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In Vivo Analysis of Uropod Function during Physiological T Cell Trafficking

Silvia F. Soriano,*1 Miroslav Hons,*1 Kathrin Schumann,† Varsha Kumar,* Timo J. Dennier,* Ruth Lyck,* Michael Sixt,†‡ and Jens V. Stein*

Migrating lymphocytes acquire a polarized phenotype with a leading and a trailing edge, or uropod. Although in vitro experiments in cell lines or activated primary cell cultures have established that Rho-p160 coiled-coil kinase (ROCK)-myosin II-mediated uropod contractility is required for integrin de-adhesion on two-dimensional surfaces and nuclear propulsion through narrow pores in three-dimensional matrices, less is known about the role of these two events during the recirculation of primary, nonactivated lymphocytes. Using pharmacological antagonists of ROCK and myosin II, we report that inhibition of uropod contractility blocked integrin-independent mouse T cell migration through narrow, but not large, pores in vitro. T cell crawling on chemokine-coated endothelial cells under shear was severely impaired by ROCK inhibition, whereas transendothelial migration was only reduced through endothelial cells with high, but not low, barrier properties. Using three-dimensional thick-tissue imaging and dynamic two-photon microscopy of T cell motility in lymphoid tissue, we demonstrated a significant role for uropod contractility in intraluminal crawling and transendothelial migration through lymph node, but not bone marrow, endothelial cells. Finally, we demonstrated that ICAM-1, but not anatomical constraints or integrin-independent interactions, reduced parenchymal motility of inhibitor-treated T cells within the dense lymphoid microenvironment, thus assigning context-dependent roles for uropod traction during lymphocyte recirculation. The Journal of Immunology, 2011, 187: 000–000.

The high diversity of the Ag-receptor repertoire and the resulting low frequency of Ag-specific T cells in an organism pose an important logistic challenge, which is to quickly allow for efficient encounters between APCs and rare specific T cells close to potential microbial entry sites. To meet this challenge, strategically positioned secondary lymphoid organs, including spleen, peripheral lymph nodes (PLNs), and bone marrow (BM), have evolved, and these secondary lymphoid organs serve as meeting points for tissue-borne APCs and continuously passing lymphocytes (1–3). Recruitment of blood-borne lymphocytes in PLNs occurs in high endothelial venules (HEVs), specialized postcapillary venules characterized by their expression of peripheral node addressin (PNAd). PNAd serves as a ligand for L-selectin expressed on naive lymphocytes, allowing cells to roll along the luminal surface of HEV, which is covered with high levels of the CCL21 chemokine (4). Binding of CCL21 to its receptor CCR7 induces rapid (>1 s) conformational activation of the LFA-1 integrin required for firm arrest of rolling lymphocytes, and it induces over the next tens of seconds to few minutes the acquisition of a polarized phenotype (5–7). Polarized lymphocytes then crawl in an ICAM-1–dependent manner along the HEV surface prior to transendothelial migration (TEM) through narrow pores of the endothelial barrier and negotiate their passage through the cuff of basement membrane meshworks surrounding fibroblast reticular cells (FRCs) and occasional pericytes (8–12). FRCs constitute the stromal backbone of the T cell area in the PLN parenchyma and form a three-dimensional (3D) network, which T cells use as contact guidance cues during their scanning of APCs inside the tightly packed lymphoid microenvironment (13). This migration is mediated, in part, by ICAM-1 and CCL21 presumably presented on the FRC surface, before T cells exit PLNs through efferent lymphatic vessels after an average dwelling time of a few hours (14–16). CXCL12 and α4 integrins are likely candidates for inducing firm adhesion and guiding naive lymphocytes through BM sinusoids into BM parenchyme (17).

The chemokine-driven rapid transition of spherical blood-borne lymphocytes to fully polarized cells with a leading edge and a uropod is orchestrated by the concerted activation of small GTPases of the Ras and Rho family (7, 18). Although Rac1 and Rac2 direct T cell protrusion at the leading edge (19), the formation of the uropod depends on the activity of Rho, and, at least in part, of its downstream effector Rho-p160 coiled-coil kinase (ROCK) (5, 20). Nonmuscle myosin IIA also localizes to the uropod, where it promotes actin–myosin contractions downstream of ROCK-dependent phosphorylation of the myosin L chain, either directly or indirectly by inactivating MLC phosphatase (21, 22). In vitro experiments support an important role for the Rho–ROCK–myosin signaling axis and uropod contraction during leukocyte migration in at least three processes. First, in two-
diminished (2D) systems, Rho–ROCK activity limits the adhesion interface, probably by promoting cortical tension. Therefore, upon blocking ROCK activity using the pharmacological inhibitor Y27632 or myosin II ATPase activity with blebbistatin, cells spread more, even on nonadhesive substrates, thereby limiting migration speed (23–27). Second, myosin contractility is required for LFA-1 de-adhesion during migration on ICAM-1–coated 2D surfaces. Upon cell treatment with Y27632 or blebbistatin, leukocytes remain attached with their uropod, restraining net cell displacement promoted by the probing leading edge (28–30). Finally, uropod contractility is required for integrin-independent dendritic cell squeezing through 3D meshworks. This is mainly due to the trapping of the relatively large nucleus in narrow pores; thus, it can be modulated by increasing the pore size of the 3D network (31). Accordingly, T cell blast migration to CCL19 and CCL21 in chemotaxis assays using 3-μm pores and dense collagen matrices is significantly diminished by ROCK inhibition (25, 32).

