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Dendritic Cell Motility and T Cell Activation Requires Regulation of Rho-Cofilin Signaling by the Rho-GTPase Activating Protein Myosin IXb

Yan Xu,*1 Stefanie Pektor,†1 Sandra Balkow,† Sandra A. Hemkemeyer,* Zhijun Liu,* Kay Grobe,*2 Peter J. Hanley,* Limei Shen,† Matthias Bros,† Talkea Schmidt,† Martin Bähler,*3 and Stephan Grabbe†,3

Direct migration of stimulated dendritic cells (DCs) to secondary lymphoid organs and their interaction with Ag-specific T cells is a prerequisite for the induction of primary immune responses. In this article, we show that murine DCs that lack myosin IXB (Myo9b), a motorized negative regulator of RhoA signaling, exhibit increased Rho signaling activity and downstream actomyosin contractility, and inactivation of the Rho target protein cofilin, an actin-depolymerizing factor. On a functional level, Myo9b−/− DCs showed impaired directed migratory activity both in vitro and in vivo. Moreover, despite unaltered Ag presentation and costimulatory capabilities, Myo9b−/− DCs were poor T cell stimulators in vitro in a three-dimensional collagen matrix and in vivo, associated with altered DC–T cell contact dynamics and T cell polarization. Accordingly, Myo9b−/− mice showed an attenuated ear-swelling response in a model of contact hypersensitivity. The impaired migratory and T cell stimulatory capacity of Myo9b−/− DCs was restored in large part by pharmacological activation of cofilin. Taken together, these results identify Myo9b as a negative key regulator of the Rho/RhoA effector Rho-kinase [Rho-associated coiled-coil-forming kinase (ROCK)]/LIM domain kinase signaling pathway in DCs, which controls cofilin inactivation and myosin II activation and, therefore, may control, in part, the induction of adaptive immune responses. The Journal of Immunology, 2014, 192: 3559–3568.

Dendritic cells (DCs) are professional APCs with the unique property to prime and polarize naïve T cells toward a tolerogenic or immunogenic state (1, 2). In peripheral tissues and nonlymphoid organs, immature DCs sample Ags and present them to T cells in draining lymph nodes (LNs) (3). In this respect, DCs undergo a maturation process that is associated with massive cytoskeletal rearrangements and directed migration toward secondary lymphoid organs.

The establishment and maintenance of cell polarity is controlled by members of the Rho family of small, monomeric GTPases, especially RhoA, Rac, and Cdc42 (4, 5). Activation of Cdc42 and Rac is part of a positive feedback loop generating robust membrane protrusive activity restricted to the front, and segregating RhoA activity to the sides and back of the cell (6). RhoA activity stimulates the RhoA effector Rho-kinase [Rho-associated coiled-coil-forming kinase (ROCK)] that augments the phosphorylation of regulatory myosin L chain (MLC), thereby inducing acto-myosin II contraction (7). In parallel, ROCK activates LIM domain kinase (LIMK), which, in turn, inactivates actin-depolymerizing factor and cofilin proteins that serve to depolymerize and repolymerize actin filaments (8). Acto-myosin II contraction and inhibition of cofilin prevent the formation of additional protrusions, and help to retract the rear end and to propel the nucleus forward (9, 10).

Besides enabling cell migration, regulated cytoskeletal rearrangements are also important to establish proper DC–T cell interaction between stimulated DCs and Ag-specific T cells in regional LNs. The interaction between a DC and a T cell generates a specific physical site, termed the “immunological synapse,” to which signaling and costimulatory molecules are recruited to trigger T cell polarization and proliferation (11). Contact formation of DCs with T cells and subsequent T cell activation is an important step in the induction of an adaptive immune response. This step is critically regulated by the small G proteins RhoA, Cdc42, and Rac (12).

We have recently found that, by coordinating the activity of Rho, the cytoskeletal motor protein myosin IXB (Myo9b) controls the cell shape and migratory activity of macrophages, and thereby contributes to innate immune responses (13). Myo9b belongs to the myosin superfamily of actin-based molecular motors and encompasses in its tail region a Rho-GTPase activating protein domain (14). Both in vitro and in vivo, Myo9b preferentially inactivates the closely related RhoA, RhoB, and RhoC Rho GTPases (13, 15, 16). Myo9b is recruited to regions of active actin polymerization such as lamellipodia, ruffles, and filopodia (17).
but it remains to be determined whether Myo9b negatively regulates Rho signaling specifically in these regions. Myo9b is abundantly expressed in various cell types of the immune system (13, 18) and appears to contribute to the development of autoimmune diseases (19, 20). Thus, we explored the relevance of Myo9b for DC functions using Myo9b-deficient mice. The results presented in this article indicate that Myo9b+/- DCs have increased levels of RhoA activity and phosphorylated MLC and coflin, resulting in both impaired migratory capacity and altered DC-T cell interaction. These effects were largely rescued by treatment with ROCK or LIMK inhibitors. Thus, Myo9b plays an important yet previously unknown role in DC biology, not only as an actin-based motor protein, but based on its role as a negative key regulator of Rho/ROCK/LIMK signaling, which, in turn, limits coflin activity. Our studies show that coflin, which has been recognized in T cells as essential for migration (21) and immunological synapse formation (22), exerts similar functions also in DCs.

