Rho-mDia1 pathway is required for adhesion, migration, and T cell stimulation in dendritic cells

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Running title: Essential role of mDia1 in DC functions

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Abstract

Dendritic cells (DCs) are essential for the initiation of acquired immune responses through antigen acquisition, migration, maturation, and T cell stimulation. One of the critical mechanisms in this response is the process actin nucleation and polymerization, which is mediated by several groups of proteins, including mammalian Diaphanous-related formins (mDia). However, the role of mDia in DCs remains unknown. Herein, we examined the role of mDia1 (one of the isoforms of mDia) in DCs. Although the proliferation and maturation of marrow-derived dendritic cells (BMDCs) were comparable between control C57BL/6 and mDia1-deficient (mDia1−/−) mice, adhesion and spreading to cellular matrix were impaired in mDia1−/− BMDCs. In addition, FITC-induced cutaneous DC migration to draining lymph nodes in vivo and invasive migration and directional migration to CCL21 in vitro were suppressed in mDia1−/− DCs. Moreover, sustained T cell interaction and T cell stimulation in lymph nodes were impaired by mDia1 deficiency. Consistent with this, the DC-dependent delayed hypersensitivity response was attenuated by mDia1-deficient DCs. These results suggest that actin polymerization, which is mediated by mDia1, is essential for several aspects of DC-initiated acquired immune responses.
Introduction

When external antigens invade the body, professional antigen-presenting dendritic cells (DCs) capture them, upregulate the expression levels of co-stimulatory molecules and chemokine receptors such as CCR7 and CXCR4, and migrate to draining lymphoid tissues via the lymphatic vessels. During the initial phase of T cell priming within the T cell areas of lymph nodes (LNs), DCs project polarized membrane extensions that facilitate repeated and, under certain conditions, sustained DC-T cell contacts called immunological synapses, which facilitate T cell proliferation and differentiation.

The actin cytoskeleton undergoes continuous remodeling and serves as the machinery for antigen acquisition, cell migration, and sustained cell contact. A critical step in this remodeling process is the formation of actin oligomers, which serve as nuclei for further polymerization. Actin nucleation and polymerization in mammalian cells is catalyzed by several groups of proteins. Two of the most important catalyst groups here are the mammalian Diaphanous-related (mDia) formins, which produce long and straight actin filaments, and the Wiskott-Aldrich syndrome protein (WASP)-Arp2/3 system, which produces a branched actin meshwork. Lack of WASP expression results in defects in T cell proliferation, T cell receptor capping, phagocytosis of macrophages, and migration of immune cells, all of which are involved in chemotaxis, chemokinesis, and adhesion.

The mDia family formins are among the effectors of Rho GTPase. The family is composed of three isoforms: mDia1, mDia2, and mDia3. The ability of mDia proteins to promote rapid assembly of actin filaments appears to be crucial to cell polarity, morphogenesis, and cytokinesis. Most studies on the mDia family have used cultured cells. As a result, the roles of mDia proteins in vivo remain unclear. Recently, we and other researchers have generated mice deficient...
in mDia1 and reported on its role in T cell proliferation, trafficking, and interaction with DCs 16,17.

Since the mDia family drives actin polymerization, mDia proteins may contribute to the functions of DCs 18, but the roles of mDia in DCs are still unclear. In this study, we studied mDia1-deficient (mDia1<sup>−/−</sup>) mice and the cells derived from these mice, and found that mDia1 in DCs is essential for adhesion to fibronectin, transmigration through small pore membranes, directional migration, and sustained interaction with T cells.

**Methods**

**Mice, reagents, flow cytometry and cell sorting**

We generated mDia1<sup>−/−</sup> mice 16 and backcrossed them for more than 10 generations onto C57BL/6J Slc (B6) mice (Japan SLC Inc., Shizuoka, Japan). OT-I, OT-II (Jackson Laboratory, Bar Harbor, ME, USA) and BALB/c (Japan SLC Inc.) mice were bred at the Institute of Laboratory Animals at Kyoto University on a 12-h light/dark cycle under specific-pathogen-free conditions. Eight- to 10-week-old female mice were used for all experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Carboxyfluorescein succinimidyl ester (CFSE) and CellTracker<sup>TM</sup> Red CMTPX were purchased from Invitrogen (Carlsbad, CA, USA). FITC, PE, PE-Cy5, PE-Cy7, APC, Pacific Blue-conjugated RM4-5 (anti-CD4), 53-6.7 (anti-CD8), HI111 (anti-CD11a), M1/70 (anti-CD11b), HL3 (anti-CD11c), HM40-3 (anti-CD40), YN1/1.7.4 (anti-CD54), Ly-22 (anti-CD62L), 16-10A1 (anti-CD80), 24-31 (anti-OX40L), GL1 (anti-CD86), M5/114.15.2 (anti-MHC class II), 4B12 (anti-CCR7) monoclonal antibodies (Abs) and PE-Cy7-conjugated
streptavidin were purchased from eBioscience (San Diego, CA, USA). Biotin–conjugated eBioRMUL2 (anti-Langerin) and PECy-7-conjugated H1.2F3 (anti-CD69) Ab were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Flow cytometry was performed using FACSCantoII (BD Biosciences) and analyzed with FlowJo (TreeStar, San Carlos, CA, USA). Cell sorting was performed using an autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) or a cell sorter (FACS Aria II, BD Biosciences) 19.