Thus far, most studies addressing the role of uropod contraction and de-adhesion in lymphocytes were performed in vitro with preactivated cells and on 2D surfaces, in the absence of shear forces. Because T cell activation dramatically alters nuclear size, actocytoskeletal activity, and overall integrin avidity, the role of ROCK–myosin II activity in the dynamic behavior of recirculating, nonactivated lymphocytes on different vascular beds under shear has not been thoroughly investigated. Therefore, it is not known whether ROCK activity is required for TEM in vivo or whether cells are trapped in the perivascular or parenchymal space when uropod contractility and integrin de-adhesion are impaired. Similarly, although a recent study revealed that myosin II is required for efficient lymphocyte migration within lymphoid parenchyma (27), it remains unclear whether this is a consequence of ICAM-1–induced retention, impaired nuclear propelling owing to the dense microenvironment, or nonspecific overadhesiveness to adjacent cells present within lymphoid tissue. Taken together, the importance of ROCK–myosin function in naive lymphocytes during their trafficking routes through various endothelial barriers and lymphoid parenchyma displaying site-specific microarchitectural constraints is not well described.

In this study, we analyzed the impact of impaired ROCK and myosin II activity on transmigration of primary mouse lymphocytes in vitro and in vivo, using pharmacological inhibitors combined with live imaging. Our data suggested that, similar to other cell types, the in vitro efficiency of integrin-independent directed T cell migration depends on intact uropod contractility when the pore diameter is small. Under flow conditions, CCL21-induced crawling and transmigration are impaired in Y27632-treated T cells in an endothelial cell type-dependent manner in vitro, correlating with the endothelial barrier properties. To investigate the function of ROCK in vivo, we applied a novel thick-tissue–imaging approach to examine T cell entry into PLN and BM parenchyme and found an organ-specific defect in T cell homing in the presence of Y27632. Finally, two-photon microscopy (2PM) experiments of T cell behavior within PLNs of live, anesthetized mice revealed that TEM and parenchymal motility were impaired in the absence of ROCK activity, the latter in an ICAM-1–dependent manner.

Materials and Methods

Mice

Five- to ten-week-old male and female C57BL/6 mice were purchased from Harlan and used for all experiments. ICAM-1-deficient mice on the C57BL/6 background were described previously (33). All experiments were performed with the approval of and in accordance with kantonal and federal animal-experimentation regulations.

Reagents

Y27632 and blebbistatin were purchased from Calbiochem (San Diego, CA). CCL21 and CXCL12 were provided by PeproTech (London, U.K.), and CXCL13 was from R&D Systems (Minneapolis, MN). CFSE, 5-chloromethylfluorescein diacetate (CMFDA; CellTracker Green), 7-amino-4-chloromethylcoumarin (CMAC; CellTracker Blue), and 5-(and-6)-((4-chloromethylbenzoyl)amino) tetramethylrhodamine) (CMTMR; CellTracker Orange) were purchased from Molecular Probes (Eugene, OR).

Lymphocyte-polarization assay

T cells were treated with DMSO (wild type [WT]), Y27632 (20 μM), or blebbistatin (30 μM) for 1 h; stimulated in suspension with 100 nM CCL21 final concentration; and immediately plated on fibronectin-coated chamber slides for incubation at 37°C. After 1 h, T cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, permeabilized in PBS/0.2% Tween-20 for 20 min, and blocked in PBS/5% BSA for 20 min. Immunostaining was performed with biotinylated anti-CD44 mAb (BioLegend, San Diego, CA) or PKCζ mAb (Santa Cruz Technology, Santa Cruz, CA), followed by labeling with Alexa Fluor 488–conjugated streptavidin or Alexa Fluor 555-conjugated anti-rabbit pAb, respectively (Invitrogen). Fluorescence and phase-contrast images were acquired with a Zeiss AxiosObserver microscope equipped with an Apotome, using a 40× objective. The T cell length and width were measured using Zeiss imaging software and expressed as the polarity index (i.e., the ratio of length/width of the cell).

Chemotaxis assay

T or B cells were purified by negative immunomagnetic cell sorting (Dynal, Oslo, Norway) with purity yields >95%. Chemotaxis assays were performed using Transwell chambers (3- or 5-μm pore size, Costar, Amsterdam, The Netherlands), with 100 μl cell suspension (5 × 105 cells/ml) in the top chamber and indicated concentrations of CCL21, CXCL12, or CXCL13 in the bottom chamber. After 2 h at 37°C, 7% CO2, the percentage of migrated cells was calculated by flow cytometry after comparing with a precalibrated bead standard (Sigma-Aldrich) and correcting for variations in input concentrations.