Materials and Methods

Mice

The generation of Myo9b+/- mice (C57BL/6 background) has been described previously (13). All mice were housed under specific pathogen-free conditions according to the guidelines of the regional animal care committee. All experiments were performed in accordance with national and European (86/609/EEC) legislation, and in accordance with the Central Laboratory Animal Facility of the University Medical Center of Mainz (approved by the license of animal testing 13177-07/G081-1-008).

Isolation of primary immune cells

Bone marrow–derived dendritic cells (BMDCs) were generated by a modified protocol of Inaba et al. (23). In brief, bone marrow cells derived from C57BL/6 and Myo9b+/- mice were cultured in DMEM with 1% FCS and 100 U/ml of IL-4 (both from eBioscience, San Diego, CA). On day 7, anti-CD40 Ab (clone 1C10; eBioscience) was added with 150 U/ml GM-CSF and 75 U/ml IL-4 (both from eBioscience, Hamburg, Germany), and the LIMK inhibitor S3 (10 

DC migration in three-dimensional collagen gels

DC migration was analyzed in three-dimensional collagen gels as described previously (27). PureCol bovine collagen I (Life Technologies, Carlsbad, CA) was prediluted in 1 X MEM, 0.42% Na2CO3 and was mixed with cell suspensions, resulting in a gel mixture with a final collagen concentration of 1.8 mg/ml and a final DC concentration of 3 x 10^5 cells/ml. After gelatinization of the gel mixture at 37˚C, the migration of DC populations was monitored by time-lapse microscopy (10 h, 1 frame/1.5 min) using an Olympus BX6 microscope with an UAP0 lens (20 x/0.75; Olympus, Hamburg, Germany) equipped with an FView camera controlled by Cell^P software (SIS, Münster, Germany).

Chemotaxis assay

BMDCs were mixed with PureCol bovine collagen I and loaded into the narrow channel (observation area) of an uncoated μ-slide chemotaxis slide (Ibidi, Martinsried, Germany). The narrow channel separates two 60-μl reservoirs, one of which contained chemokine CCL21 (600 ng/ml; R&D Systems, Minneapolis, MN). Cells were imaged by phase-contrast microscopy (5 h, 1 frame/min) via a 10x/0.3 objective (Carl Zeiss, Jena, Germany). Two-dimensional chemotaxis assays were performed in a μ-slide chemotaxis two-dimensional chambers (Ibidi) that were coated with 20 μg/ml of collagen (Hamburg, Germany) and 10 μg/ml of fibronectin (Abcam, Cambridge, UK). For 1 h at room temperature. Slides were washed two times with chemotaxis medium (RP1M 1640, 10% FCS) before chemokine CCL21 (final concentration 600 ng/ml; R&D Systems, Minneapolis, MN) was applied to one of the reservoirs below or under the observation area. Finally, cells were loaded into the observation area with a final concentration of 3 x 10^5 cells/ml and left to adhere for 1 h. Cell migration tracks were analyzed with ImageJ (National Institutes of Health) using the “manual tracking plugin” and the “chemotaxis and migration tool plugin” from Ibidi. Chemotactic efficiency was quantified by calculating the forward migration index (FMI) and the directionality of migrating cells. The FMI is the ratio of the net distance the cell progressed in the direction of the chemotactic gradient to the total distance traversed by the cell. Directionality denotes the ratio of the linear distance from the starting point to the end point and the total distance traversed by the cell.

Cell stimulation with CCL21

Matured day 9 WT and Myo9b+/- BMDCs were washed twice with PBS, resuspended in RPMI 1640/10 mM Hepes pH 7.4, and incubated for 10 min before stimulation for 0, 2, or 5 min with 250 ng/ml CCL21. Cells were lysed and prepared for immunoblotting. The Abs against phospho-p44/42 MAPK (p-Erk1/2, catalog no. 4370; 1:2000; Cell Signaling, Danvers, MA), p44/42 MAPK (Erk1/2, catalog no. 4370; 1:1000; Cell Signaling), and β-actin (clone AC-15, catalog no. A1978; 1:2500; Sigma) were used to detect the corresponding proteins.

Skin explant culture

Ears of WT and Myo9b+/- mice were split into dorsal and ventral halves, and were floated with the dermal side downward on DC medium. After 24 h, ear sheets were transferred to fresh DC medium containing 100 ng/ml CCL21. After 24 h, nonadherent cells in the medium were characterized by flow cytometry.

