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Control of Homeostasis and Dendritic Cell Survival by the GTPase RhoA

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Tissues accommodate defined numbers of dendritic cells (DCs) in highly specific niches where different intrinsic and environmental stimuli control DC life span and numbers. DC homeostasis in tissues is important, because experimental changes in DC numbers influence immunity and tolerance toward various immune catastrophes and inflammation. However, the precise molecular mechanisms regulating DC life span and homeostasis are unclear. We report that the GTPase RhoA controls homeostatic proliferation, cytokinesis, survival, and turnover of cDCs. Deletion of RhoA strongly decreased the numbers of CD11b−/CD8+ and CD11b+/Esamhi DC subsets, whereas CD11b+/Esamhi DCs were not affected in conditional RhoA-deficient mice. Proteome analyses revealed a defective prosurvival pathway via PI3K/protein kinase B (Akt1)/Bcl-2–associated death promoter in the absence of RhoA. Taken together, our findings identify RhoA as a central regulator of DC homeostasis, and its deletion decreases DC numbers below critical thresholds for immune protection and homeostasis, causing aberrant compensatory DC proliferation.

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onventional dendritic cells (cDCs) are professional APCs, which, depending on the stimulus, initiate either adaptive immunity or immune tolerance (1). Although cDCs represent only a very low percentage of all immune cells, the presence of appropriate numbers of cDCs with defined life spans in lymphoid organs is crucial for a balance between immunity and tolerance. Accordingly, elevated numbers of cDCs and experimentally prolonged dendritic cell (DC) life spans caused exaggerated T and B cell responses, defective tolerance induction, and autoimmunity (2–5). Conversely, experimentally reduced cDC frequencies or life spans led to defective priming of T cell responses (6–10), as well as defective DC-mediated negative selection, T cell–mediated autoimmunity (9), inflammation, and myeloproliferation (11). Although these examples show the importance of maintaining precise physiological numbers of cDCs, it remains unclear which signals and molecular mechanisms control cDC homeostasis and turnover in vivo.

Initially cDCs in lymphoid organs were thought to be nondividing and short-lived (2, 12–14). However, more refined analyses of cDCs in parabiont experiments indicated that ≥5% of cDCs in spleen are in the cell cycle (15, 16). The constant replenishment of cDC pools by DC precursors from the circulation maintains the homeostasis of a defined number of cDCs in lymphoid organs (16). Therefore, cell cycle control and in situ proliferation of cDCs play important roles in the maintenance of correct cDC numbers and immune homeostasis. Control of cytokinesis might also regulate precursor–product relationships between circulating precursor DCs (preDCs) and cDCs, which occupy distinct niches and have different proliferative capacities. For cDCs, these mechanisms are not described; knowledge of cytokinesis regulation in DCs may increase our knowledge of the regulation of key checkpoints of immune homeostasis.

The two best-studied cytokines controlling the DC lineage are GM-CSF and Flt3 ligand (Flt3L). Although lack of GM-CSF does not abrogate generation of cDCs in mice (17), it caused a reduction in CD103+ and CD11b+ cDC subsets in various nonlymphoid organs by apoptosis, suggesting its role as a DC survival factor (18). The receptor for Flt3L, Flt3 (CD135), is expressed on various DC progenitors and drives differentiation of cDCs from hematopoietic stem cells (HSCs) (18). It also contributes to maintenance of homeostatic DC numbers by regulating proliferation (19). GM-CSF signals involve RhoA GTPase pathways in various cell types (20, 21). Also, signaling via mutated Flt3 in acute myeloid leukemia exploits a pathway involving RhoA for survival and proliferation of tumor cells (22). Therefore, RhoA might be involved in the integration of signals through GM-CSFR and Flt3, and it could play a pivotal role in the control of DC proliferation, longivity, and homeostasis.

RhoA is a member of the Rho GTPase family, which cycles between active GTP-bound and inactive GDP-bound states in response to various external stimuli. RhoA plays a central role in regulating cytoskeleton organization, adhesion, proliferation, migration, cytokinesis, and cell survival (23). However, most of our knowledge about RhoA function is based on overexpression of dominant-negative or constitutively active mutants in cell lines with little physiological relevance and nonspecific effects on other...
GTases (23, 24). Cell-specific RhoA deletion demonstrated that its effects vary greatly in different cell types. For example, RhoA deficiency results in cytokinesis failure in keratinocytes (25), migration defects in mouse embryonic fibroblasts (26), and necrotic loss of HSCs (27).

In this study, we used CD11c-Cre mice crossed with RhoAIffl/II mice to study the role of RhoA in cDCs in vivo. We demonstrate that RhoA controls DC homeostasis, and loss of RhoA results in a subset-specific decrease in cDC numbers in lymphoid organs. RhoA deficiency causes cytokinesis failure that is accompanied by increased rates of apoptosis via inhibition of the prosurvival pathway PI3K/Akt/Bcl-2-associated death promoter (BAD), which likely causes a reduction in DC numbers. Our results indicate that RhoA controls DC homeostasis by regulating cytokine and cell survival.

Materials and Methods

Mice

RhoAIffl/II mice were described previously (25). Six- to eight-week-old CD11c-Cre and CD11c-Cre RhoAIffl/II mice were used for this study. Thy1.1+ OT-I and OT-II mice with a transgenic TCR recognizing pOVA257-264 or pOVA323-339, respectively, were originally obtained from The Jackson Laboratory. All mice were bred and maintained at the animal facilities of the Institute for Immunology, Ludwig-Maximilians-University, in accordance with established guidelines of the Regional Ethics Committee of Bavaria.