Collagen matrix assay

Three-dimensional collagen gel chemotaxis assays were done as described previously (31). Briefly, PureCol (INAMED, Fremont, CA) in 1× Eagle’s MEM (Sigma-Aldrich) and 0.4% sodium bicarbonate (Sigma-Aldrich) was mixed with OTII T cell blasts in RPMI 1640 (Invitrogen) and 10% FCS (Invitrogen) at a ratio resulting in gels with the indicated concentration. Final cell concentrations in the assay were 100 cells/ml gel. Collagen–cell mixtures were cast in custom-made migration chambers with a thickness of 0.5–1 mm. After 30-min assembly of the gels, cells were recorded with custom-built brightfield video microscopy setups using a 10× time lapse. Cells were manually tracked using the ImageJ chemotaxis plug-in.

Flow chamber assays

These assays were essentially carried out as described, using primary mouse brain microvascular endothelial cells (pBMMECs) or the endothelial cell line bEnd5 (19). In brief, a custom-built laminar flow chamber was placed on the CCL21-coated pBMMEC or bEnd5 monolayer or on ICAM-1–coated slides and mounted on the stage of a computer-controlled inverted microscope equipped for automated fluorescence and phase-contrast imaging (Axi-oObserver Z1; Zeiss, Feldbach, Switzerland). All flow experiments were conducted inside a heat-controlled chamber at 37°C. DMSO- and Y27632-treated T cells (5 × 105 cells/ml) labeled with CellTracker dyes (CFSE, 2.5 μM and CMTMR, 5 μM; 20 min at 37°C) were perfused with an automated syringe pump (Harvard Apparatus) at a wall shear stress of 0.24 dyne/cm² for 1–2 min to allow cells to accumulate on the endothelium or on ICAM-1. Subsequently, shear flow was increased to 1 dyne/cm² and kept constant during the remaining recording period, while time-lapse images (one frame every 20 or 30 s) were taken. All images were recorded through a 20× or 40× objective (LD Plan-Neofluar; Zeiss, Feldbach, Switzerland) with phase or differential interference contrast and fluorescence illumination. Image analysis was performed using ImageJ software using the manual tracking, chemotaxis and migration tools, and color-merging plug-ins.

Short-term homing and 3D immunofluorescence

These assays were essentially carried out as described (12). Purified T cells (1 × 10³ cells/ml) in RPMI 1640/10% FCS were labeled with CellTracker Blue (CMAC; 20 μM), CellTracker Green (CMFDA; 3 μM), or CellTracker Orange (CMTMR; 5 μM) for 20 min at 37°C. CMAC-labeled...
T cells were additionally treated with DMSO, whereas CMFDA- and CMTMR-labeled cells were incubated with Y27632 (20 μM) or blebbisatin (30 μM) for 1 h. After washing, 1 × 10^7 cells/ml in RPMI 1640/10% FCS were labeled with CMTMR (5 μM) for 20 min at 37˚C and additionally treated with DMSO or Y27632 (20 μM) for 1 h. A total of 1 × 10^7 cells was injected i.v. into age- and sex-matched C57BL/6 recipient mice. Twenty minutes after cell transfer, 100 μg Mel-14 mAb (anti-i–selectin) and 5 μg Alexa Fluor 633-conjugated MECA-79 mAb (anti-β2m) were injected i.v. 20 min later, mice were killed using iso-flurane (Minrad, Buffalo, NY), perfused with 5 ml cold PBS to wash off blood-borne cells, and fixed in 10 ml cold PBS/1% PFA. PLNs were collected and carefully cleaned from surrounding fat tissue under a stereomicroscope. After fixation for 1 h in PBS/1% PFA, PLNs were rinsed in PBS and embedded in 1.3% low melting point agarose (Sigma). Agarose blocks containing fixed PLNs were excised and dehydrated with 100% MeOH for 6 h before tissue clearing in benzyl alcohol–benzyl benzoate (1:2 ratio) for ≥12 h. Blocks (500 × 500 × 400–800 μm) of agarose-embedded PLNs were imaged using a 2PM setup (TrimScope; LaVision, Biotec, Bielefeld, Germany) and, in some cases, were spectrally unmixed using ImScope software. The absolute number and localization of differentially labeled T cells within a tissue block were determined using Volocity (Improvision; PerkinElmer, Cambridge, U.K.).

For 3D immunofluorescence (3-DIF) of BM, purified T cells (1 × 10^7 cells/ml in RPMI 1640/10% FCS) were labeled with CMTMR (5 μM) for 20 min at 37˚C and additionally treated with DMSO or Y27632 (20 μM) for 1 h. A total of 1 × 10^7 cells was injected i.v. into age- and sex-matched C57BL/6 recipient mice. Twenty minutes after cell transfer, 300 μg Alexa Fluor 633-conjugated tomato lectin (Sigma) was injected i.v. to label the vasculature. Twenty minutes later, mice were killed and perfused as above. Long bones (femur and tibia) were collected, frozen, and cut in pieces 1–2 mm in length. Still-frozen bone pieces were fixed in 10 ml PFA for 12 h. Blocks (500 × 500 × 300–500 μm) of agarose-embedded bone pieces were imaged and analyzed as above.