**Cell culture, mixed lymphocyte reaction (MLR), and ELISA**

The complete RPMI (cRPMI) culture medium consisting of RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS), 5 x 10⁻⁵ M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin, was used, unless otherwise indicated.

For bone marrow-derived DCs (BMDC) culture, 5 x 10⁶ BM cells were cultured in 10 mL of cRPMI supplemented with 5 ng/ml recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ, USA) for 5 to 7 days 20.

For spleen cell suspensions, spleens were cut into small pieces and digested by being mixed for 25 min at 37°C with RPMI-1640 containing 1.6 mg/ml type II collagenase (Worthington Biochemical, Freehold, NJ, USA) and 100 µg/ml DNase I (Sigma-Aldrich).

For epidermal cell suspensions, ear skin sheets were floated on 0.25% trypsin in phosphate-buffered saline (PBS) for 30 min at 37°C 21. The epidermis was separated from the dermis with forceps in PBS supplemented with 10% FCS. The epidermis was filtered through a 40-µm cell strainer. For dermal cell suspensions, the dermis was further digested with 1.6 mg/ml type II collagenase and 100 µg/ml DNase I in cRPMI for 25 min at 37°C. The cells were then
filtered through a 40-µm cell strainer.

For allogeneic MLR, 1 x 10³ or 10⁴ BMDCs with a B6 background were pretreated with
mitomycin C and incubated with 5 x 10⁵ CD4⁺ T cells from BALB/c mice in 200 µl of cRPMI
for 3 days. The cells were pulsed with 0.5 µCi ³H-thymidine for the last 24 h, and subjected to
liquid scintillation counting. For the measurement of cytokine production, culture supernatants
were collected 72 h after the start of cultivation and measured with an ELISA kit (BD Biosciences).

Chemotaxis, TAXIScan, adhesion, cell polarity, and fluorescein isothiocyanate
(FITC)-induced DC migration assays

For the chemotaxis assay, suspensions of epidermal cells were prepared and incubated overnight,
then 1 x 10⁶ cells were transferred into the upper chamber of the transwell with 3 or 8-µm pore
size filters (BD Biosciences) and 10 or 100 ng/ml of CCL21 (R&D systems, Minneapolis, MN,
USA) was added to the lower chamber, and allowed to incubate for 3 h or 24 h at 37°C. The cells
that migrated to the lower chambers were stained with MHC class II and counted by flow
cytometry (32). The migration index was presented as a percentage of input: the number of cells
in each lower chamber was divided by the total number of cells placed in the corresponding
upper chamber.

For the evaluation of invasive migration, the upper chambers (8-µm pore size) were coated
with Matrigel (BD Biosciences), which consists of laminin, collagen IV, heparin sulfate
proteoglycans, and entactin/nidogen. After 24 h of incubation, the upper chambers were washed
three times with 500 µl of PBS. The upper chambers were treated with 500 µl of dispase (0.15
mg/ml; Gibco, Grand Island, NY, USA) cRPMI for 60 min at 37°C to collect the cells in the
For the TAXIScan assay, time-lapse images of BMDCs during chemotaxis were obtained using TAXIScan (GE Healthcare Piscataway, NJ, USA) as described previously. First, 2 x 10^6 BMDCs were applied to the lower side of the chamber. Data were collected at 60-sec intervals for 6 h, starting at 10 min after addition of 1 μl of CCL21 (10 μg/mL) to the upper side of the chamber, and analyzed with the TAXIScan Analyzer 2 (GE Healthcare).

For the adhesion assay, 4 x 10^4 CFSE-labeled BMDCs were incubated in a 96-well plate that had previously been coated overnight with PBS with or without 10 μg/ml of fibronectin (FN), and incubated for 45 min at 37°C. The plate was read in a fluorescent plate reader (Wallac 1420 ARVOsx, Perkin Elmer, Waltham, MA, USA) at 485 nm excitation and 535 nm emission, both immediately after incubation, to determine the original number of cells (the fluorescent total or FT). The plate was read again after it had been washed twice by vigorous pipetting with 200 μl of PBS and decanting, to determine the number of cells that had adhered (the fluorescent adhesion or FA). The percentage of adherence was calculated as the ratio of FA/FT.

To evaluate the cell polarity induced by the chemokine gradient, 25 μl of CCL21 (10 ng/mL) was added to the edge of the BMDC culture in 24-well plates. Thirty minutes later, the culture medium was washed out, and the cells were fixed, permeabilized and stained with phalloidin as described below.

For FITC-induced cutaneous DC migration, the footpads of mice were painted with 50 μl of 2% FITC (Sigma-Aldrich) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma-Aldrich) mixture; 72 h later, the number of cutaneous DCs that had migrated into skin-draining popliteal and inguinal LNs was counted by means of flow cytometry.
**Immunostaining**

BMDCs were plated on coverslips coated with or without 10 µg/ml FN for 16 h. The cells were fixed for 15 min with 3.7% formalin (Wako, Osaka, Japan), and permeabilized with 0.1% triton-X (Sigma-Aldrich) in PBS for 7 min at room temperature. Next, slides were incubated with the anti-mDia1 or anti-vinculin Ab (BD Biosciences) and Alexa Fluor 488-conjugated phalloidin (Invitrogen) at room temperature for 1 h, then stained with Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen) for 30 min at room temperature. The slides were mounted in ProLong Gold Antifade reagent (Invitrogen) and fluorescence images were obtained using a BIOREVO BZ-9000 system (Keyence, Osaka, Japan).