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For quantification of Langerhans cells (LCs) in the epidermis, epidermal sheets were separated from the underlying dermis, fixed in 4% PFA for 30 min at 37°C, followed by incubation with 0.15 M glycine. All reagents were buffered with PBS. After permeabilization and blocking (0.05% saponin/5% NGS) for 30 min, sheets were stained with a rat anti-MHC II Ab (Sigma-Aldrich, St. Louis, MO), followed by rhodamine-conjugated anti-rat Ab (Jackson Immunoresearch, West Grove, PA). Staining was visualized using either the LSM 510 confocal microscope (Carl Zeiss) with LSM Release 4.2 software or an Axioskop fluorescence microscope (Carl Zeiss) with Wasabi software (version 1.4; Hamamatsu, Japan).

Ear painting assay

The dorsal and ventral ear skins of WT and Myo9b<sup>−/−</sup> mice were painted with 15 μl of 33 mg/ml FITC (Sigma-Aldrich) dissolved in 1:1 (v/v) acetonitrile-butylphthalate, or an equal volume of PBS alone as control. Draining auricular LNs were excised 24 h after exposure and digested by collagenase III (20 U/ml; Cell System)/EDTA (2.5 mM)/HBSS treatment. Details of the ear painting assay are published elsewhere (28). The Ab titer was defined as the photocounting of Langerhans cells (LCs) in the epidermis, epidermal sheets were separated from the underlying dermis, fixed in 4% PFA for 30 min at 37°C, and the increase of ear thickness was measured after 24 h using an engineers’ micrometer (Oditest; Messwellk, Klein-Ostheim, Germany).

Model of type I allergy

Four mice per group were immunized by i.p. injection of 100 μl OVA (10 μg) adsorbed to an equal volume of aluminum hydroxide (containing 40 mg/ml aluminum hydroxide and 40 mg/ml magnesium hydroxide) on days 0 and 14. Sera were collected 7 d after the last immunization and stored at −20°C until determination of OVA-specific Ab titers.

Determination of OVA-specific Ab titers by ELISA

Anti-OVA IgE, IgG1, and IgG2a Ab concentrations in the serum were measured by ELISA as described previously, with the modification that OVA (25 μg/ml) was used as Ag (28). The Ab titer was defined as the reciprocal serum dilution, yielding an absorbance reading of an OD of 0.2 after linear regression analysis.

Analysis of cell–cell interactions in three-dimensional collagen gels

DC–T cell interactions in three-dimensional collagen gels were determined and analyzed as described by Gunter et al. (27). In brief, BMDCs pre-incubated with 1 μg/ml OVA<sub>323–339</sub> for 1 h were cocultured overnight with CD4<sup>+</sup> T cells at a ratio of 1:10. The number and duration of physical contacts of randomly selected individual DCS with T cells was monitored by time-lapse microscopy (10 h, 1 frame/2 min). Contacts that lasted longer than three times the median contact time between WT BMDCs and CD4<sup>+</sup> T cells were classified as long contacts.

Immunofluorescent staining of DC–T cell interactions

BMDCs loaded with OVA<sub>323–339</sub> and CD4<sup>+</sup> T cells were cocultured (ratio 1:2) for 1 h in chamber slides (Nunc, Rochester, NJ), fixed with 4% PFA at 37°C for 10 min, incubated with 0.15 M glycine for 10 min at room temperature, and then permeabilized with 5% rat serum/0.5% saponin/2% PBS for 10 min. To block nonspecific binding, we incubated cells with PBS containing 5% rat serum/PBS for 20 min. Cells were incubated with Texas Red–conjugated phalloidin (Life Technologies) at room temperature for 30 min and analyzed by confocal laser-scanning microscopy (LSM510-ultraviolet; Zeiss, Jena, Germany).

Quantification of actin content in DC–T cell cocultures

A two-dimensional maximal intensity profile image was calculated out of a three-dimensional Z-stack by ZEN software. The fluorescence intensity over the synaptic cleft between DC and T cell was then quantified.

Ag-specific T cell proliferation

OVA peptide-pulsed DCs (see earlier) were titrated in triplicates in three-dimensional collagen gels or in liquid culture, and Ag-specific CD4<sup>+</sup> OT-II T cells were added. Cocultures were incubated for 3 d at 37°C, and [<sup>3</sup>H]thymidine (1 μCi/well) was added for the last 16 h. T cell proliferation was assessed by incorporation of [<sup>3</sup>H]thymidine as measured by liquid scintillation counting.

Multiplex Cytometric Bead Assay

Cytokine concentrations in cell culture supernatants were detected by Multiplex Cytometric Bead Assay (BD Biosciences). In brief, bead populations with distinct fluorescence intensities were coated with capture Abs specific for each cytokine. Bead populations were mixed and were resolved in the FL3 channel of a LSR-II. The capture beads were mixed with the recombinant standards or test samples and then incubated with PE-conjugated detection Abs. Sample results were tabulated and graphed using the BD Multiplex Cytometric Bead Assay Analysis Software (BD Biosciences).