2PM of popliteal PLNs

Purified T cells were treated with DMSO or Y27632 (20 μM) for 1 h and fluoreoscently labeled with 2.5 μM CMTMR or CFSE for 15 min at 37˚C. After washing, labeled T cells were injected i.v. into sex-matched 5–10-wk-old anesthetized C57BL/6 or ICAM-1−/− mice. After washing, labeled T cells were injected i.v. into sex-matched 5–10-wk-old anesthetized C57BL/6 or ICAM-1−/− mice. To block uropod contraction, we used the ROCK and myosin II inhibitors Y27632 and blebbistatin. Both inhibitors significantly reduced the formation of an aneuropod (31). We examined whether this was also relevant for CCR7-dependent migration of naive T cells in vitro. To block uropod contraction, we used the ROCK inhibitor Y27632 or the myosin II inhibitor blebbistatin. To verify the inhibitory activity, we analyzed chemokine-induced polarization in the presence or absence of Y27632 and blebbistatin. Both inhibitors significantly reduced the formation of an elongated uropod in naive T cells, without affecting the polar distribution of leading and trailing edge markers (Fig. 1A).

In chemotaxis experiments, we observed a pore size-dependent effect on T cell migration after inhibition of ROCK or myosin II. Control and inhibitor-pretreated T cells migrated comparably toward CCL21 through filter inserts with 5-μm pores. In contrast, the migration of Y27632 or blebbistatin–treated T cells was reduced by half compared with control T cells when the pore diameter was only 3 μm (Fig. 1B). We observed a similar reduction when the inhibitors were present throughout the chemotaxis assay (data not shown). In B cells, Y27632 and blebbistatin inhibited migration through 5-μm pore sizes but with less efficacy than when using inserts with 3-μm pore diameters (Fig. 1C). When we examined the reversibility of both inhibitors, we observed continuous inhibition of migration through 3 μm pores for up to 2 h after treatment in naive, non-activated T cells, whereas the inhibitory action of both drugs was rapidly reversed in B cells (data not shown).

To mimic more complex migration conditions, we performed migration experiments in 3D collagen matrices with varying collagen densities (31). In low-density collagen matrices, Y27632 blocked the migratory speed of T cell blasts toward CCL19 by 32% compared with control T cells. With increased collagen density and reduced pore size, Y27632 inhibited the T cell velocity by 52% (Fig. 1D).

In summary, our data showed a pore size-dependent role for ROCK and myosin II–triggered uropod contraction or maintenance of cortical tension in T and B cells, in the absence of integrin-mediated retention. The lack of inhibition of T cell migration through large pores also indicated unimpaired promigratory capabilities in the presence of Y27632 and blebbistatin. Based on the reversibility of Y27632 and blebbistatin inhibition in B cells, we focused additional analyses on ROCK and myosin II function in naive T cells.

T cell crawling and TEM under flow are inhibited by Y27632 in an endothelium-specific manner

We next investigated the requirement of ROCK activity for TEM under flow, to mimic physiological barrier conditions encountered in vivo. Based on the above findings, we set out to examine dynamic T cell crawling and transmigration through endothelial beds showing a complete, or incomplete, tight junction organization and overall permeability. First, we superfused control or Y27632-pretreated T cells over TNF−α–activated and CCL21-coated pMBMECs, which possess a low permeability rate (35). Under these conditions, control T cells exhibited dynamic motility and transmigration (Fig. 2A, 2B, Supplemental Video 1), with a mean luminal crawling velocity of 5.7 ± 0.2 μm/min (Fig. 2B). Occasionally, we observed a sudden change in the directionality of crawling T cells in line with the direction of shear flow, whereas the uropod anchored crawling T cells on the pMBMECs (data not shown).

Contrary to T cell blasts (29, 30), we rarely observed naive T cells with elongated tails in the presence of Y27632 (data not shown). However, compared with control T cells, Y27632-treated T cells moved more slowly on pMBMECs (3.4 ± 0.2 μm/min; Fig. 2B, Supplemental Video 1). The overall percentage of Y27632-treated T cells undergoing transmigration in the recording period was reduced by 74% (from 51.1 ± 18.4% to 13.5 ± 3.4%; Fig. 2C). Occasionally, we observed Y27632-treated T cells protruding a lamellipodium underneath the endothelium in an apparent attempt to transmigrate but apparently were unable to translocate the main cell body containing the nucleus through the endothelial pore (Fig. 2A, Supplemental Video 1). These data suggested that uropod contraction is central for efficient passage through a tight endothelial cell layer. Furthermore, Y27632-treated T cells, which managed to transmigrate, moved more slowly underneath pMBMECs compared with control cells, indicating a role for uropod contraction or cortical tension in this constrained environment (Fig. 2D).