**Antigen acquisition, DC kinetics, T cell priming and delayed-type hypersensitivity (DTH) model**

The activity of antigen acquisition by DCs was measured with 2 mg/ml FITC-OVA (Molecular Probes, Eugene, OR, USA). After incubation for 45 min at 4°C or 37°C, the cells were washed 4 times with PBS and the mean fluorescence intensity (MFI) of FITC was measured by flow cytometry.

For the evaluation of the kinetics of DCs in the LNs, BMDCs were cultured for 7 days, stimulated with 200 ng/ml of LPS for 2 h, and labeled with 5 µM CFSE or 3.3 µM CMTPX at 37°C for 20 min.

For the assessment of the interaction between CD4⁺ T cells and BMDCs, BMDCs were cultured for 7 days, pulsed with 1 µg/ml of OVA (Sigma-Aldrich) for 30 min at 37°C, stimulated with 200 ng/ml of LPS for 2 h, and labeled with 5 µM CFSE at 37°C for 20 min. Three million B6 or mDia1⁻/⁻ BMDCs were injected into the footpads of B6 mice. Twenty-four hours later,
CD4⁺ T cells from OT-II mice purified with CD4⁺ T cell negative separation kits (Miltenyi Biotech) were labeled with 3.3 μM CMTPX and transferred into B6 mice intravenously (1 x 10⁷ cells per mouse). Two hours later, the popliteal and inguinal LNs were harvested and observed for 90 min under two-photon microscopy to measure the period of conjugate formation between T cells and DCs.

For assessing the ability of DCs to induce OT-II CD4⁺ T cell proliferation or OT-I CD8⁺ T cell proliferation, 20 μL of 0.5 μg/ml OVA emulsified with complete Freund’s adjuvant (CFA; Difco, Detroit, MI, USA) and PBS (1:1) was injected into mouse footpads. CD4⁺ T cells from OT-II mice and CD8⁺ T cells from OT-I mice were isolated using magnetic bead separation (Miltenyi Biotech) and labeled with 8 μM CFSE. One day after injection of OVA-CFA emulsion, 1 x 10⁷ CFSE-labeled OT-II CD4⁺ T cells or OT-I CD8⁺ T cells were transferred via the tail vein. After 48 h, draining popliteal and inguinal LNs and non-draining axillary LNs were collected and analyzed by flow cytometry.

For DTH response, mice were sensitized with 20 μl of 0.5 mg/ml OVA in CFA or with 5 x 10⁵ LPS-pretreated and 1-μg/ml-OVA-pulsed BMDCs in 20 μl PBS subcutaneously injected into the front footpads. Five days later, the mice were challenged with an injection of 20 μl of 0.5 mg/ml OVA in CFA into the hind footpads. Footpad swelling was measured before and 48 h after the challenge. Controls consisted of CFA injections containing no OVA or sensitization with non-OVA pulsed DCs.

**Two-photon imaging**

Two-photon imaging was performed as previously described 23 with some modifications. Briefly, we used a custom-built, video-rate multiphoton microscope: an upright BX61WI microscope
(Olympus, Tokyo, Japan) fitted with a x20 water-immersion objective lens (numerical aperture: 0.95), a Spectra-Physics Mai Tai femtosecond laser (Newport, Santa Clara, CA, USA), and a resonant-mirror scan head. The image acquisition process, the z-axis stepper, shutters, and the emission filter wheel were under software control (FluoView10-ASW1.6, Olympus). LNs were cemented to the base of the imaging chamber with a thin film of veterinary-grade superglue and were continuously superfused with warm (35°C to 37°C) RPMI 1640 medium bubbled with 95% O2 and 5% CO2.

Quantitative RT-PCR analysis
Total RNA was isolated using an RNeasy kit (QIAGEN, Hilden, Germany) with an on-column DNase I digestion kit (QIAGEN). cDNA was synthesized using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative PCR analysis was carried out with a Light Cycler™ 480 (Roche Diagnostics, Foster City, CA, USA) using SYBR Green I (Takara Bio) with 40 cycles of 5 sec at 95°C, 15 sec at 60°C, and 10 sec at 72°C. Primer sequences used in this study are as follows: 

- **mDia1**, 5’- CGA CGG CGG CAA ACA TAA G-3’ (forward) and 5’- TGC AGA GGA GTT TCT ATG AGC A-3’ (reverse);
- **mDia2**, 5’- GAG AAG CGA CCC AAG TTG CAT-3’ (forward) and 5’- GAA GGG GAG GTC TCT CTT TCT T-3’ (reverse);
- **mDia3**, 5’- AAT CTT CTG GAA GCC CTA CAG T-3’ (forward) and 5’- GGC CGT CTG TTA TCT GGA TTT C-3’ (reverse);
- **Gapdh**, 5’-AAA CCC ATC ACC ATC TTC CA -3’ (forward) and 5’-GTG GTT CAC ACC CAT CAC AA-3’ (reverse).

The reaction protocol included preincubation at 95°C to activate FastStart DNA polymerase for 1 min, 40 cycles of amplification each consisting of 5 sec at 95°C, annealing for 15 sec at 60°C and elongation for 10 sec at 72°C. Relative quantification was performed after determining the cycle threshold (Ct) values for reference gene **Gapdh**, and
target genes in each sample sets according to the 2−ΔΔCt method\textsuperscript{24}. We verified that all primer sets used in this study showed similar amplification efficiency. For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were used.