In vivo T cell proliferation assay

CFSE-labeled OT-I Ly5.1 and OT-II Ly5.1 cells were mixed (1:1) and injected i.v. into C57BL/6 mice (2 × 10<sup>6</sup> cells/animal). After 24 h, mice were immunized i.v. with 10<sup>6</sup> BMDCs loaded with OVA peptides (1 μg/ml OVA<sub>323–339</sub> plus 10 μg/ml OVA<sub>27–39</sub>). Three days later, CD4<sup>+</sup> and CD8<sup>+</sup> cells from spleens and LNs were analyzed by flow cytometry for proliferation as reflected by dilution of CFSE-fluorescence intensity.

Statistical analysis

The unpaired two-tailed Student t test was used to determine statistical significance. Calculations were performed using Prism software (GraphPad), and data are presented as mean ± SEM.

Results

The Rho-GTPase activating protein Myo9b negatively regulates RhoA in BMDCs

Myo9b protein was abundantly expressed in WT BMDCs (Fig. 1A). GST pull-down assays of active GTP-bound Rho revealed that non-stimulated and stimulated Myo9b<sup>−/−</sup> BMDCs had markedly increased RhoA-GTP levels, whereas no differences in the amounts of Cdc42-GTP or Rac1-GTP levels were detected (Fig. 1A, 1B, Supplemental Fig. 1). Consistent with augmented Rho signaling (Fig. 1C), Myo9b<sup>−/−</sup> BMDCs exhibited increased amounts of phosphorylated MLCK phosphorylated collagen, relative to WT cells (Fig. 1C, Supplemental Fig. 1). These findings demonstrate that Myo9b is a critical negative regulator of Rho signaling in BMDCs.

The morphology of BMDCs was assessed in liquid culture, as well as in collagen gels that allow for active cell migration in a three-dimensional environment. Whereas activated BMDCs (WT and Myo9b<sup>−/−</sup>) showed a mature morphology and cluster formation in liquid culture, in collagen matrices, Myo9b<sup>−/−</sup> BMDCs lost their dendritic morphology and were spherical with very short dendrites (Fig. 1D, Supplemental Fig. 2A). Thus, Myo9b<sup>−/−</sup> BMDCs exhibited a defect in actin polymerization and translocation of extracellular matrix proteins. Myo9b colocalized with F-actin at the leading edge of migratory WT BMDCs (Fig. 1E), suggesting a functional role of Myo9b for BMDC motility as well.

Mobility of Myo9b<sup>−/−</sup> DCs is severely impaired

To investigate the role of Myo9b in DC migration, we cultured stimulated WT and Myo9b<sup>−/−</sup> BMDCs in three-dimensional collagen gel matrices and measured their spontaneous migration by time-lapse microscopy. The spontaneous migration velocity of Myo9b<sup>−/−</sup> BMDCs was significantly reduced in comparison with WT BMDCs (Fig. 2A). Because BMDCs may display a migration behavior distinct from primary DCs, we next analyzed the migration of primary DC populations isolated from spleen in three-dimensional collagen gels. Lymphoid-tissue–resident CD8<sup>+</sup> and CD8<sup>+</sup> DCs, and plasmacytoid DCs (29) were obtained from the spleens of WT and Myo9b<sup>−/−</sup> mice. All three types of Myo9b<sup>−/−</sup> splenic DCs migrated significantly slower than their WT counter-
Myo9b REGULATES DC FUNCTIONS VIA COFILIN ACTIVATION

FIGURE 1. Rho signaling activity is enhanced in Myo9b−/− BMDCs. (A) Expression of Myo9b was detected in WT BMDCs, but not Myo9b−/− BMDCs. (A and B) Levels of active (GTP-bound) and total RhoA (A), Cdc42, and Rac1 (B) in WT and Myo9b−/− BMDCs were measured by GST pull-down assays and immunoblotting. β-Actin served as a loading control. Representative immunoblots are shown in the left panel, and quantifications of phospho-specific Abs. (C) Schematic diagram of Rho signaling through Rho kinase (ROCK). Amounts of phosphorylated MLC (p-MLC) and coflin (p-cofilin) were determined by Western blot analysis using phospho-specific Abs. Quantifications of the results are shown. (A–C) Error bars indicate ± SEM. **p < 0.01, ***p < 0.001 (unpaired t test; data obtained from three independent experiments each; see also Supplemental Fig. 1). (D) The morphology of stimulated WT and Myo9b−/− BMDCs was analyzed on day 9 by microscopy in liquid culture and in collagen gels (see also Supplemental Fig. 2A). (E) Double-immunofluorescence labeling of WT and Myo9b−/− BMDCs in a three-dimensional collagen matrix for Myo9b (Ab FP3/F8) and F-actin. Scale bar, 5 μm.

parts (Fig. 2B). Supplemental Fig. 2B shows a comparison of single migratory paths of WT and Myo9b−/− DCs of either subtype.