Flow cytometry and sorting

Spleens or lymph nodes were digested in a solution of Liberase and DNase I (Roche) in serum-free medium for 30 min at 37˚C. Unless stated otherwise, we used pooled axillary, brachial, and inguinal lymph nodes for analyses. BRCs were removed with ACK buffer (0.15 M NHCl, 1 mM KHCO3, and 0.1 mM Na2EDTA [pH 7.4]) for 5 min at room temperature, and cells were filtered through a 70-µm nylon mesh strainer (BD Biosciences). The following Abs were used for flow cytometry: CD3 (145-2C11), CD4 (GK1.5), CD11b (M1/70), CD11c (N418), MHCII (M5/114), Esam (IG8), F4/80 (BM8), and Flt3 (A2F10) (eBio-Science); CD43 (S7), B220 (RA3-682) CD45RA (14.8), CD103 (M290), MHCII (M5/114), Esam (1G8), F4/80 (BM8), and Flt3 (A2F10) (eBio-Science); CD43 (S7), B220 (RA3-682) CD45RA (14.8), CD103 (M290), NK1.1 (PK136), Sirpα (P84), and Kit67 (BD Pharmingen); CD80 (MCD0826) (Invitrogen); and CD45.1 (A20), CD45.2 (104), and PDCA-1 (129c1) (BioLegend). Dead cells were identified by Aqua LIVE/DEAD dye (Invitrogen). Progenitors of preDCs (proDCs) and preDCs were sorted from Flt3L culture on day 3 using a FACSARia. ProDCs were identified as LinCD11c+MHCII CD43+Ly6C−, whereas preDCs were identified as LinCD11c+MHCII+CD43+Ly6C+. To sort preDCs from spleen, total splenocytes were coated with biotin-conjugated CD3, B220, NK1.1, and CD19 Abs, and Lin− cells were depleted by Streptavidin MicroBeads (Miltenyi Biotech)-mediated column sorting. The flow-through Lin− cells were used for preDC sorting using a FACSARia.

OT-I and OT-II T cell adoptive transfer

OT-I (CD8+) and OT-II (CD4+) T cells were isolated from lymph nodes and spleens of OT-I or OT-II donor mice using magnetic CD8 or CD4 negative selection (>96% purity; MACS; Miltenyi Biotec). Either 1 × 106 OT-I or 2 × 106 OT-II CFSE-labeled T cells were injected i.v. into the lateral tail veins of age- and sex-matched recipient mice. One day later, recipient mice were immunized by injecting 100 µg OVA protein in PBS (10 µg LP5) into the tail vein. OT-I and OT-II T cell proliferation was analyzed by CFSE dilution 3 d after immunization.

Generation of Flt3L T cells and bone marrow–derived DCs

DCs were generated from bone marrow (BM) in the presence of Flt3L. BM was extracted from femur and tibiae, and erythrocytes were lysed with ACK buffer. BM cells were cultured at 1 × 106 cells/ml in RPMI 1640 medium (with 10% FCS, Pen-Streptomycin, and 50 µg 2-ME) in the presence of 20 ng/ml homemade recombinant murine Flt3L. At day 3, cells were collected and used for proDC and preDC sorting, as described (28). For BM-derived DC (BMDC) culture, BM cells were cultured at 1 × 106 cells/ml in IMDM (Sigma) (with 10% FCS, Pen-Streptomycin, and 50 µg 2-ME) in the presence of 20 ng/ml homemade rGM-CSF. The medium was refreshed on days 3, 7, and 10. BMDCs can be used from days 10 to 13.

Histology

For preparation of cryosections, spleen was embedded in O.C.T. compound (no. 4583; Sakura Finetek) and snap frozen; 5-mm sections were cut with a cryostat (Jung Frigocut 2800 E; Leica), air-dried overnight, fixed in acetone (~20˚C for 10 min), and air-dried for a minimum of 12 h. Sections were rehydrated for 15 min in PBS containing 0.25% BSA and blocked for 15 min in PBS containing 0.25% BSA and 10% mouse serum. Rat anti-mouse CD8-FITC, CD11c-PE, MOMA-allophycocyanin (all from BD Pharmingen), and DAPI (1 mg/ml; Molecular Probes) diluted in blocking buffer were added to the sections and incubated for 30 min. After washing, sections were mounted in Fluoromount-G (Southern Biotech). Sections were analyzed on an Olympus BX41T-F 5-microscope (Olympus Cell-BND-F software) equipped with an F-View II Digital Micro Camera (Olympus).

Image stream

For cell mitosis analysis, 3 × 106 BMDCs or splenic DCs isolated by MACs were stained with CD11c1 and MHC class II Abs. Cells were fixed in BD Cytofix/Cytoperm buffer for 30 min at 4˚C, washed with Perm/Wash buffer, and stained with DAPI in Perm/Wash buffer at 4˚C for 10 min. After washing, cells were resuspended with 50 µl PBS. Mitosis was analyzed using ImageStream X Mark II (Amnis, Seattle, WA). Flow cytometry and image data were acquired and analyzed by INSPIRE and IDEAS software, respectively. Different populations were discriminated based on combined features of size, shape, and fluorescent intensity.

BrDU labeling in vivo

Mice were injected i.p. with 1 mg BrdU in PBS and then continuously received drinking water containing 0.8 mg/ml BrdU for 3 d. After various times, splenocytes were harvested and processed using a BrdU flow kit (BD Pharmingen).

Generation of BM chimeras

BM was harvested from femurs and tibiae of 6–8 wk-old wild-type (wt; CD45.1) and DC-specific RhoA-deficient (DCRhoa-ko) donor (CD45.2) mice. BRCs were lysed with ACK buffer, and 5 × 106 total BM cells (1:1 ratio of BM cells from both donors) was injected i.v. into lethally irradiated (600 rad; split dose at days −1 and 0) recipient mice (age 10–12 wk). Chimeras were analyzed 8–10 wk after BM reconstitution.