Statistical analysis

Unless otherwise indicated, data are presented as means ± standard deviations (SD). \(P\)-values were calculated with the two-tailed Student’s \(t\)-test after data were confirmed to meet the criteria of normal distribution and equal variance using Lilliefors test.

Results

mRNA expression of the mDia family in DCs

We examined expression of mRNA for the mDia isoforms in DCs through a quantitative RT-PCR analysis. We prepared B6 and mDia1\textsuperscript{-/-} BMDCs, epidermal LCs, dermal and splenic DCs, and CD4\textsuperscript{+} T cells of B6 mice and found that transcripts encoding all three isoforms were detected in all DC populations (Figure 1). The expression levels of the mDia isoforms in BMDCs were not affected by the maturation stimulated by LPS. In BMDCs from mDia1\textsuperscript{-/-} mice, mDia1 expression was not detected as expected and, interestingly, mDia2 mRNA levels were higher than those of WT mice.

Impaired interstitial migration of cutaneous DCs in mDia1\textsuperscript{-/-} mice

Initially we evaluated the role of mDia1 in the development of DCs. The numbers of skin-derived DCs, resident DC subsets, splenic DC subsets, and skin DC subsets, and the maturation status of DCs in skin-draining LNs were comparable between B6 and mDia1\textsuperscript{-/-} mice (Supplemental Fig. 1). In addition, the number and maturation rates of mDia1\textsuperscript{-/-} BMDCs were
similar to those of B6 BMDCs in the absence (Supplemental Fig. 2A-D) or presence (Supplemental Fig. 2E) of 200 ng/mL LPS for the last 2 h of 5-day cultivation period. These results suggest that mDia1 deficiency does not affect the development and maturation of conventional DCs.

We evaluated the significance of mDia1 with regard to DC functions in vivo. We used FITC, a fluorescent hapten, to induce DC maturation and mobilization. FITC applied topically to the footpads of mice is taken up by cutaneous DCs, which then migrate to draining popliteal and inguinal LNs as MHC class II$^+$ FITC$^+$ cells. The number of MHC class II$^+$ FITC$^+$ DCs that accumulated in draining LNs was significantly lower in mDia1$^{-/-}$ mice than in B6 mice (Fig. 2A), and the numbers of Langerin$^+$ DCs (including both LCs and Langerin$^+$ dermal DCs) and Langerin$^-$ dermal DCs among the FITC$^+$ population of mDia1$^{-/-}$ mice were lower in mDia1$^{-/-}$ mice than in B6 mice (Fig. 2B). On the other hand, FITC$^+$ mDia1$^{-/-}$ DCs were localized appropriately in the T zone of draining LNs, as observed using fluorescent microscopy (data not shown).

To assess the role of mDia1 in DC mobility, we performed a transwell assay with 3- and 8-µm pore sizes. Epidermal cell suspensions from the ears of B6 and mDia1$^{-/-}$ mice were applied to the upper transwell chambers, and the numbers of MHC class II$^+$ LCs in lower chambers with or without CCL21 were measured. The chemotaxis of mDia1$^{-/-}$ LCs to lower chambers containing CCL21 through the 8-µm transwell tended to be low, but not significantly so, compared to that of B6 LCs (Fig. 2C, right panel). The impaired chemotaxis of LCs by mDia1 deficiency became more distinct when transwells with a smaller pore size (3-µm) were used (Fig. 2C, left panel), suggesting that mDia1 is more essential to cell polarity-dependent transmigration. Next, we evaluated the invasion and directional movement of LCs by coating the polycarbonate filters of
the 8-µm transwell with Matrigel. Three hours after application, the number of LCs in the Matrigel was increased by the addition of CCL21 to the lower chamber, which was further increased by deficiency of mDia1 (Fig. 2D, right panel). On the other hand, the number of LCs in the lower chamber was decreased by deficiency of mDia1 (Fig. 2D, left panel). Similar results were obtained 24 h after the chemotaxis assay (Fig. 2E). These results suggest that mDia1 in DCs is indispensable for migration into the extracellular matrix protein-enriched area. We further evaluated the directional migration of BMDCs using TAXIScan and found that migration towards a gradient of the chemokine CCL21 was impaired by mDia1 deficiency in BMDCs (Fig. 2F).

**DC adhesion to FN was partially impaired by mDia1 deficiency**

The interaction of DCs with extracellular matrix proteins such as FN is essential for their migration and formation of adhesion with integrins. We evaluated localization of mDia1 in DCs, and the effect of the deficiency of mDia1 on morphology and adhesions of BMDCs. We incubated BMDCs on plates coated with FN for 30 min and stained them with Alexa Fluor 488-labelled phalloidin and non-conjugated mDia1 Ab, followed by Alexa Fluor 546 goat anti-mouse IgG. Microscopic examination revealed that endogenous mDia1 was localized in the cytoplasm diffusely, and around the leading edge of B6 BMDCs strongly, and this localization was abolished in mDia1−/− BMDCs (Fig. 3A).

