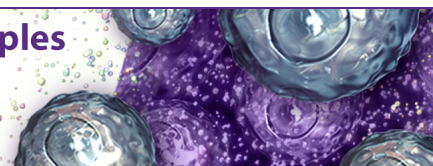


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SWAP-70 Restricts Spontaneous Maturation of Dendritic Cells

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SWAP-70 Restricts Spontaneous Maturation of Dendritic Cells

Carlos Ocaña-Morgner,¹ Anne Götz,¹ Christine Wahren, and Rolf Jessberger

Spontaneous maturation observed in dendritic cell (DC) cultures has been linked to their capacity to induce immune responses. Despite several recent studies, the mechanisms and signals triggering spontaneous maturation of DCs are largely unknown. We found that the absence of SWAP-70 causes spontaneous maturation of spleen- and bone marrow–derived DCs and, in vivo, of spleen-resident CD11c⁺CD11b⁺CD8α[−] DCs. Activation markers, cross-presentation of exogenous Ags, and activation of CD8⁺ T cells are much increased in *Swap-70*^{−/−} DCs. Spontaneous maturation of *Swap-70*^{−/−} DCs depends on cell–cell contact and does not involve β-catenin signaling. SWAP-70 is known to regulate integrin activity. Signaling through the integrin CD11b (α_M) subunit increases spontaneous maturation of wild-type (wt), but not of *Swap-70*^{−/−} DCs. Signaling through the CD18 (β₂) subunit decreases spontaneous maturation of wt and *Swap-70*^{−/−} DCs. Constitutive activation of RhoA in *Swap-70*^{−/−} DCs was determined as a key mechanism causing the increased spontaneous maturation. Inhibition of RhoA early, but not late, in the activation process reduces spontaneous maturation in *Swap-70*^{−/−} DCs to wt levels. Inhibition of RhoA activation during CD11b integrin activation had a significant effect only in *Swap-70*^{−/−} but not in wt DCs. Together, our data suggest that integrin-mediated spontaneous maturation of wt DCs does not depend on active RhoA, whereas the increase in spontaneous maturation of *Swap-70*^{−/−} DCs is supported by integrin CD11b and by hyperactive RhoA. Thus, SWAP-70 deficiency reveals two pathways that contribute to spontaneous maturation of DCs. *The Journal of Immunology*, 2013, 190: 5545–5558.

Of all APCs, dendritic cells (DCs) are the most potent in initiating a cellular immune response by activating CD4⁺ and CD8⁺ T cells (1). Upon encounter with microbial products and/or inflammatory molecules in the skin and organs, DCs begin a maturation process characterized by the onset of a number of functions—for example, migration to lymph nodes, Ag presentation, expression of costimulatory molecules, and secretion of inflammatory cytokines (2, 3). DCs also mature in vitro and in vivo without a microbial or inflammatory stimulus (4–6). This so-called “spontaneous maturation” indicates alternative pathways of DC maturation. Deciphering such pathways is important for understanding the immune regulation of DCs. Recently, “spontaneous maturation” was observed in bone marrow–derived DC (BMDC) cultures after mechanical disruption of cell clusters or after integrin signaling through Abs. This type of adhesion-related maturation was regulated by β-catenin signaling (7–9). However, spontaneous maturation by cluster disruption of β-catenin–deficient spleen DCs suggests regulation by different signaling pathways (10). The processes and factors that initiate

spontaneous maturation of DCs are incompletely understood, but at least some forms of spontaneous maturation involve adhesion molecules, for example, E-cadherin and integrins (7, 9, 11) and secreted factors (10). It should be noted that in some of these instances “spontaneous maturation” does not imply maturation triggered only by cell-intrinsic properties but also by extrinsic factors that are distinct from infection or inflammation. The mechanisms that lead to spontaneous maturation of DCs are central to the regulation of the maturation state of DCs and thus for maintaining the critical balance between inflammation and tolerance.

SWAP-70 is expressed in DCs and contains a putative N-terminal EF hand domain, a pleckstrin homology (PH) domain in the center region, a Dbl-homology/coiled-coil domain, and a C-terminal F-actin binding domain. DC upregulation of surface MHC class II (MHCII) molecules after TLR signaling and migration toward S1P are governed by SWAP-70 (12, 13). Control of RhoA activation by SWAP-70 in DCs is responsible for the regulation of these processes (12, 13). Activation of RhoA in DCs controls cytoskeleton rearrangements, which regulate a number of processes connected to DC maturation (14–18). Nonactivated *Swap-70*^{−/−} BMDCs show significantly increased levels of active RhoA when compared with their wild-type (wt) counterparts. SWAP-70 interacts with RhoA-GTP and thus represents a novel element controlling important DC functions (12, 13).

In the current study, we show that SWAP-70 restricts spontaneous maturation of DCs. The absence of SWAP-70 causes increased numbers of DCs expressing high levels of T cell stimulatory molecules and an increase in Ag cross-presentation. Consequently, *Swap-70*^{−/−} DCs are more potent in activating CD8⁺ T cells than wt cells. Spontaneous maturation of *Swap-70*^{−/−} DCs was triggered by cell–cell contact, which involves integrins, and was not accompanied by an increase in β-catenin signaling. Constitutively active RhoA is responsible for the increase in spontaneous maturation of *Swap-70*^{−/−} DCs. Abolishing RhoA activation early but not late in BMDC cultures reduced the number of spontaneously matured *Swap-70*^{−/−} DCs to wt levels. Similarly, inhibiting constitutively active RhoA in *Swap-70*^{−/−} DCs had a significant effect in integrin-mediated spontaneous maturation. These findings suggest a novel control

Institute of Physiological Chemistry, Faculty of Medicine Carl Gustav Carus, Dresden University of Technology, D-01307 Dresden, Germany

¹C.O.-M. and A.G. contributed equally to this work.