TUNEL assay

TUNEL+ DCs in spleens were detected using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore, Billerica, MA), per the manufacturer’s instructions. Briefly, splenocytes were labeled with mAbs specific for surface markers, and cells were fixed in 1% paraformaldehyde in PBS for 30 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 on ice for 2 min. Then, cells were incubated with working-strength TdT enzyme at 37˚C for 30 min. Adding stop/ wash buffer stopped the reaction. After three washes in PBS, cells were incubating with anti-digoxigenin fluorescein conjugate for 30 min at room temperature, and DNA of cells was stained with DAPI. TUNEL+ DCs were determined by flow cytometry.

Proteome analysis

Cell pellets were mixed with 400 µl lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris [pH 7.6]), incubated for 5 min at 95˚C, sonicated for 5 min in a sonication bath, and incubated again for 5 min at 95˚C. The samples were cleaned using a phenol:chloroform mixering as described previously (29). In brief, seven volumes of 8 M urea were mixed with the lysate, and the sample was loaded onto a spin column (30 kDa nominal m.w. cut off; Sartorius Stedim). Alkylation was performed with iodoacetamide, and samples were digested using trypsin (Promega) for 16 h. Peptides were eluted in double-distilled water and subsequently fractionated into three fractions using strong anion exchange (30 kDa nominal m.w. cut off; Sartorius Stedim) for 16 h. Peptides were eluted in double-distilled water and subsequently fractionated into three fractions using strong anion exchange in a pipet tip-based format, as described (30). Fractions were generated using elution buffers with a pH of 11, 6, and 3. Sample clean-up and desalting were achieved on homemade C18 STAGE tips (31). Samples were analyzed in liquid chromatography—mass spectrometry—mass spectrometry mode on the LTQ Orbitrap Velos Pro Mass Spectrometer coupled to an Easy-nLC II Liquid Chromatograph (both from Thermo Fisher Scientific) using homemade 15-cm columns (2.4 mm resin) and 3-h gradients. A total of 3 technical replicates were generated per sample. Five biological replicates with two technical replicates each were analyzed, alternating control and knock-out (ko) samples. Label-free quantification and data analysis were performed using the freely available MaxQuant and Perseus software.
(http://www.proteomexchange.org). Only proteins that were quantifiable with two or more unique peptides in at least three of five biological samples were included in the dataset. The p value was corrected after false discovery rate-based multiple hypothesis testing. The mass spectrometry proteomics data were submitted to the ProteomeXchange Consortium (32) (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD001636.

Quantitative PCR

Total RNA was isolated from 1 × 10^6 BMDCs or splenic DCs with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using a Quantitect Reverse Transcription Kit (QIAGEN). The TaqMan assay was performed with the LightCycler TaqMan Master Kit and the Universal Probe Library (Roche), according to the manufacturer’s instructions, on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). The housekeeping gene HPRT was used for normalization. The mRNA level of each gene was measured by quantitative PCR (qPCR), according to the Universal Probe Library system (Roche) with the following primers and Universal Probe Library Probes (Roche): HPRT: forward, 5'-TCTCTCAGACCCGGTTTTGTT-3', reverse, 5'-CTCTGGGAACTGGTCCTT-3', and probe #95; RhoA: forward, 5'-CCTGTGTTGTTTTCGACACCTT-3', reverse, 5'-ACCTCTGGAAGCTGTGCTTT-3', and probe #46; PSK-γ: forward, 5'-GGAGACGTAAACTCTGGACCTG-3', reverse, 5'-GTCGACATCTTACATGCTG-3', and probe #29; Ripk1: forward, 5'-TACCTCCGAGCAGGTCTGAAAT-3', reverse, 5'-AAACCAGGACTCCTCCACAG-3', and probe #50; Ripk3: forward, 5'-CCACAGGATGTCTGCTTGAG-3', reverse, 5'-TCAGTGTCGTAACCGAGATACCTC-3', and probe #76; TNF-α: forward, 5'-CCCCAAAGGGTAGTGAGTG-3', reverse, 5'-CTAACCCGTCTTGCTTGTGAG-3', and probe #104; Pib1: forward, 5'-GCCGAGACCTACACCACCGACG-3', reverse, 5'-CCCCACATCAGTGGTCCTGAAAT-3', and probe #75; TNFR1: forward, 5'-TGTCATTGCTGGCCCTGG-3', reverse, 5'-GATGATCCCCATCAACGACAG-3', and probe #40. The relative expression of target genes was calculated using the 2^(-ΔΔct) method.

Western blots

The following Abs were used for Western blot: actin, RhoA (67B9), PSK-γ (DS5DS), Akt, phospho-Akt (Ser473), Bad (D24A9), and phospho-Bad (D55D5) (Cell Signaling). Proteins from BMDCs and splenic DCs were separated by SDS-PAGE (12%). After transferring to a nitrocellulose membrane, proteins were incubated with primary Ab, washed, and incubated with secondary HRP-labeled Ab. Then, membranes were visualized using luminescent substrate ECL (Amersham, Piscataway, NJ). Western blot bands were quantified by ImageJ software.

Detection of Flt3L in serum

Serum concentrations of Flt3L were tested by mouse Flt3L ELISA (R&D Systems), according to the manufacturer’s instructions.

Statistical analysis

Statistical differences between the experimental groups were determined by the Student two-tailed t test. The p values <0.05 were considered significant.