We then evaluated the morphology and adhesion of BMDCs. We incubated BMDCs for 30 min, 4 h, and 18 h, and stained them with Alexa Fluor 488-labelled phalloidin and non-conjugated mDia1 Ab, followed by Alexa Fluor 546 goat anti-mouse IgG. Most B6 DCs exhibited membrane protrusions at 30 min after incubation. The cells spread out 4 h after
incubation, and stress fibers were clearly formed 18 h after cultivation (Fig. 3B). In contrast, almost all mDia1−/− BMDCs maintained their round shape without the formation of distinct membrane protrusions, but with distinct filopodia formation 30 min after incubation; and this apparent filopodia formation was still visible 4 h, and even 18 h after incubation (Fig. 3B). To further evaluate podosome/focal adhesion formation, BMDCs were stained with Alexa Fluor 488-labelled phalloidin and non-conjugated vinculin Ab 4 h after incubation, followed by Alexa Fluor 546 goat anti-mouse IgG. Although adhesion structures were detected in both B6 BMDCs and mDia1+/− BMDCs (Supplemental Fig. 3A), the frequencies of cells with podosome and focal adhesion formation were significantly lower in mDia1-deficient cells (podosome formation: 61.3±4.3 versus 52.9±2.6%; focal adhesion: 62.9±3.1% versus 44.1±3.1%; for B6 versus mDia1+/− BMDCs, respectively; data presented as average±SEM from 5 independent experiments, with 20 cells from each group evaluated). We then examined the surface expression levels of integrins and adhesion molecules, such as CD11a (LFA-1), CD11c, CD54 (ICAM-1), CD62L (L-selectin) and CD11b, and found that expression levels of these molecules were comparable between B6 BMDCs and mDia1+/− BMDCs (Supplemental Fig. 3B). Lastly, we evaluated the cell polarity of BMDCs exposed for 30 min to CCL21 chemokine by staining with F-actin (Supplemental Fig. 3C). A chemokine gradient induced cell spreading of both B6 and mDia1−/− BMDCs with a visible leading edge (Supplemental Fig. 3C). We evaluated the cell polarity and cell spreading by scoring BMDCs for polarized accumulation of actin and evaluated the major/minor axis ratio. Both the cell polarity and cell spreading of mDia1−/− BMDCs were less prominent than those of B6 BMDCs (Supplemental Fig. 3D and E). These results were consistent with the previous finding that the mean velocity of BMDCs to CCL21 was partially attenuated by mDia1 deficiency in a TAXIScan assay (Fig. 2F).
We further evaluated the major/minor axes of BMDCs as indicators of cell polarity and protrusion after the BMDCs had been incubated on the coverslips for 16 h. This parameter was significantly lower in mDia1<sup>−/−</sup> BMDCs than in B6 BMDCs (Fig. 3C). We next assessed the adhesion of BMDCs after a 45-min incubation period. The incidence of adhesion of BMDCs to the plates, indicated as a percentage, was higher when the plate was coated with FN, and the incidence of adhesion was significantly higher in B6 BMDCs than in mDia1<sup>−/−</sup> BMDCs (Fig. 3D). These results suggest that mDia1 is an essential factor for the adhesion of DCs to FN, as well as for the establishment of cell polarity.

**mDia1 deficiency in DCs impairs DC interaction with T cells**

T cell activation and differentiation require sustained interaction with DCs via integrins<sup>5</sup>. To investigate whether mDia1 deficiency in DCs impairs their interaction with T cells, we performed 3D live-imaging of DCs using two-photon microscopy. To avoid environmental interference resulting from mDia1 deficiency in cells other than DCs, experiments were conducted using B6 mice as recipients for the transfer of B6 and mDia1<sup>−/−</sup> BMDCs. The dynamics of DC behavior in draining popliteal LNs were evaluated through the injection of CFSE- or CMTPX-labeled BMDCs pretreated with LPS into the footpads of B6 mice (Supplemental Video 1). The mean velocities, turning angles (i.e., deviations from the previous orientation), and displacements (i.e., distances between starting and ending positions) were comparable between B6 DCs and mDia1<sup>−/−</sup> DCs (Supplemental Fig. 4A-C).

We next evaluated DC-T cell interaction in the LNs. T cells slow down their active migration and interact with DCs for up to several hours during the initial phase of T cell priming<sup>31,32</sup>. BMDCs from B6 and mDia1<sup>−/−</sup> mice were pulsed with or without OVA, pretreated with LPS,
labeled with CFSE, and injected into the footpads of mice. Twenty-four hours later, we intravenously transferred CMTPX-labeled CD4+ OT-II T cells, whose T cell receptors specifically recognize OVA. An additional 2 h later, we measured the duration of contacts formed between DCs and T cells in popliteal LNs during a 90-min period of observation using two-photon microscopy (Supplemental Videos 2 and 3). The addition of OVA induced long-lasting contacts between B6 DCs and OT-II T cells; the length of these contacts was attenuated by mDia1 deficiency in DCs (Figs. 4A, B). Our measurement of the durations of DC-T cell conjugations revealed that mDia1−/− DCs exhibited higher rates of shorter-duration contacts, but lower rates of longer-duration contacts, than B6 DCs did (Fig. 4B). Consistent with this, the overall mean duration of individual DC-T interactions was shorter in mDia1−/− DCs than in B6 DCs (Fig. 4C). We also evaluated whether the impaired T cell-DC interaction brought about by mDia1 deficiency was not merely due to impaired antigen acquisition by mDia1 BMDCs. The activity of temperature-dependent antigen acquisition of DCs was assessed by measuring FITC-labeled OVA in the cells using flow cytometry, and it was found that the activity levels were comparable between B6 and mDia1−/− mice (Fig. 4D). Taken together, these results indicate that mDia1 expression in DCs is required to maintain sustained interactions with T cells.