An important hallmark of mature DCs is their ability to migrate vigorously from the periphery into the T cell area of draining LNs in response to CCL21 ligands such as CCL21 (30, 31). We exposed WT and Myo9b−/− BMDCs to diffusion gradients of CCL21 to test whether Myo9b is required for the directed migration of DCs (Fig. 2C). WT cells displayed highly persistent chemotactic movement (1.89 μm/min), whereas Myo9b−/− DCs migrated with a strikingly reduced velocity (0.95 μm/min) and impaired chemotaxis efficiency, as illustrated by a significantly reduced directionality and y-FMI (Fig. 2C–E). Defective chemotactic migration of Myo9b−/− cells was not due to impaired surface expression of CCR7, the receptor for CCL21 (Fig. 2F). Interestingly, on a two-dimensional fibronectin substrate, only chemotactic efficiency of Myo9b−/− BMDCs was significantly impaired, but not migration velocity (Fig. 2G, 2H). Using transwell filters, it has been reported that CCL21-mediated chemotaxis of DCs matured with TNF-α, but not DCs matured with LPS, depends on the activity of ERK1/2 (32–34). A comparison of the ERK1/2 phosphorylation levels in WT and Myo9b−/− BMDCs after stimulation with CCL21 did not reveal any significant differences (Supplemental Fig. 1C).

Myo9b−/− mice exhibit diminished migratory activity of cutaneous DCs and attenuated contact hypersensitivity reaction responses

Based on the critical role of Myo9b for DC migration in vitro, we explored the migratory ability of resident cutaneous DCs both ex vivo and in vivo. In freshly isolated epidermal sheets, the distribution, shape (Fig. 3A, upper panel, 0 h), and density (Fig. 3B, 0 h) of LCs from Myo9b−/− mice were similar to that of WT mice. To investigate potential differences in the emigration of LCs from the epidermis, we floated skin explants on CCL21 containing medium for 48 h. Despite morphological signs of LC activation, the number of remaining LCs in epidermal sheets derived from Myo9b−/− mice was significantly exceeding that in WT epidermal sheets (249 ± 13/mm² versus 96 ± 8/mm²; Fig. 3A, lower panel [48 h], 3B). Accordingly, the number of Langerin+ MHC II+ DCs that emigrated from the skin sheets into the culture medium was 2.2-fold lower in Myo9b−/− compared with WT samples (Fig. 3C).

The migration of cutaneous DCs was further investigated in vivo using a contact sensitization model (35). The ear skin of WT and Myo9b−/− mice was painted either with FITC to induce DC migration or with PBS alone as control, and 24 h later, DC migration was evaluated by measuring FITC+CD11c+MHCII+ DCs in the auricular draining LNs. Consistent with impaired ex vivo migration of Myo9b-deficient LCs, the mean number of FITC-bearing DCs in the draining LNs of Myo9b−/− mice was significantly lower compared with WT mice (Fig. 3D). Notably, the migration of both Langerin+ DCs (including LCs and Langerin+ dermal DCs) and Langerin− dermal DCs was impaired to a similar extent (Fig. 3E). In accordance with these findings, Myo9b−/− mice produced an attenuated allergic contact hypersensitivity reaction (CHS), as measured by the ear-swelling response after sensitization and challenge with TNCB (Fig. 3F). This result suggested that
FIGURE 2. Spontaneous and directed migration of Myo9b \(^{-/-}\) DCs in collagen gels is impaired. (A and B) Mature BMDCs (A), splenic CD11\(^{+}\) CD8\(^{-}\), CD11\(^{+}\) CD8\(^{+}\), and plasmacytoid DCs (B) from WT and Myo9b \(^{-/-}\) mice were cultivated in collagen gels overnight, and cell movements were monitored by live cell imaging and quantified using CellP software (Olympus; see also Supplemental Fig. 2B). (C, left panel) Schematic diagram of the μ-slide chemotaxis chamber. Cells were embedded into a gel inside the narrow observation area that separates the two reservoirs, one of which contained the chemoattractant CCL21 (600 ng/ml). Directed migration of WT (middle panel) and Myo9b \(^{-/-}\) (right panel) BMDCs along the chemoattractant gradient was recorded by live cell imaging and analyzed with the “Chemotaxis and Migration tool plugin” (Ibidi) for ImageJ. Individual cell tracks are indicated in different colors. (D) The migration tracks of 40 WT and 40 Myo9b \(^{-/-}\) BMDCs in collagen gels were plotted after normalizing the start point to \(x = 0\) and \(y = 0\). The y-axis represents the direction toward the source of chemoattractant. (E) Quantification of migration velocity, y-FMI, and directionality of BMDCs in a three-dimensional chemotactic CCL21 gradient. (F) CCR7 expression of CD11c\(^{+}\) BMDCs was analyzed by flow cytometry. Representative data from one of three independent experiments are shown. (G) Representative migration plots of WT and Myo9b \(^{-/-}\) BMDCs on a two-dimensional substrate (20 μg/ml murine fibronectin) in a gradient of CCL21 (highest concentration at the top). (H) Plots of cell velocity, directionality, and y-FMI in the two-dimensional chemotaxis assay. (A, B, and E) Data indicate mean ± SEM of three independent experiments each. The significance of (Figure legend continues)
adaptive cellular immune responses are affected by the loss of Myo9b. To address the question of what kind of Ab response Myo9b-/- mice were able to mount, we immunized mice with OVA in alum, and the humoral immune response was monitored thereafter. As shown in Supplemental Fig. 3, before immunization, both groups of mice produced negligible amounts of OVA-specific Abs (IgG1, IgG2, IgE). After immunization, mice sera contained well-detectable amounts of either class of OVA-specific Abs, whose concentration increased over time. Interestingly, in most cases, Ab titers were largely similar between WT and Myo9b-/- mice, except for IgG1, whose content was higher in sera of Myo9b-/- than in WT mice assessed 3 wk after immunization. These findings suggest that under these experimental conditions, Myo9b-/- mice develop largely unaltered humoral immune responses.