Results

RhoA is essential for the homeostasis of DCs in spleen

To explore the function of RhoA in DCs, we crossed mice carrying loxP-flanked RhoA alleles (RhoAfl/fl) (25) with CD11c-Cre mice (33) to generate DCRhoA-ko mice. We confirmed RhoA deficiency by qPCR and Western blot analyses of MHCII^+CD11c^+ cDCs (Fig. 1A). Flow cytometry analyses revealed that frequencies of cDC populations were significantly decreased in spleens of DCRhoA-ko mice (Fig. 1B). The percentages of CD11c^+MHCII^+ cDCs were reduced by >50% compared with controls (Fig. 1B). Further analyses of CD11b^+ and CD8^+ DC subsets of the spleen showed that both subsets were substantially reduced (Fig. 1B). CD11b^+ DCs of the spleen can be subdivided into Esam^lo DCs, whereas the total number of Esam^lo DCs is unaffected and, therefore, seems independent of RhoA (Fig. 1B, 1C). Similar results were obtained when CD4 was used as a marker for DCs, with CD4^+ DCs being more affected than CD4^- DCs (Fig. 1B). In lymph nodes, the total numbers of migratory and resident RhoA-ko DCs were substantially reduced (Fig. 1D). Although migratory DC subpopulations (CD103^+CD11b^+ and CD103^-CD11b^-) were equally affected by lack of RhoA, in the resident DC subpopulation primarily CD8^- DCs were reduced in frequency and total number; reductions in RhoA-deficient CD11b^- DCs were not significant (Fig. 1D). A similar picture was found in mesenteric lymph nodes draining the intestine, with migratory CD103^-CD11b^- and CD103^-CD11b^- DC subsets and CD8^- resident DCs being the most affected by RhoA deficiency, whereas the numbers of CD103^+CD11b^- migratory and CD11b^- resident DCs were normal (Fig. 1E). However, the numbers of other cell types, such as CD4^- T cells, B220^- B cells, Ly6c^-CD11b^- monocytes, F4/80^- macrophages (Fig. 1F), and NK cells (data not shown), were unaltered. CD8^- T cells, which partially express CD11c-driven transgenes, were also reduced in number to some extent (Fig. 1F). Histological analyses revealed that DCRhoA-ko mice have an intact overall architecture of the spleen with normal white/red pulp organization (Fig. 1G). As previously described, CD8^- DCs are primarily located in T cell zones (36), as well as in marginal zones, and fewer numbers were found in the red pulp of the spleen (37), whereas CD11b^- DCs are preferentially located in the bridging channels (10) and red pulp of the spleen. Although both DC subsets could be identified in spleens of DCRhoA-ko mice in their proper locations, their total numbers were markedly decreased in the absence of RhoA (Fig. 1G), confirming the flow cytometry data (Fig. 1B, 1C). As a consequence, priming of adoptively transferred CD8^-OT-I or CD4^-OT-II T cells with OVA protein was strongly impaired in DCRhoA-ko mice (Fig. 1H). Neither OT-I nor OT-II T cells proliferated to the same extent as observed in control animals (Fig. 1H). Taken together, our results indicate that RhoA controls homeostasis of the majority of DC subsets; upon deletion of RhoA, DC numbers decrease beyond a critical level for T cell priming.

Deletion of RhoA does not affect generation of cDCs from progenitors

cDCs develop from preDCs, which originate from hematopoietic progenitor cells in the BM (38-40). It was reported recently that RhoA deficiency in hematopoietic progenitor cells causes multi-lineage hematopoietic failure (27). To control whether the observed decrease in cDC numbers in DCRhoA-ko mice was due to defects in preDCs or occurred later in cDC development, we next analyzed preDCs from BM and spleen. Because preDCs constantly leave the BM and enter lymphoid organs (40), we also analyzed preDCs after their migration to spleens of DCRhoA-ko mice. The frequency or total cell number of preDCs (Lin^-CD11c^+MHCII^-CD43^-Sirp^lo) in BM and spleen was not affected by RhoA deficiency (Fig. 2A). PreDCs were identified according to their expression of CD11c, the regulatory elements of which control expression of Cre recombinase and, therefore, deletion of the floxed RhoA gene. Despite CD11c expression, preDCs from BM did not show reduced levels of RhoA mRNA (Fig. 2B). Only those preDCs that had emigrated from the BM and reached the spleens of DCRhoA-ko mice showed significantly lower expression levels of RhoA mRNA (Fig. 2B). However, this loss of RhoA mRNA apparently did not affect the size of the preDC pool in spleens in vivo (Fig. 2A). To control the abilities of preDCs to generate cDCs in vitro, we analyzed Flt3L culture-enriched proDCs (Lin^-CD11c^-CD43^-Ly6^-) and preDCs (Lin^-CD11c^-MHCII^-CD43^-Sirp^lo), as described previously (28), and
FIGURE 1. RhoA is essential for the homeostasis of DCs. (A) RhoA deficiency is confirmed in mRNA and protein levels by qPCR and Western blot in spleen cDCs isolated via MACS. (B) The frequency of cDCs (CD11c+MHCII+) is significantly decreased in the spleen of DCRhoA-ko mice. cDCs can be separated into CD8+ and CD11b+ DC subsets. Expression of Esam and CD4 was further analyzed in CD11b+ DCs. The percentages represent mean ± SEM (n = 3). (C) Bar graph shows total cell number of spleen cDCs and different spleen DC subsets (n = 3). (D) Surface lymph node DCs from pooled inguinal, brachial, and axillary lymph nodes were analyzed based on the expression of CD11c and MHC class II. Resident DCs (MHCIIint) were further analyzed for expression of CD8 and CD11b. Migratory DCs (MHCIIhi) were analyzed for expression of CD103 and CD11b. Percentages represent mean ± SEM. Bar graphs show the total cell number of DCs and DC subsets in lymph node (n = 3). (E) Same analysis as shown in (D) for resident and migratory DCs from mesenteric lymph nodes. (F) Bar graph shows total number of CD19+ B cells and CD4+ and CD8+ T cells in spleen (Figure legend continues).
monitored their abilities to generate cDCs in vitro (Fig. 2C). ProDCs first develop into CD11c\(^+\) preDCs and differentiate further into CD11c\(^+\)MHCII\(^+\) cDCs (Fig. 2C). These analyses showed that proDCs, as well as preDCs, from BM and spleen of DCRhoA-ko mice could differentiate normally into preDCs and cDCs or cDCs, respectively (Fig. 2C). ProDCs isolated from BM and preDCs from BM or spleen of DCRhoA-ko mice did not show any deficiency with regard to the generation of their expected progeny in vitro. This suggests that, in DCRhoA-ko mice, neither the seeding of preDCs from BM to spleen nor the development of cDCs from their precursors is affected (Fig. 2B, 2C).

