered by signaling via chemoattractant and adhesion molecules, which facilitates rapid detection of infected cells. More recent evidence has indicated that T_{RM} within submandibular salivary glands display different motility patterns exclusive of chemosensing. Ruef *et al.* now show that SMG T_{RM} from virally infected mice display spontaneous retrograde F-actin flow as a means of force-generated translocation. Similar patterns of locomotion were detected in T_{RM} from other exocrine glands and were dependent on the sensing of changes in mechanical loads through signals triggered by nuclear deformation. These findings provide critical insight into how T_{RM} can surveil tissue independent of chemosensing. —Christiana Fogg

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