Loss of Myo9b in DCs alters their interaction with T cells

The formation of cell–cell contacts, such as the immunological synapse, is associated with cytoskeletal rearrangements. Because Myo9b-/- DCs exhibited overactive Rho signaling (Fig. 1) and a pronounced migratory defect (Fig. 2), we next assessed their capability to communicate with T cells in three-dimensional collagen gels by live cell imaging microscopy. On average, significantly fewer CD4+ OT-II T cells contacted a given Myo9b-/- BMDC than a WT BMDC (mean 3.4 versus 7.5 OT-II T cells per DC) within an observation period of 10 h (Fig. 4A). In contrast, the contact duration between BMDCs and T cells was significantly longer for Myo9b-/- than for WT BMDCs (median 27 versus 8 min; Fig. 4B, Supplemental Videos 1, 2). This difference was the result of an increased frequency of long contacts between Myo9b-/- BMDCs and T cells (Fig. 4C).

Because of the pronounced colocalization of Myo9b with dynamic F-actin (Fig. 1E) and the role of Myo9b in regulating the activity of the actin-binding proteins myosin II and cofilin via the Rho signaling axis (Fig. 1C), we monitored the distribution of F-actin in the immunological synapse in WT and Myo9b-/- BMDC/T cell cocultures. Interestingly, we observed a higher accumulation of F-actin at the synaptic cleft on the DC side in WT BMDCs than in Myo9b-/- BMDCs (Fig. 4D, 4E). The marked Myo9b-dependent effects on BMDC–T cell interaction were not due to an altered expression of MHC or costimulatory and adhesion molecules (Fig. 4F). Not only did the secretory pathways seem to be unaffected in Myo9b-/- BMDCs, but also the endocytic pathways including phagocytosis, macropinocytosis, and receptor-mediated differences was tested by unpaired t test. (G and H) Results of three independent experiments are shown. In each experiment, 20–25 individual cells were tracked. The significance of differences was tested by nonparametric Mann–Whitney U test. Medians are depicted.
endocytosis were unaltered in Myo9b<sup>−/−</sup> BMDCs (Supplemental Fig. 4). Also, the ability of Myo9b<sup>−/−</sup> BMDCs to process internalized OVA Ag was comparable with WT BMDCs (Supplemental Fig. 4).

Next, we assessed the functional consequences of the significantly altered DC–T cell interaction profile of Myo9b<sup>−/−</sup> BMDCs. In liquid cocultures containing WT or Myo9b<sup>−/−</sup> BMDCs as stimulators, T cells revealed no differences in the expression of activation-dependent surface molecules CD25 and CD69 (Fig. 5A). However, in three-dimensional collagen matrices, BMDCs from Myo9b<sup>−/−</sup> mice were less capable of inducing these activation molecules on T cells (Fig. 5B). Likewise, in liquid culture, either BMDC population stimulated the proliferation of OT-II T cells to a similar extent (Fig. 5C, left panel). In contrast, in three-dimensional collagen gels, OT-II proliferation was significantly lower when Myo9b<sup>−/−</sup> BMDCs were used as stimulators compared with WT BMDCs (Fig. 5C, right panel). In these three-dimensional cultures, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells was diminished to a proportional extent when Myo9b<sup>−/−</sup> BMDCs were used as stimulators (Fig. 5D). Consistent with our in vitro results, the proliferation of CFSE-labeled OVA peptide–specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells transferred to C57BL/6 mice, subsequently immunized with OVA peptide–loaded BMDCs, was significantly lower in spleen (Fig. 5E, left panel) and in LNs (Fig. 5E, right panel) when Myo9b<sup>−/−</sup> BMDCs were used for immunization.

**Discussion**

Tight regulation of immune cell motility and cell–cell interactions is of major importance for host immunity. Both processes depend on the spatiotemporal coordination of actin cytoskeleton dynamics by the monomeric G protein Rho. In this study, we show that the “motorized Rho inhibitor” Myo9b is an important signaling component required for the trafficking and T cell stimulatory capacity of DCs by controlling coflin and myosin II activities via the Rho/ROCK/LIMK signaling axis.