**DCRhoA-ko phenotype is DC intrinsic**

We next assessed whether the reduction in cDCs found in DCRhoA-ko mice is cell intrinsic or caused by external or secondary factors. To this end, we reconstituted lethally irradiated B6 mice with a 1:1 BM mixture derived from CD11c-Cre (CD45.1) and DCRhoA-ko (CD45.2) mice. In this competitive situation, DCRhoA-ko–derived DCs reconstituted the cDC pool in spleens very inefficiently (13%, Fig. 3A). Reductions in different cDC subsets were also detected (Fig. 3A); similar to nonchimeric DCRhoA-ko mice (Fig. 1C), the total cell numbers of RhoA-deficient CD8\(^+\) and CD11b\(^+\)Esam\(^+\) DCs were markedly decreased (Fig. 3B). In contrast, CD11b\(^+\)Esam\(^+\) DCs showed only a slight decrease in cell number (Fig. 3B). In addition, a distinct population of CD8\(^+\)CD11b\(^+\) cDCs, the natural immature stages of CD11b\(^+\) and CD8\(^+\) DCs (35), accumulated in DCRhoA-ko–derived DCs proportionally compared with the other DC populations (Fig. 3A) but were strongly reduced with regard to total cell number (Fig. 3B). B and T cells served as internal controls because, as a result of the specificity of the CD11c promoter RhoA, they should not be deleted. Accordingly, both lymphocyte subsets were not reduced when derived from DCRhoA-ko BM; rather, they were present in slightly increased numbers (Fig. 3B). Taken together, these results suggest that the reduction in cDCs is cell intrinsic in DCRhoA-ko mice and directly due to the loss of RhoA.

**RhoA-deficient cDCs show decreased long-term survival**

The above experiments showed that lack of RhoA caused lower numbers of DCs. To determine whether this is the result of altered survival and turnover of cDCs, we next performed in vivo BrdU-labeling studies (Fig. 4). Three days of continuous BrdU labeling revealed a statistically significantly higher BrdU incorporation rate into cDCs and CD8\(^+\) and CD11b\(^+\) DCs in DCRhoA-ko mice (Fig. 4A). When we analyzed the Esam\(^lo\) and Esam\(^hi\) DC subsets at day 9 of the experiment, we detected significantly reduced frequencies of BrdU\(^+\) DCs in CD8\(^+\), Esam\(^hi\), and Esam\(^lo\) subsets, indicating faster turnover of all subsets analyzed (Fig. 4B). A more refined analysis was then performed after a 2-h short-term BrdU pulse in vivo (Fig. 4C); this revealed strongly elevated incorporation of BrdU into RhoA-ko DC subsets, suggesting

![Images](http://www.jimmunol.org/)
increased proliferation (Fig. 4C). CD8+ and CD11b+Esamhi DCs were most affected and showed the highest BrdU-incorporation rates (Fig. 4C). However, these DCRhoA-ko–derived cDC subsets showed normal BrdU incorporation rates during the 3-d pulse phase in a competitive transplantation model in mixed BM chimeras, indicating normal proliferative behavior, despite the absence of RhoA, when the cDC pool was filled up with competitor control cDCs (Fig. 4D). BrdU analyses during the 12-d chase period revealed that survival and turnover of RhoA-deficient cDCs were much shorter compared with control DCs (Fig. 4A, 4D, days 6–12). In mixed BM chimeras, a strongly reduced survival was apparent, and CD11b+ cDCs were most affected and showed the highest BrdU-incorporation rates (Fig. 4C). However, these DCRhoA-ko–derived cDC subsets showed normal BrdU incorporation rates during the 3-d pulse phase in a competitive transplantation model in mixed BM chimeras, indicating normal proliferative behavior, despite the absence of RhoA, when the cDC pool was filled up with competitor control cDCs (Fig. 4D). BrdU analyses during the 12-d chase period revealed that survival and turnover of RhoA-deficient cDCs were much shorter compared with control DCs (Fig. 4A, 4D, days 6–12). In mixed BM chimeras, a strongly reduced survival was apparent, and CD11b+ cDCs were most dramatically affected, showing a >50% reduced half-life compared with the internal competitor control CD11b+ cDCs (Fig. 4D). When we analyzed DC subsets at day 9 of this experiment, we found that CD8+ and Esamhi DCs, but not the Esamhi subset, were affected (Fig. 4E, left panel). However, 3 d later, after 9 d of BrdU-free chase, differences also became apparent in Esamhi DCs, which showed significantly higher turnover without RhoA (Fig. 4E, right panel). This suggests that the reduced half-life of all DC subsets was a direct consequence of RhoA deficiency and demonstrates that RhoA controls survival of cDCs.