The brain-derived endothelial cell line bEnd5 is characterized by its >10-fold greater permeability rate and its less elaborate tight
FIGURE 1. ROCK and myosin inhibition reduces chemokine-induced in vitro migration of naive T and B cells through narrow pores. A, Left panels, Y27632 and blebbistatin block CCL21-induced uropod induction in naive T cells. Although uropod formation is inhibited by Y27632 and blebbistatin, the CCL21-induced segregation with PKCζ at the leading edge and CD44 at the trailing edge is largely intact in these cells. Scale bar, 10 μm. Right panel, Polarity index in control and Y27632- and blebbistatin-treated T cells. The data are from one experiment with three individual slides per condition. We analyzed 134 individual control T cells, 137 Y27632-treated T cells, and 123 blebbistatin-treated cells. **p < 0.001. B, T cell migration to CCL21 through Transwell membranes with pore diameters of 5 μm (left panel) or 3 μm (right panel). Purified DMSO-, blebbistatin-, or Y27632-pretreated naive T cells were allowed to migrate for 2 h toward 100 nM CCL21. C, B cell migration to CCL21 through Transwell membranes with pore diameters of 5 μm (left panel) or 3 μm (right panel). Purified DMSO-, blebbistatin-, or Y27632-pretreated B cells were allowed to migrate for 2 h toward 100 nM CXCL13. Data represent mean ± SD of three independent experiments. D, 3D migration velocity of T cell blasts in collagen matrices of low (1.6 mg/ml) and high (4.27 mg/ml) density, corresponding to large and small pore sizes, respectively. Data are from four experiments. *p < 0.05, ***p < 0.001, compared with DMSO-treated cells (ANOVA). n.s., not significant.

junction organization compared with pMBMECs (35). Superfused control T cells moved efficiently on and through CCL21-coated bEnd5 cells (Fig. 2E, 2F, Supplemental Video 2), with a mean migration velocity of 3.9 ± 0.3 μm/min. Inhibition of ROCK by Y27632 reduced luminal T cell crawling velocity to 3.3 ± 0.3 μm/min (Fig. 2F, Supplemental Video 2). Nonetheless, in marked contrast to pMBMECs, both T cell populations showed a comparable TEM efficiency (Fig. 2G), indicating that uropod contractility is not absolutely required for crossing a “permissive” endothelial cell layer. Because activated endothelial cells express high surface levels of ICAM-1, and based on the observed anchoring of luminal crawling T cells, we reasoned that the reduced luminal crawling velocity in Y27632-treated T cells might be due, at least in part, to deficient LFA-1 de-adhesion. To directly test this hypothesis, we superfused control and Y27632-treated T cells on coated rICAM-1 and CCL21. After allowing their accumulation at low shear, we increased the shear rates to physiological levels and analyzed the dynamic crawling behavior of control and inhibitor-treated cells. Y27632 significantly reduced T cell migration velocity on purified ICAM-1 + CCL21 from 9.2 ± 6.2 to 4.7 ± 2.4 μm/min (Fig. 2H), supporting the notion that the reduced motility on endothelial cells in the presence of Y27632 is due to reduced detachment during crawling.

In summary, although efficient cell crawling on endothelium seemed to require LFA-1 de-adhesion, defective TEM of Y27632-treated T cells through pMBMECs was reminiscent of impaired propulsion of the main cell body through the endothelial pore.

T cell homing through HEVs, but not BM sinusoids, is reduced by inhibition of ROCK and myosin II

The data accumulated thus far argued for a role for uropod contractility in the passage of naive T cell through narrow pores in vitro. We next wished to determine, in vivo, whether the importance of ROCK function for TEM differs in vascular beds with variable permeability characteristics. We chose to compare HEV and BM sinusoids, because the latter are fenestrated with a high level of permeability and, therefore, may present an incomplete barrier for transmigrating T cells (36). We performed short homing assays of fluorescently labeled control or blebbistatin- or Y27632-treated T cells, followed by thick-tissue imaging of PLNs and BM slices using 3-DIF reconstructions (Fig. 3A) (12). The short total transfer time (40 min) ensured continued efficacy of the inhibitors, whereas the large tissue volume permitted the detection of a sufficient number of adoptively transferred cells for a meaningful analysis. Furthermore, the analysis of single z planes permitted the precise intra- or perivascular localization of transferred T cells (Fig. 3B, 3C).

When we analyzed thick BM sections, we observed an efficient accumulation of control T cells in BM parenchyma, with 10.1 ± 4% of transferred T cells being intravascular and 22 ± 3.8% being perivascular (Fig. 3D). Y27632 pretreatment did not alter the distribution of transferred T cells (9.7 ± 2.6% intravascular and 24.3 ± 6.4% perivascular; Fig. 3D), indicating that ROCK function is not required for efficient TEM through the single layer of BM sinusoidal cells.