**Migratory and T cell stimulatory defects of Myo9b<sup>−/−</sup> BMDCs are rescued by activation of coflin**

We have shown that Myo9b acts as a negative regulator of Rho/ROCK signaling, and thereby controls the downstream targets coflin and MLC, both of which mediate cytoskeletal rearrangements. Furthermore, our results suggest that the functional defects of Myo9b<sup>−/−</sup> BMDCs are a consequence of deregulated cytoskeletal reorganization. To analyze the potential involvement of either actin-binding protein, we mimicked Myo9b activity by applying suitable pharmacological agents (Fig. 6A). Pharmacological inhibition of ROCK activity in Myo9b<sup>−/−</sup> BMDCs was sufficient to restore, in large part, the migratory (Fig. 6B) and T cell stimulatory (Fig. 6C) capabilities of Myo9b<sup>−/−</sup> BMDCs. However, the ROCK inhibitor did not significantly affect the behavior of WT BMDCs. An inhibitor of LIMK (S3) exhibited comparable activities (Fig. 6), which suggests that restoration of coflin activity is sufficient to revert the observed functional defects of Myo9b<sup>−/−</sup> BMDCs.

**FIGURE 4.** Myo9b<sup>−/−</sup> BMDCs show an altered interaction with CD4<sup>+</sup> T cells. (A–C) The number of CD4<sup>+</sup> OT-II T cells contacted by a single stimulated BMDC pulsed with OVA-peptide (A), individual DC–T cell contact duration (B), and the percentages of long DC–T cell contacts (C) were determined. One hundred to 250 contacts per group were recorded over 10 h of DC–T cell coculture in collagen gels by live cell imaging microscopy (see also Supplemental Videos 1, 2). The black line in (B) indicates the median contact duration (10 and 24 min, respectively). (C) The relative frequency of long contacts between BMDCs and CD4<sup>+</sup> T cells. (D) F-actin (Texas Red phalloidin) staining in immunological synapse areas. (E) Quantification of F-actin staining in the immunological synapse. (A–C and E) *p < 0.05, **p < 0.001 (unpaired t test; data obtained from two independent experiments). (F) FACS analysis of the expression of CD11c, CD11a, MHCII, CD40, CD54, CD80, CD86, and CD18 by WT and Myo9b<sup>−/−</sup> BMDCs at stimulated state. Graphs are representative of five independent experiments each.
Specifically, genetic deletion of Myo9b resulted in increased levels of activated (GTP-bound) RhoA in BMDCs. Elevated Rho activity, in turn, caused increased phosphorylation of MLC and of cofilin that result in increased acto-myosin contractility and reduced F-actin dynamics, respectively. In line with these molecular alterations, Myo9b-deficient BMDC and primary splenic DC populations exhibited a significant inhibition of spontaneous and chemotactic motility in a three-dimensional collagen matrix. We have recently reported similar functional defects for Myo9b-deficient mouse macrophages that adhered to a two-dimensional substrate (13). When Rho was inhibited with the toxin C3 in that study, Myo9b−/− macrophage cell velocity, but not chemotactic efficiency, was rescued. In this study, inhibition of downstream Rho effector molecules, namely, ROCK and LIMK, reverted the migratory defects of Myo9b−/− BMDCs in a three-dimensional collagen matrix. Because cofilin is the only known LIMK target, our results indicate that cofilin plays an important role in DC motility. In agreement with this notion, cofilin−/− bone marrow-derived macrophages were recently shown to display migratory defects that adhered to a two-dimensional substrate (13).

In support of the in vitro data, Myo9b-deficient cutaneous DCs showed impaired emigration from epidermal sheets in response to CCL21 in an ear explant model and reduced migration to the draining LNs after ear sensitization with FITC, indicating that Myo9b is required for regulating DC homing during the adaptive immune response. Because epidermal LCs and dermal DCs play critical roles in the processing of allergens and in the stimulation of hapten-specific T cells in the draining LNs (35, 40), our findings suggest a critical role for Myo9b in cutaneous immune reactions. Indeed, Myo9b was critically involved in eliciting a CHS. This was not due to an altered density of LCs in the epidermis of Myo9b−/− mice. Rather, the impaired contact hypersensitivity dermatitis response may be linked to the reduced migration of Myo9b−/− LCs and dermal DCs to the draining LNs and their impaired T cell stimulatory capacity. Thus, this study provides the
first evidence, to our knowledge, that Myo9b regulates certain DC-mediated immune responses.

Although the function of RhoA in the process of Ag uptake remains controversial and presumably depends on the respective endocytic pathway (41), it was somewhat surprising to find that endocytic pathways were not affected in Myo9b\(^{-/-}\) BMDCs. Because the activities of Rac and Cdc42 in the Myo9b\(^{-/-}\) cells remained unchanged, they may functionally antagonize the increased Rho activity during endocytosis. In support of this hypothesis, Blöcker et al. (38) reported that Pasteurella multocida toxin stimulated RhoA in DCs but did not influence Rac activity and overall macrophagocytic activity.