**RhoA deficiency indirectly enhances DC proliferation but controls DC cytokinesis intrinsically**

DCs were thought to be nondividing cells, until recent studies showed that ∼5% of cDCs were dividing at any time in the spleen (15, 16). Therefore, cDC proliferation is considered an important mechanism to maintain homeostatic cDC numbers in lymphoid organs. RhoA inhibition by dominant-negative mutants was previously reported to enhance proliferation of hematopoietic stem and progenitor cells (41). Furthermore, RhoA controls cytokinesis by regulation of cortical contractility and cleavage furrow formation during proliferation (42, 43). To test whether RhoA controls DC proliferation, we analyzed spleen cDCs for DNA content and expression of the proliferation marker Ki67 (Fig. 5). As described previously, ∼5% of CD11c+ MHCII+ spleen cDCs were in the cell cycle (DAPI+Ki67+) in control mice (Fig. 5A). However, two-fold more cDCs were dividing in DCRhoA-ko spleens compared with DCs in control mice (Fig. 5A). As a consequence of such compensatory proliferation, we found similar total numbers of Ki67+ DCs in controls and DCRhoA-ko mice (data not shown). To test whether such increased proliferation was caused by deletion of RhoA itself or a secondary effect, we generated mixed BM chimeras, as described above (Fig. 3). Cre control–derived cDCs should develop in normal numbers in the same host as RhoA-deficient cDCs and fill the cDC pool in a competitive situation. Analyses of cDCs in mixed chimeras showed that Cre control and RhoA-deficient cDCs had the same normal proliferation rate (∼5%) (Fig. 5B). As a consequence of this normal proliferation rate of RhoA-ko DCs in the competitive condition in mixed chimeras, the total numbers of Ki67+ RhoA-ko DCs were strongly decreased (data not shown). These results indicate that the compensatory proliferation of cDCs in DCRhoA-ko mice (Fig. 5A) was most likely due to secondary effects caused by disturbed cDC homeostasis and reduced cDC numbers in the absence of RhoA but was not due to RhoA deficiency itself. It was reported that the reduction in cDC numbers as a result of diptheria toxin–mediated ablation causes myeloproliferation because of increased levels of Flt3L (11). Because cDCs are major “consumers” of Flt3L among peripheral hematopoietic cells (44), loss of cDCs might cause elevated levels of Flt3L, triggering increased cDC proliferation. Therefore, we tested for levels of Flt3L and found significantly elevated concentrations in the serum of DCRhoA-ko mice compared with control animals (Fig. 5C). In contrast, when wt DCs could fill up the DC compartment in mixed BM chimeras, Flt3L levels returned to normal and were comparable to those in wt mice (Fig. 5C). This finding suggests that reductions in cDC numbers as a result of the absence of RhoA leads to elevated Flt3L concentrations in serum, which, in turn, induces enhanced cDC proliferation in DCRhoA-ko mice but not in mixed chimeras. These results suggest that deficiency of RhoA does not indirectly lead to enhanced DC proliferation and that RhoA is even dispensable for proliferation of cDCs.

We next analyzed the distribution of RhoA-ko cDCs in the different stages of the cell division cycle (G1, S, G2/M) (25, 26). CD11c+MHCII+ cDCs from spleens of DCRhoA-ko mice (Fig. 5D) or mixed BM chimeras (Fig. 5E) showed a similar cell cycle distribution, with significantly lower frequencies of DCs in the G1 phase and more DCs in the S/G2-M phases in the absence of RhoA (Fig. 5D, 5E). These data suggest that RhoA regulates cell cycle progression in cDCs. We next analyzed mitosis of spleen cDCs using ImageStream analyses (Fig. 5F). Ex vivo–isolated spleen cDCs from DCRhoA-ko mice showed a statistically significant accumulation of both telephase-like and anaphase-like cells, which normally coincide with cytokinesis (45). Also, the fact that frequencies of DCs in metaphase were not significantly altered and the nuclei were properly separated (Fig. 5F) suggests that RhoA controls cytokinesis in cDCs.
RhoA controls GM-CSF–induced cDC survival

To test whether loss of cDCs in the absence of RhoA was due to apoptosis, we next performed TUNEL assays of spleen cDCs. DCRhoA-ko cDCs had significantly higher frequencies of TUNEL+ cDCs in spleen, where deletion of RhoA resulted in 2-fold increased rates of apoptosis (Fig. 6A). Primarily, the CD8+ and Esamhi subpopulations were affected (Fig. 6A, bar graph). In mixed BM chimeras, the competitive condition between Cre control cDCs and RhoA-ko cDCs led to a 4-fold increase in TUNEL+ cDCs from DCRhoA-ko mice compared with control cDCs within the same chimeras (Fig. 6A, lower panels), and the frequencies of TUNEL+ Esamhi cDCs were even more pronounced (Fig. 6A). Also, when we quantified apoptotic cDCs using Annexin V and 7-aminoactinomycin D (7-AAD), we detected a statistically significant increase in apoptotic (AnnexinV+7-AAD+) CD8+ and CD11b+ Esamhi cDCs, whereas CD11b+Esamlo cDCs did not show significant changes (Fig. 6B). As controls, we analyzed MHCII+CD11c+ cells, including B cells, and CD8+CD11c+ cells, including CD8+ T cells, which did not contain increased frequencies of AnnexinV+7-AAD+ apoptotic cells (Fig. 6B). To further confirm that RhoA promotes DC survival, we generated DCs in vitro by culturing BM cells from wt and DCRhoA-ko mice in the presence of GM-CSF. Upon GM-CSF removal, DC survival was monitored by flow cytometry. Consistent with the in vivo findings (Fig. 6A, 6B), RhoA-deficient BMDCs showed significantly impaired survival kinetics in the absence of the growth factor compared with BMDCs from Cre control mice (Fig. 6C). Together, these findings indicate that RhoA is critical for the homeostasis of DCs because it regulates cDC survival, and lack of RhoA leads to cell death by apoptosis.