**mDia1 deficiency attenuates T cell stimulatory capacity of DCs in vitro**

Based on the results we had thus far obtained, we sought to clarify whether mDia1 deficiency in DCs attenuated T cell stimulatory capacity by means of MLR. Alloreactive CD4+ T cells were purified from BALB/c mice and stimulated with B6 or mDia1−/− BMDCs. Proliferation of T cells was lower in response to stimulation with mDia1−/− BMDCs than in response to stimulation with B6 BMDCs (Fig. 5A). Consistently, levels of IFN-γ were lower in the supernatants of T cell
cultures stimulated with mDia1−/− BMDCs than in those stimulated with B6 BMDCs (Fig. 5B).

We further examined the expression levels of MHC class II, CD40, and CD54 as indicators of DC maturation 24 and 48 h after cultivation, and found that MFIs of all surface markers of mDia1−/− BMDCs were lower than those of B6 BMDCs 48 h after cultivation (Figs. 5C-E). We assume that DCs undergo a further maturation upon sustained interaction with T cells in an mDia-1 dependent manner, therefore, mDia1 deficiency in DCs leads to impaired T cell stimulatory capacity in MLR.

**Priming of T cells is reduced by mDia1 deficiency in vivo**

Based on these results, we hypothesized that mDia1 deficiency in DCs leads to the attenuation of T cell priming in vivo. We immunized B6 and mDia1−/− mice with OVA emulsified in CFA and transferred CFSE-labeled OT-II CD4+ T cells intravenously. Forty-eight hours later, draining popliteal and inguinal LN cells were dissociated, and dividing cells were measured with flow cytometry (Figs. 6A, B). The numbers of total and dividing OT-II CD4+ T cells per draining LNs were significantly attenuated in mDia1−/− mice compared to B6 mice (Fig. 6C). We also examined non-draining axillary LN cells for comparison purposes, and found that the frequencies of CFSE-positive OT-II CD4+ T cells to total cells were comparable in mDia1−/− and B6 mice (Supplemental Fig. 5). Cells in the process of division were not detected (Supplemental Fig. 5). In addition, the numbers of activated CD69+ CFSE+ cells and the relative amounts of IFN-γ mRNA were lower in the draining LNs of mDia1−/− mice than in those of B6 mice (Figs. 6D, E).

We next repeated the above experiments using OT-I CD8+ T cells instead of OT-II CD4+ T cells, in order to evaluate the capacity of DCs to stimulate CD8+ T cells. Like that of OT-II CD4+
T cells, stimulation of OT-I CD8 T cell proliferation was impaired by mDia1 deficiency in DCs (Fig. 6F). These findings indicate that DCs require mDia1 in order to prime both CD4+ and CD8+ T cells in vivo.

Finally, we evaluated the impact of mDia1 deficiency in DCs on immune responses by using a DTH model. B6 and mDia1−/− mice were subcutaneously sensitized with OVA in CFA in the front footpads, and challenged with OVA in CFA in the hind footpads. Mice not subjected to OVA sensitization were used as a negative control. The hind footpad swelling change during the 48 h after challenge was attenuated in mDia1−/− mice compared to B6 mice (Fig. 6G), providing further evidence that mDia1 is critical for the T cell-priming ability of DCs in vivo. To avoid environmental interference resulting from mDia1 deficiency in cells other than DCs, we transferred B6 or mDia1−/− BMDCs pulsed with OVA into the front footpads and challenged the mice with OVA in CFA into the hind footpads. Mice injected with B6 BMDCs that had not been pulsed with OVA during sensitization were used as a negative control. The footpad thickness change was almost entirely abolished by mDia1 deficiency in DCs (Fig. 6H). Thus, mDia1 in DCs is critical for the initiation of DTH response via T cell priming in vivo.

Discussion

The role of mDia1 in the reorganization of the DC cytoskeleton has not yet been fully studied. In this report, we demonstrated that the number and maturation of DC subsets in the LNs, spleen, and the skin in the steady state, the development and maturation of BMDCs in vitro, and the motility of BMDCs in the LNs were not affected by mDia1 deficiency. However, DCs from mDia1−/− mice exhibited impaired transmigration of LCs through transwells with small pore size, directional migration towards a chemokine gradient, cell adhesion to FN, and interaction with T
cells. Consistent with this, the CD4+ and CD8+ T cell-stimulating capacity of DCs, as well as their OVA-induced DTH response *in vivo*, were attenuated as a result of mDia1 deficiency. The above results demonstrate the involvement of mDia1 at each step of DC functioning, as well as the importance of actin cytoskeletal reorganization and cognate interaction through mDia1 in DCs.

Cell migration is frequently described as a multistep cycle. F-actin polymerization at the cell surface induces a membrane protrusion, which is subsequently anchored to the extracellular matrix by integrins, which couple with the cytoskeleton and transduce the internal force via the actin cytoskeletal network \(^{33,34}\). Although motility in the LNs and invasion into the extracellular matrix-rich area remained mostly intact in mDia1 \(^{-/-}\) BMDCs, the transmigration to CCL21 through transwells with small pore size, migration of mDia1 \(^{-/-}\) BMDCs across the Matrigel-coated transwell, and directional migration to chemokine on TAXIScan were all impaired. In addition, cell spreading and membrane protrusion were attenuated in mDia1 \(^{-/-}\) BMDCs. These findings are likely to explain the *in vivo* finding that FITC-induced cutaneous DC migration from the skin to the draining LNs was impaired. Using rat C6 glioma cells, Yamana et al. demonstrated that mDia1 is required for cell polarization, cell focal adhesion turnover, and microtubule stabilization, possibly through the collaboration with Rac and Cdc42 \(^{14}\). Therefore, the role of Rac/Cdc42 in mDia1 \(^{-/-}\) DCs in cell spreading and adhesion is worthy of future study.