We suspected that the reduced T cell proliferation induced by Myo9b\(^{-/-}\) DCs in a three-dimensional matrix and in vivo was simply a consequence of impaired DC migration. However, when we took a closer look at the interaction of knockout versus WT BMDCs with CD4\(^+\) T cells, we observed that within the same period of time, significantly more T cells interacted with each WT DC than with each Myo9b\(^{-/-}\) DC, and that the frequency of long DC–T cell contacts was elevated in Myo9b\(^{-/-}\) DCs. Because we did not observe differences in overall surface molecule expression between WT and Myo9b\(^{-/-}\) DCs, our data suggest that the altered spatiotemporal organization of the actin cytoskeleton within the immunological synapse may explain the impaired T cell stimulatory capacity of Myo9b\(^{-/-}\) BMDCs. Inhibition of LIMK restored this functional defect, suggesting that the downstream Rho target cofilin is important for immunological synapse formation. Subject to the cofilin/actin ratio, cofilin may serve as an F-actin polymerizing or depolymerizing factor (8). Because Myo9b\(^{-/-}\) BMDCs displayed a much lower F-actin density at the immunological synapse site than WT BMDCs, active cofilin appears to be required for local submembranous F-actin polymerization upon T cell engagement. It is tempting to speculate that this kind of cofilin-mediated cytoskeletal rearrangements, controlled by Myo9b as an actin-binding motorized negative Rho regulator, may enable local concentration of surface receptors within the immunological synapse in DCs and, as a consequence, may determine the outcome of DC–T cell interaction as well. In support of this view, Eibert and coworkers (22) identified cofilin as a key factor in immunological synapse formation on the T cell side, whose inhibition interfered with T cell activation. Interestingly, in the same study, cofilin was not enriched within the immunological synapse in B cells. However, Verdijk et al. (42) reported translocation of cofilin toward submembranous regions in DCs in a stimulation-dependent manner, whereas we have observed an even distribution of cofilin within the cytosol of stimulated BMDCs (data not shown). Together, these findings suggest that cofilin may contribute to different degrees to immunological synapse formation in distinct APC populations. It will be of interest to correlate the cell-type–specific relevance of cofilin for immunological synapse formation with immunological synapse structural composition, shown to clearly differ between DC and other types of APCs (43, 44).

In summary, we have identified Myo9b as a key regulator of spatiotemporal Rho activity in DCs with cofilin as a target protein of major importance for the regulation of DC migration and T cell stimulatory capacity. Future work will have to determine the precise pathways and factors that control localized Myo9b activity in DCs. Based on our findings and the notion that Myo9b may play a role in inflammatory bowel disease and other autoimmune diseases (19, 20), it will be of interest to determine to which extent the loss of Myo9b affects peripheral tolerance under homeostatic conditions and immune responses toward infections and allergic reactions that are mediated by DCs.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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**FIGURE 6.** Inhibition of Rho signaling at downstream checkpoints restores the migratory and T cell stimulatory capacity of Myo9b\(^{-/-}\) BMDCs. (A) Schematic overview of the Myo9b/Rho/ROCK/LIMK signaling cascade and inhibitors applied. (B) Mature WT and Myo9b\(^{-/-}\) BMDCs, pretreated with the inhibitors Y-27632 or S3-fragment of actin-depolymerizing factor/cofilin or left untreated, were cultivated in collagen gels pretreated with the inhibitors Y-27632 or S3-fragment of actin-depolymerizing factor (8). Because Myo9b\(^{-/-}\) BMDCs displayed a much lower F-actin density at the immunological synapse site than WT BMDCs, active cofilin appears to be required for local submembranous F-actin polymerization upon T cell engagement. It is tempting to speculate that this kind of cofilin-mediated cytoskeletal rearrangements, controlled by Myo9b as an actin-binding motorized negative Rho regulator, may enable local concentration of surface receptors within the immunological synapse in DCs and, as a consequence, may determine the outcome of DC–T cell interaction as well. In support of this view, Eibert and coworkers (22) identified cofilin as a key factor in immunological synapse formation on the T cell side, whose inhibition interfered with T cell activation. Interestingly, in the same study, cofilin was not enriched within the immunological synapse in B cells. However, Verdijk et al. (42) reported translocation of cofilin toward submembranous regions in DCs in a stimulation-dependent manner, whereas we have observed an even distribution of cofilin within the cytosol of stimulated BMDCs (data not shown). Together, these findings suggest that cofilin may contribute to different degrees to immunological synapse formation in distinct APC populations. It will be of interest to correlate the cell-type–specific relevance of cofilin for immunological synapse formation with immunological synapse structural composition, shown to clearly differ between DC and other types of APCs (43, 44).

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