Proteome analyses of RhoA-deficient cDCs

To explore the survival pathway controlled by RhoA in cDCs, we performed proteome analyses to compare BMDCs from Cre control and DCRhoA-ko mice (Fig. 7A). A total of 2699 proteins was quantifiable, with inclusion criteria being quantification of at least two unique peptides/protein in at least three biological replicates.
Fifty-four proteins were upregulated and 39 proteins were downregulated 3-fold in RhoA-deficient BMDCs (Fig. 7A). Among those, several proteins related to gene ontology (GO) terms “cell cycle regulation” (GO:0022402) and “regulation of programmed cell death” (GO:0043067), such as E-cadherin, α- and β-catenin, NEDD4, and FLNA, were deregulated. The second messenger P13K-γ was reduced >6-fold in RhoA-ko BMDCs (Fig. 7A). This was of potential interest because it plays an important role in promoting cell survival and growth through activation of protein kinase B (or AKT) (46, 47). Active AKT phosphorylates...
proapoptotic protein BAD, thereby blocking BAD-induced apoptosis (48). To investigate whether RhoA deletion deregulates the PI3K > AKT > BAD signaling pathway in DCs, we verified the levels of PI3K, AKT, and BAD by Western blot of lysates from BMDCs and purified spleen cDCs. As shown in Fig. 7B, RhoA was effectively deleted from both sources of RhoA-ko DCs, with some residual RhoA protein remaining in the BMDCs of DCRhoA-ko mice (Fig. 7B). PI3K levels decreased in BMDCs and spleen cDCs of DCRhoA-ko origin compared with wt controls (Fig. 7B), confirming proteomics data (Fig. 7A). This decrease was also confirmed on the mRNA level for DCRhoA-ko DCs, indicative of an influence of RhoA on PI3K gene transcription (Fig. 7C). Although total amounts of Akt protein were not altered in DCRhoA-ko DCs (Fig. 7B), phospho-AKT was strongly decreased (Fig. 7B), possibly due to the reduction in the upstream activator PI3K. Consequently, RhoA-ko DCs contained decreased levels of phospho-BAD, whereas total protein levels of BAD were not altered (Fig. 7B). These results suggest that deletion of RhoA leads to reduced mRNA and protein levels of PI3K, causing inefficient engagement of the PI3K/AKT/BAD survival pathway, which could be responsible for loss of cDCs in the absence of RhoA. It was reported that RhoA is implicated in necrotic cell death rather than apoptosis (27). In RhoA-deficient HSCs, the TNF receptor-interacting protein (RIP) kinase pathway was shown to regulate programmed necrosis upon upregulation of TNF-RIP–associated genes (49). Therefore, we also analyzed necrosis-relevant genes, including *Tnfa*, *Ripk3*, *Fh1*, and *Gld1* (27, 50), but could not detect significant alterations in gene expression (Fig. 7D). Therefore, our findings suggest that, in cDCs, RhoA induces survival by inhibiting apoptosis. Lack of RhoA permits the induction of apoptosis via the PI3K > AKT > BAD signaling pathway.

**FIGURE 6.** RhoA deficiency leads to increased apoptosis. (A) Apoptotic spleen cDCs were detected by TUNEL assay in the steady-state and in mixed BM chimeras. Cells not treated with TdT enzyme were used as negative controls. Bar graphs show the percentage of TUNEL− splenic cDCs, as well as distinct DC subpopulations (n = 3). (B) Annexin V and 7-AAD were used to detect apoptotic cells in CD8+, Esamhi, and Esamlo spleen DC subsets. Bar graphs show the percentage of all Annexin V+ cells in DC subsets, as well as B cells and CD8+ T cells as internal control (n = 3). (C) Apoptosis of BMDCs was induced by removing GM-CSF from the medium. BMDC survival was monitored over 4 d (n = 3). Data are representative of two (A) or three (B and C) experiments with similar outcome. Asterisks indicate statistical significance. *p < 0.05, **p < 0.01; ***p < 0.001.
The Rho GTPase family has several members, with RhoA, Cdc42, and Rac1 being the best characterized (51). They are known to integrate signals for regulation of diverse cellular processes, such as cell cycle progression, cytoskeleton rearrangement, and gene expression (52). Furthermore, they control the Ag-presenting functions of DCs, as well as their maturation and migration capacities (53–58). However, little is known about the DC-specific functions of RhoA. Studies on SW AP70, a regulator of RhoA, indirectly showed its involvement in motility, endocytosis (59), and MHC class II surface localization in DCs (60). Our findings in DCRhoA-ko mice suggest that RhoA regulates DC survival by inhibiting apoptosis.

Constant replenishment of DC precursors from blood, DC division, and cell death maintain DC homeostasis in lymphoid organs in a dynamic balance (16). Deletion of RhoA in DCs led to a significant decrease in DC numbers in lymphoid organs. Both CD8+ DCs and CD11b+Esam+ DCs were dramatically decreased, whereas the total number of CD11b+Esam- DCs did not change. Even in the competitive chimera model, in which the decrease in RhoA-deficient CD8+ DCs and CD11b+Esam+ DCs became more severe, the Esam- population was affected much less than the other DC subsets. The marker Esam was recently used to subdivide CD11b+Esam+ DCs into Esam+ and Esam- populations, which are either more specialized for CD4+ T cell priming or secrete proinflammatory cytokines, respectively (35). In contrast to the other DC subsets, CD11b+Esam- DCs are most likely derived from monocytes (35, 61). This subset of DCs also was shown to proliferate less than other DCs (35). Eventually, the low intrinsic

FIGURE 7. RhoA promotes DC survival through PI3K/Akt/Bad pathway. (A) BMDCs of wt controls and DCRhoA-ko mice were used for proteome analysis. A total of 2699 proteins was quantified by label-free quantification. Each black circle represents a protein that was quantifiable in at least three biological replicates. The log2-fold change in the mean DCRhoA-ko/CD11c-CRE ratio is plotted against the −log10 of the p value. The vertical dashed lines mark a fold change ≫ 3. The dashed horizontal line shows the cutoff for statistical significance (p value < 0.05). Proteins that are up- or downregulated >3-fold in DCRhoA-ko mice compared with wt controls are considered significantly altered. Among those proteins, cell survival–related proteins (red color) and cell cycle–related proteins (green color) are indicated. (B) The downregulation of PI3K-γ was confirmed in BMDCs and spleen DCs by Western blot. Spleen DCs were isolated by CD11c mAb-coated magnetic beads. Phosphorylation of Akt and BAD was confirmed by Western blot. Bar graphs show quantification of Western blot bands done using ImageJ software. (C) PI3kg mRNA was determined in spleen cDCs by qPCR. (D) qPCR to examine expression of necrosis-induced genes from spleen cDCs. (E) RhoA signaling cascade regulating apoptosis of DCs. RhoA deletion caused downregulation of PI3K-γ, and the phosphorylation of its downstream effector Akt and BAD was inhibited. Inefficient phosphorylation of proapoptosis protein BAD results in active BAD, leading to apoptosis. Asterisks indicate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.
pansion rate and the constant replenishment of this subset from monocytes or some earlier monocytic precursors might be the reason why deletion of RhoA did not affect Esam\(^{18}\) DCs in the steady-state.