Although the total number of T cells per PLN volume, as detected by 3-DIF, was not affected by inhibition of ROCK or myosin II (data not shown), we observed a shift in the microcompartamental distribution of adoptively transferred Y27632- or blebbistatin-treated T cells within lymphoid tissue. A significantly higher percentage of transferred inhibitor-treated T cells was present in the intraluminal compartment and the perivascular space (Fig. 3E) compared with control T cells. Similar results were obtained when the short-term homing assay was limited to a total of
In summary, analogous to our results from the in vitro experiments, our thick-tissue–image analysis uncovered a role for ROCK during T cell homing through the more restrictive endothelium of lymphoid tissue, although numerous T cells were still able to cross HEVs.

20 min, with anti-L-selectin mAb treatment 10 min after adoptive transfer (data not shown).

In summary, analogous to our results from the in vitro experiments, our thick-tissue–image analysis uncovered a role for ROCK and myosin II activity during T cell homing through the more restrictive endothelium of lymphoid tissue, although numerous T cells were still able to cross HEVs.

Y27632-treated T cells showed reduced luminal crawling velocity and prolonged TEM and perivascular dwelling time on HEVs in vivo

T cell homing to PLNs is a multistep process in which T cells adhere via LFA-1–ICAM-1–mediated interactions, followed by an optional ICAM-1–dependent crawling step and a rapid TEM event, which occurs efficiently in the absence of ICAM-1 or ICAM-2 (10–12). Successfully transmigrated T cells remain in the perivascular space for a few minutes, presumably negotiating their passage into the lymphoid parenchyma (12). Based on the observation of impaired T cell accumulation within parenchymal lymphoid tissue after Y27632 and blebbistatin treatment, we set out to delineate, in more detail, the role for ROCK functionality during intraluminal crawling, transmigration, and perivascular motility of single cells. We adoptively transferred fluorescently labeled control and Y27632-treated T cells into recipient mice surgically prepared to expose the popliteal PLN and that had received fluorescently labeled MECA-79 to identify the HEV outline 15 min prior to T cell transfer, as well as fluorescent dextran as plasma marker in some experiments. Using 2PM of popliteal PLNs, we carefully tracked the transferred T cells from the site of adhesion until their appearance in the lymphoid parenchyma (Fig. 4A, Supplemental Video 3). In accordance with published data, we found that most control T cells directly egressed from the site of adhesion into the underlying perivascular space. Similarly, the majority of Y27632-treated T cells directly egressed from the site of adhesion into the underlying tissue (Supplemental Videos 3, 4). The percentage of intraluminally crawling T cells, defined as luminal postadhesion migration for $>20 \mu m$, was not reduced by Y27632 treatment (13/46 total tracks $= 27\%$ for control versus 13/48 total tracks $= 27\%$ for Y27632-treated T cells). However, we found that the intraluminal crawling velocity was reduced significantly from $10.5 \pm 1.9 \mu m/min$ in control T cells to $6.5 \pm 0.6 \mu m/min$ (mean $\pm$ SEM) in Y27632-treated T cells (Fig. 4B). In inhibitor-treated T cells, the time required for HEV transmigration was increased significantly (Fig. 4C), whereas the perivascular velocity was decreased (Fig. 4D), and the meandering index as an indicator of directionality showed a nonsignificant increasing tendency in Y27632-treated T cells (Fig. 4E).

Taken together, our dynamic single-cell analysis uncovered multiple roles for ROCK during LFA-1–dependent intraluminal crawling and regulating the transmigration time through HEV. Similarly, the ICAM-1–dependent exit from the perivascular area was delayed in Y27632-treated T cells.

Y27632 treatment reduces parenchymal T cell motility in an ICAM-1–dependent manner

T cells rapidly migrate inside lymphoid parenchyma, guided by the FRC network (13). We explored whether ROCK function was required to move through the densely populated lymphoid microarchitecture. We transferred fluorescently labeled control and Y27632-treated T cells and observed their parenchymal motility immediately after transfer (<70 min) using 2PM of popliteal PLNs. Although both populations showed comparable directionality, as assessed by turning angles, Y27632-treated T cells moved significantly slower than did control T cells (13.2 $\pm$ 3.8 $\mu m/min$ and 15.8 $\pm$ 4.0 $\mu m/min$, respectively; Fig. 5A). Although this seems to be a modest decrease, the difference in velocity distributions resulted in a 45% reduction in the motility coefficients, from 130 $\mu m^2/min$ to 71 $\mu m^2/min$ for control and Y27632-treated T cells, respectively. We then investigated whether the reduced migration velocity was due to spatial constraints imposed by the dense lymphoid microenvironment, thus...
impairing nucleus propulsion in the absence of uropod contraction (31), or to integrin-independent promiscuous cell attachment due to reduced cortical control (27) or, alternatively, whether impaired uropod detachment from ICAM-1 caused T cell slowing in the presence of Y27632. To this end, we carried out 2PM experiments with control and Y27632-treated T cells in ICAM-1−/− PLNs, which have an overall cell density, microarchitecture, and, thus, spatial constraints comparable to wild-type PLNs (12). As reported (12, 14), the absence of LFA-1–ICAM-1 interactions resulted in reduced migration speed and increased turning angle distribution in control T cells. Of note, Y27632-treated T cells migrated with speeds and turning angles comparable to control T cells (12.8 ± 3.9 μm for control versus 12.6 ± 3.5 μm for Y27632-treated T cells; Fig. 5B). In summary, these data suggested that, somewhat unexpectedly, the dense lymphoid microarchitecture does not constitute an obstacle for the rigid nucleus of migrating T cells, rather the specific detachment from adhesive LFA-1–ICAM-1 interactions requires ROCK–myosin II action.