Recent findings have suggested that integrins are essential for crossing the endothelial or epithelial lining, but not for migration of DCs in three-dimensional environments \(^{35}\). Instead, these cells migrate solely by the force of actin-network expansion, which promotes the protrusive flow of the leading edge. In addition, the dynamics of DC behavior were not affected by
deficiency of mDia1 in LNs in the steady state, which implies that mDia1 is not necessary for DCs to perform ‘amoeboid movement’\textsuperscript{36}. On the other hand, the invasive migration through the Matrigel and the directional migration to a chemokine gradient were attenuated in mDia1\textsuperscript{-/-} BMDCs. Therefore, mDia1 appears indispensable for invasive movement in the extracellular matrix-rich area and directional movements towards a chemokine gradient, which depends on cell polarity (at least in part).

It is notable that behavior similar to that of mDia1\textsuperscript{-/-} DCs, such as decreased adhesion and defects in T cell stimulating capacity, has been observed in WASP\textsuperscript{-/-} DCs\textsuperscript{37-39}. Yet the impairments in maturation and chemotaxis, and the dislocalization of DCs in the LNs that were observed in WASP\textsuperscript{-/-} DCs\textsuperscript{38} were not observed in mDia1\textsuperscript{-/-} DCs. Yet although WASP-deficient DCs form abnormal lamellipodia resulting in hyper-elongated morphology\textsuperscript{40}, mDia1\textsuperscript{-/-} DCs exhibit a rather round morphology without stress fiber formation. Collectively, these results suggest that the two actin-nucleating systems work together to enable the functions of DCs, and that the lack of either cannot be compensated for by the other.

Given that the mDia family plays an elemental role in the actin filament dynamics in DCs, it is surprising that mDia1\textsuperscript{-/-} DCs exhibited relatively mild and limited dysfunction. We ourselves (in a previous study) and other researchers have previously found only partial impairment of T cell trafficking in mDia1\textsuperscript{-/-} mice\textsuperscript{16,17}. This may be partly explained by the fact that the roles of the other mDia isoforms, mDia2 and mDia3, overlap somewhat with those of mDia1\textsuperscript{9}. In addition, we demonstrated that the mDia2 expression level in mDia1\textsuperscript{-/-} DCs was elevated, which suggests that mDia2 might be induced to compensate for the deficiency of mDia1. Intriguingly, although mDia1 deficiency appears to cause only partial impairment of DC functioning at each step, its overall impact on the delayed-type hypersensitivity response in DCs was nearly complete. The
roles of mDia2 and mDia3 in DCs, as well as the role of WASP in relation to mDia1, are important topics for future study.

**Authorship**

Contributions: H.T. performed experiments, analyzed data and wrote the paper. G.E., K.I., T.H., S.Nakajima, C.S., A.O., T.I., M.T., T.W., Y.M., S.Narumiya, and T.O. analyzed and discussed the data. K.K. designed the research, analyzed the data, and co-wrote the paper.

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References


FIGURE LEGENDS

Figure 1. mRNA expression profiles of mDia isoforms in DCs
The mRNA expression profiles of mDia isoforms, mDia1, mDia2, and mDia3, in BMDCs from B6 and mDia1−/− mice stimulated with (+) or without (-) LPS, and LCs, dermal DCs, splenic DCs, and CD4+ T cells (T cells) from B6 mice. Data are expressed as the amounts of mRNA relative to that of GAPDH. Data are presented as the mean + SD (n=3) of three independent experiments. N.d.: not detected. *, P < 0.05 versus corresponding B6 mice.

Figure 2. Impaired cutaneous DC migration in mDia1−/− mice
(A) The number of cutaneous DCs migrating from the skin to draining LNs. The numbers of FITC+ MHC class II+ total DCs (A) and FITC+ MHC class II+ Langerin+ DC and FITC+ MHC class II+ Langerin− DC subsets (B) in draining LNs of B6 (open bars) and mDia1−/− mice (shaded bars) were analyzed 72 h after application of FITC to the footpad. Bars show the mean ± SD from at least three mice per group. (C) Mobility of LCs to CCL21. Epidermal cell suspensions were applied to the upper chamber of a transwell with 3 μm (left panel) and 8 μm (right panel) pore size without coating for 3 h. Percentages of MHC class II+ LCs that migrated from the upper chambers to the lower are shown. (D, E) Invasive movement of LCs to CCL21. Epidermal cell suspensions were applied to the upper chamber of a transwell coated with Matrigel for 3 h (D) and 24 h (E). The numbers of MHC class II+ cells in the lower chamber (left panels) or in the
Matrigel (right panels) were counted with flow cytometry. Data are presented as the mean± SD of three independent experiments. *, $P < 0.05$ versus corresponding B6 mice. (F) TAXIScan assay. BMDCs chemotaxing under the CCL21 gradient were analyzed with TAXIScan. BMDCs from B6 and mDia1$^{-/-}$ mice were compared in terms of the velocity. Data are presented as the mean ± SD of 4 independent experiments. *, $P < 0.05$ versus corresponding B6 mice.