Proliferation of cDCs was enhanced $\geq$2-fold upon deletion of RhoA, and it compensated, to some degree, for the overall loss of DC numbers. However, as shown in competitive transplantation experiments, this effect was not caused directly by lack of RhoA but, rather, by environmental factors. Flt3L was elevated in the sera of DCRhoA-ko mice and may have become available in higher amounts as a result of the loss of competitor DCs, as described in other models (11). However, although the experimental setup of Birnberg et al. (11) caused a $\geq$10-fold decrease in DC numbers in spleens and a 3-fold increase in serum Flt3L concentration, lack of RhoA only caused a 2-fold decrease in DC numbers, which was accompanied by a 1.5-fold increase in Flt3L. Yet, such a modest increase in Flt3L was sufficient to cause DC proliferation, as well as mild myeloid proliferative syndrome (data not shown), as observed upon complete deletion of DCs (11). These data illustrate that levels of DCs must be maintained at optimal numbers, and RhoA seems to play a central role in this balance. Even a subtle change in homeostasis leads to activation of the feedback loop and increased compensatory DC proliferation. Similarly, increased proliferation was observed previously upon RhoA suppression by a dominant-negative mutant approach in hematopoietic stem and progenitor cells (41), as well as in HSCs of genetically RhoA-deficient mice (27). The investigators speculated that secondary effects caused by acute hematopoietic stress were the reason for such increased proliferation (27). It is also possible that, in these models, increased availability of unidentified HSC factors/niches caused RhoA-deficient cells to proliferate more strongly. However, in another study, RhoA deletion in B cells using CD19-cre did not affect B cell proliferation, but it abrogated B cell differentiation (62). Together, these data indicate that proliferation of hematopoietic cells seems to be independent of RhoA, but lack of RhoA can cause increased compensatory proliferative responses in some, but not all, cell types.

We identified reduced survival of RhoA-ko cDCs as a RhoA-autonomous effect. In DCRhoA-ko mice, more cDCs were TUNEL\(^{+}\) and AnnexinV\(^{+}\), indicating increased apoptosis rates. In contrast, cell cycle control was deregulated in the absence of RhoA, because a high frequency of DCs accumulated in mitotic anaphase. It was proposed earlier in megakaryocytes that RhoA is needed for cleavage furrows to complete separation of daughter cells (63, 64). Defects in RhoA or its effector/regulatory proteins caused various phenotypes of failure of cell separation (65, 66), often accompanied by the presence of multinucleated cells (27, 63, 64). We found multinucleated cells in in vitro BMDC cultures (data not shown) but not in RhoA-ko DCs in vivo. This might be another example of RhoA’s effects being influenced by environmental differences, as we described recently for in vitro versus in vivo conditions (25). Eventually, compensatory effects by RhoB and RhoC might also cause such differences, as discussed previously (25). Because mitosis and apoptosis are connected, it is possible that extended mitotic arrest due to the absence of RhoA leads to mitotic cell death of cDCs. Recently, a genome-wide screen identified BAD and other components of the mitochondrial apoptosis pathway as contributors to mitotic cell death (67). We also identified lower amounts of phospho-BAD in RhoA-ko cDCs; therefore, the link among mitochondrial apoptosis, mitotic arrest, and RhoA deficiency likely contributes to the death of RhoA-ko cDCs.

In addition, protein levels of PI3K-\(\gamma\), which has well-described prosurvival functions, were significantly reduced in RhoA-ko DCs. In contrast, inhibition of PI3K alone in human cancer cell lines was insufficient to induce apoptosis via BAD inactivation, because other signaling pathways compensate for the lack of PI3K signals (48). However, recent data from PI3K-\(\gamma\)-deficient mice also showed increased rates of apoptosis in normal leukocytes (68) and strongly reduced DC numbers (69). We show that expression of PI3K was significantly reduced in RhoA-ko DCs, which led to decreased phosphorylation of its downstream effector, Akt. Thus, the proapoptotic protein BAD cannot be inhibited effectively by phosphorylation due to low levels of active Akt, resulting in apoptosis of RhoA-ko DCs. It is unclear how RhoA regulates protein levels of PI3K-\(\gamma\), but we also found that PI3K-\(\gamma\) mRNA transcripts were significantly reduced in RhoA-ko DCs.

Previous reports showed transcriptional regulation by RhoA via transcription factor GATA-4 as a nuclear mediator of RhoA signaling (70). Also, the c-Fos serum response element is used by RhoA to control transcription in some cells (71). However, it is unclear whether these or other RhoA-responsive transcription factors transcriptionally regulate PI3K. Therefore, further work is necessary to differentiate whether apoptosis of RhoA-ko DCs is caused by defective mitosis, direct regulation of PI3K by RhoA, or both. However, there is indirect evidence from the literature that receptors for DC growth factors GM-CSF (20, 21) and Flt3L (22) can signal via RhoA. In cells bearing the oncogenic form of Flt3, a signaling pathway via PI3K is activated (22). Therefore, RhoA may be a sensor for DC growth factors, and its absence could lead to cell death by apoptosis, either directly via PI3K pathways or indirectly via the block in mitotic cell division.

In summary, our findings indicate that RhoA is crucial for the homeostasis of cDCs in spleen. Deletion of RhoA caused a significant decrease in cDCs due to decreased DC survival. Further unraveling of the signaling pathways used by DC-specific growth factors might help us to understand the feedback mechanisms that control DC homeostasis in the steady-state and inflammation.

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Disclosures

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