Discussion
As an adaptation to their motile life style, recirculating lymphocytes are undergoing numerous transient adhesive interactions with other hematopoietic and nonhematopoietic cells. Within tissue, polarized lymphocytes acquire a largely integrin-independent migratory phenotype characterized by a forward-pushing lamellipodium and a contractile uropod under control of ROCK and myosin II. In this study, we analyzed the importance of a contractile uropod and the maintenance of cortical control for efficient trafficking of naive T cells under physiological conditions in vitro and in vivo, thereby indirectly examining the anatomical constraints imposed by varying endothelial barriers and lymphoid microenvironments. To distinguish between the various roles for ROCK and myosin II during lymphocyte–stroma interactions, such as integrin de-adhesion, decrease of promiscuous cell attachment, and nucleus propulsion, we carefully dissected potential pathways using a combination of in vitro and in vivo experiments in the presence or absence of shear and integrin ligands and shear. Our findings confirmed that, in the absence of shear and integrin-mediated adhesion, the pore size determined efficient lymphocyte migration toward homeostatic chemokines in vitro. Furthermore, we found that ROCK–myosin II-induced uropod contractility and preservation of cortical tension are invariably required for efficient T cell crawling on various vascular beds in vitro, probably, in part, to overcome LFA-1–ICAM-1 retention of the uropod. In contrast, efficient in vitro TEM in the
tracks for control and Y27632-treated T cells, respectively. ***

Y27632-treated T cells migrating inside lymphoid parenchyme of ICAM-type mice. Data are pooled from five mice/six videos/177 and 138 tracks for control and Y27632-treated T cells, respectively.

FIGURE 4. 2PM analysis of control and Y27632-treated T cell crawling and HEV transmigration. A, Representative images of control (green) and Y27632-treated (red) T cells transmigrating through an HEV (shown brown in the top panel and omitted for clarity in the bottom panel). Arrowheads show examples of successfully emigrated T cells. Time is shown in minutes and seconds. Scale bar, 20 μm. B, Migration velocity of control and Y27632-treated T cells crawling on the intraluminal side of HEVs for >20 μm prior to TEM. C, Transmigration time of control and Y27632-treated T cells through HEVs. D, Migration velocity of control and Y27632-treated T cells in the perivascular area. E, Meandering index of control and Y27632-treated T cells in the perivascular area. Each dot in B–E represents a single cell track. Data are pooled from three independent experiments with 13 (B) and 33–49 tracks (C–E) of control and Y27632-treated T cells. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control T cells. n.s., not significant; Y27, Y27632.

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absence of ROCK function is only affected through endothelial cells with low permeability. Similarly, in vivo T cell TEM was more dependent on ROCK function when crossing PLN HEVs than BM sinusoids. Finally, our data suggested that tissue-expressed ICAM-1 requirement for ROCK function when crossing PLN HEVs than BM sinusoids. Similarly, in vivo T cell TEM was more dependent on ROCK function than BM sinusoids. Finally, our data suggested that tissue-expressed ICAM-1

FIGURE 5. 2PM analysis of uropod contractility during parenchymal T cell migration in control and ICAM-1-deficient hosts. A, Average migration velocity (left panel) and turning angles (right panel) of control and Y27632-treated T cells migrating inside lymphoid parenchymec of wild-type mice. Data are pooled from five mice/six videos/177 and 138 tracks for control and Y27632-treated T cells, respectively. B, Average migration velocity (left panel) and turning angles (right panel) of control and Y27632-treated T cells migrating inside lymphoid parenchyma of ICAM-1−/− mice. Data are pooled from four mice/eight videos/314 and 204 tracks for control and Y27632-treated T cells, respectively. ***p < 0.001, n.s., not significant; Y27, Y27632.

chyme, whereas the tightly packed, yet dynamic, microenvironment does not strongly interfere with the propulsion of T cell nuclei.