**Figure 3. mDia localization in DCs and characterization of cell morphology and adhesion in mDia1$^{-/-}$ BMDCs**

(A) mDia1 expression in DCs. B6 and mDia1$^{-/-}$ BMDCs were incubated on FN-coated coverslips for 16 h and stained with phalloidin and mDia1 Ab to reveal F-actin (green) and mDia1 (red). The cell nucleus was detected using DAPI staining (blue). Bar, 10 μm. (B) B6 and mDia1$^{-/-}$ BMDCs incubated on FN-coated coverslips for 30 min, 4 h and 18 h were stained with phalloidin; immunofluorescent images are shown. Open and closed arrowheads indicate membrane protrusion/lamellipodia and filopodia, respectively. (C) Morphology of BMDCs. B6 and mDia1$^{-/-}$ BMDCs were incubated on coverslips coated with or without FN for 18 h, and the major/minor axis ratio of BMDCs was measured. (D) Cell adhesion. B6 and mDia1$^{-/-}$ BMDCs were labeled with CFSE and incubated in a 96-well-plate coated with or without FN for 45 min. The percentage of adherent BMDCs was measured using a fluorometer. Data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$ versus corresponding B6 mice.

**Figure 4. Impaired interaction with T cells resulting from mDia1 deficiency in DCs**

(A) Time-lapse images of DC-T cell interaction *ex vivo*. OVA-pulsed CFSE-labeled B6 or mDia1$^{-/-}$ BMDCs were injected into mouse footpads, and CMTPX-labeled OT-II CD4$^+$ T cells
were intravenously administered. The popliteal LNs were collected and the DC-T cell interaction was monitored using two-photon microscopy; typical images at 0, 14, and 25 min are shown. White and red dots depict DCs. (B) Incidence of contact. Incidence of interactions of various durations between BMDCs and OT-II CD4+ T cells at 10 min intervals. Data are presented as the mean ± SD and are representative of three experiments. (C) The overall duration of DC-T cell interaction is shown. Data points represent individual durations. *, P < 0.05 versus corresponding B6 mice. (D) Temperature-dependent activity of acquired antigens with FITC-OVA in MHC class II+ DCs of B6 mice and mDia1−/− mice. The activities of acquired antigens of DCs were observed by measuring the MFI of ingested FITC at 4°C and 37°C for 45 min. Data are presented as the mean ± SD and are representative of three experiments.

**Figure 5. Impaired T cell stimulatory capacity of DCs resulting from mDia1 deficiency**

(A, B) Alloreactive CD4+ T cells were purified from BALB/c mice and stimulated with or without B6 or mDia1−/− BMDCs in 200 μl of cRPMI for 72 h. The numbers of each cell subset are shown in the figure. 3H-thymidine incorporation in triplicate wells as an indicator of T cell proliferation (A) and levels of IFN-γ in the culture supernatant (B) were measured. (C-E) The expression levels of MHC class II (C), CD40 (D), and CD54 (ICAM-1) (E) of BMDCs at 24 h and 48 h are shown. Data are presented as the mean ± SD and are representative of three independent experiments. *, P < 0.05 versus corresponding B6 mice.

**Figure 6. Attenuated CD4+ and CD8+ T cell stimulation in mDia1−/− BMDCs**

(A-D) OT-II CD4+ T cell stimulation by DCs. B6 and mDia1−/− mice were injected with CFA in the presence or absence of OVA, and CFSE-labeled OT-II CD4+ T cells were transferred
intravenously. Forty-eight hours later, skin-draining popliteal and inguinal LN cells were collected and stained with CD4. Flow cytometry profiles (A), histograms of CFSE+ CD4+ cells (B), the numbers of CD4+ T cells per draining LNs in each division (C), and the numbers of CD69+ activated T cells (D) per draining LNs are shown. The numbers indicated in A are the ratio (%) of CD4+ CFSE+ cells (right) and CD4+ CFSE- cells to total cells. The numbers indicated in B are the ratio of each fraction to CD4+ CFSE+ cells. (E) IFN-γ mRNA expression was evaluated through quantitative RT-PCR analysis. (F) OT-I CD8+ T cell stimulation by DCs. CFSE-labeled OT-I CD8+ T cells were transferred intravenously to mice that had been pretreated as described above (A-E), and the distribution of cell numbers in draining LNs is shown. (G) DTH induced by injection with OVA plus CFA. B6 and mDia1-/- mice were sensitized with OVA in CFA subcutaneously at the front footpads, and challenged with OVA in CFA in the hind footpads. B6 and mDia1-/- mice that had not received OVA sensitization were used as negative controls. Footpad thickness change over 48 h is shown. S: sensitization; C: challenge. (H) DTH induced by OVA-pulsed BMDCs. We injected B6 or mDia1-/- BMDCs pulsed with (BMDC+) or without (BMDC-) OVA into the front footpads, and challenged the mice by injecting OVA in CFA into the hind footpads. Administration of B6 BMDCs not-pulsed with OVA during sensitization was used as a negative control. Footpad swelling data over 24 h and 48 h after challenge with OVA in CFA are shown. Data are presented as the mean ± SD and are representative of three independent experiments with similar results.
Figure 3
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Rho-mDia1 pathway is required for adhesion, migration, and T-cell stimulation in dendritic cells

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