In line with a central role for actin polymerization at the leading edge of migrating cells, intraluminal crawling and interstitial motility are virtually abolished in the absence of the Rac guanine exchange factor DOCK2 or its downstream effectors Rac1 and Rac2, although the shear-induced transmigration capacity was less affected (12, 19, 37, 38). Although it has long been recognized that ROCK or myosin II inhibition resulted in impaired crawling of activated T cells on 2D surfaces coated with integrin ligands, resulting in a strongly elongated cell shape (29, 30), the requirement for ROCK–myosin II functionality during physiological migration of nonactivated naive T cells has only recently started to be investigated (27). In this study, we followed up in vitro observations to investigate whether ROCK–myosin II function is also central during recirculation pathways of naive T cells. To this end, we used the ROCK inhibitor Y27632 and the myosin II inhibitor blebbistatin, and exploited the fact that these compounds remained active in naive T cells for up to 2 h after washing off. This allowed us to follow inhibitor-treated naive T cells immediately after superfusion onto endothelial cells in vitro or posttransfer into recipient hosts in vivo. After adhesion to a CCL21-loaded endothelial cell surface, blood-borne non-polarized T cells undergo a rapid polarization, which is characterized by the acquisition of a protruding lamellipodium and a contractile uropod. Although naive T cells crawling on ICAM-1 do not show an elongated cell shape, as observed in T cell blasts, perhaps owing to reduced overall adhesiveness and lower lamellipodia protrusive activity, our findings suggested that uropod contractility facilitates trailing edge release during physiological migration on 2D surfaces. We also showed that Y27632 reduced crawling velocity on HEVs, supporting a general role for ROCK function during intraluminal crawling.

Our data uncovered an endothelial-dependent requirement for intact ROCK–myosin II signaling for efficient TEM, which correlated with the degree of tight endothelial junction formation. In the bEnd5 endothelial cell line, the tight junction formation is less prominent than in pMBMECs, with the latter expressing higher levels of the tight junction protein occludin-1 (35). We took advantage of these differences to correlate their permeability properties with the requirement of ROCK activity for TEM. A novel finding reported in this article is that endothelial properties impose differing constraints on efficient lymphocyte TEM. Although this suggests that lymphocytes use the paracellular, rather than the transcellular, TEM pathway in vitro, we cannot exclude that factors in addition to the increased tight junction functionality of primary endothelial cells compared with immobilized cell lines affect the transcellular TEM efficiency. This is particularly relevant because pMBMECs display different F-actin networks, which may influence transcellular TEM (35). Irrespective of the TEM pathway, we hypothesize that the pore size is the decisive factor for the requirement of ROCK activity. Because anatomical studies identified a pore size of 2.2–2.5 μm through HEVs (9), and our in vitro studies indicated a strong reduction in directed motility of Y27632-treated T cells through 3-μm pores, we anticipated a strong inhibition through Y27632 for TEM through HEVs. Although we observed significant defects in T cell crawling, TEM, and passage of the perivascular space by ROCK inhibition, the blocking effect was less pronounced than predicted. This indicated that, under physiological conditions, HEVs are more flexible than anticipated by morphological studies and/or the reduced thickness of HEVs compared with Transwell filter inserts masks the requirement of ROCK–Rho–myosin II-mediated uropod contractility for squeezing the rigid
leukocyte nucleus through narrow pores in reconstituted 3D microenvironments (25, 31). Furthermore, efficient dendritic cell entry into afferent lymphatic vessels requires myosin II function (39), and Y27632 reduces neutrophil motility in inflamed tissue (40). In line with these observations, the increased transmigration time through HEVs and the delayed motility of Y27632-treated cells in the perivascular space, which is characterized by a porous and thick basement membrane and stromal cuffs (12, 13), support the concept of nuclear trapping in environments with small pores. Furthermore, our data indicated that Y27632 reduces parenchymal T cell motility. A recent study showed that myosin II deficiency induced the overall adhesiveness of preactivated T cells through increased contact areas with surrounding interfaces, rather than through specific ICAM-1–mediated interactions, thereby causing defects in T cell motility (27). However, in this study, naïve T cells adhered well to ICAM-1 but not to other proteins tested. Within the dynamic and highly motile microenvironment of the T cell area that is difficult to recreate in simplified in vitro systems, we were unable to identify a role for integrin-independent promiscuous cell attachment in the absence of ROCK function. In ICAM-1–deficient PLNs, Y27632 did not reduce lymphocyte-migration parameters compared with control T cells, despite similar overall cell density of the lymphoid parenchyma. This observation indicated that anatomical constraints within lymphoid parenchyma (outside the perivascular space) are less stringent than predicted from the high cell density, perhaps owing to the intrinsic robust lymphocyte motility and widely spaced FRC network. Based on the observation that ICAM-1 on stroma is the major binding partner for lymphocyte-expressed LFA-1 (12), our data suggested that reduced de-adhesion from ICAM-1 on stromal elements slows the migration of Y27632–treated T cells, whereas other integrins and integrin ligands do not seem to be involved (12, 14). Taken together, ICAM-1 represents a nonredundant parenchymal adhesion molecule for non-activated lymphocytes, presumably retaining T cells on the FRC network in close proximity to surface-bound CCL21 in a haptokinetic fashion. Nonetheless, it is conceivable that, during effector cell migration in nonlymphoid stroma, a careful balance between netic fashion. Nonetheless, it is conceivable that, during effector lymphocyte motility at sites of inflammation. Studies will be required to investigate the role of uropod contraction in vitro and in vivo, correlating with endothelial barrier properties, and territoriality in lymph nodes.