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Address correspondence and reprint requests to Prof. Carlos Ocaña-Morgner and Prof. Rolf Jessberger, Institute of Physiological Chemistry, Faculty of Medicine Carl Gustav Carus, Dresden University of Technology, Fiedlerstrasse 42, Medizinisch-Theoretisches Zentrum, D-01307 Dresden, Germany. E-mail addresses: Carlos.Ocana-Morgner@mailbox.tu-dresden.de (C.O.-M.) and rolf.jessberger@mailbox.tu-dresden.de (R.J.).

The online version of this article contains supplemental material.

Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; cDC, classical dendritic cell; DC, dendritic cell; PH, pleckstrin homology; PI, propidium iodide; PIP₃, phosphatidylinositol 3,4,5-triphosphate; SDC, spleen-derived dendritic cell; wt, wild-type.

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mechanism of spontaneous maturation of DCs. This mechanism depends on integrin and RhoA activation, controlled by SWAP-70.

Materials and Methods

Mice

Swap-70^{-/-} and isogenic *Swap-70*^{+/+} mice of two different strains were used: 129/SvEMS and C57BL/6 as well as C57BL/6 expressing the congenital markers CD45.1 (*Swap-70*^{-/-}) or CD45.2 (*Swap-70*^{+/+}). For CD45.1 *Swap-70*^{-/-} mice, *Swap-70*^{-/-} C57BL/6 were crossed with CD45.1 C57BL/6 (B6.SJL-*Ptprca*^u *Pepc*^b/BoyJ) and genotyped. OTI mice expressing the OTI TCR (Va2/Vb5) transgene (19), encoding a TCR specific for the OVA epitope (SIINFEKL-H-2K^b) used for the MHC class I (MHCI)-restricted Ag presentation assay, were kindly provided by Dr. K. Kretschmer (Center for Regenerative Therapies Dresden, Dresden, Germany) or obtained from Charles River. Animals were bred and maintained under pathogen-free conditions in the Animal Facility of the Medical Faculty of Dresden University of Technology (TU Dresden) according to approved animal welfare guidelines.

BMDCs

Murine BMDCs were obtained by differentiation of bone marrow-derived precursors as described previously (20), with small modifications. Femora and tibiae were dissected from male *Swap-70*^{+/+} and *Swap-70*^{-/-} mice (6–10 mo of age, with strain background of 129/SvEMS if not indicated otherwise), and bone marrow was flushed with supplemented RPMI 1640, using a 29-gauge syringe. Bone marrow cells were harvested by centrifugation and seeded at 0.5×10^6 cells per well of a six-well plate (non-tissue culture-treated; Sarstedt) in 3 ml supplemented RPMI 1640. In addition, 10% of a GM-CSF-containing cell culture supernatant was added to the culture. GM-CSF supernatant was routinely produced using the stably transfected cell line J558 (21) and generally led to a GM-CSF concentration of ~1 µg/ml, as tested by ELISA (BD Biosciences). In some experiments, different concentrations of recombinant murine GM-CSF (PeproTech) were used instead of the homemade supernatant. At day 3 of culture, an additional 3 ml medium was added. Then half of the medium was changed every 2–3 d. BMDCs were analyzed at day 11 of culture unless otherwise stated, at which point > 90% of the cells were CD11c positive, as analyzed by FACS. For some experiments BMDCs were activated in a TLR-dependent manner by incubating the cells with 1 µg/ml LPS (*Salmonella enterica*; Sigma-Aldrich) overnight before analysis. Supernatants of LPS-stimulated BMDCs were also collected for analysis of inflammatory cytokines IL-1α, IL-6, IL-10, and TNF-α, using FlowCytomix Multiplex (eBioscience) according to the manufacturer's instructions.

DCs were also generated in vitro using Fms-like tyrosine kinase 3 ligand (Flt3-L) (R&D Systems), as described (22). Briefly, total bone marrow cells (2×10^6 /ml) were cultivated with 400 ng/ml Flt3-L for 9 d. CD24^{hi} CD11b^{lo} (CD8⁺ DCs equivalent) and CD24^{lo} CD11b^{hi} (CD8⁻ DCs equivalent) were analyzed by FACS.

To address the influence of *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs cultured together, we used BMDCs from the bone marrow of C57BL/6 mice expressing CD45.1 (*Swap-70*^{-/-}) or CD45.2 (*Swap-70*^{+/+}). To this end, *Swap-70*^{+/+} and *Swap-70*^{-/-} bone marrow cells were mixed at a 1:1 ratio at day 0 and cultured as described. At day 11, BMDCs were then stained for the respective congenital markers and analyzed by FACS.

In some experiments, 0.1 µg/ml C3 transferase (CT04 Rho Inhibitor I; Cytoskeleton) or 1 µg/ml anti-integrin Abs (see Table I) were added at different days and kept as indicated. The compounds were added fresh with each medium change, under the assumption that remaining molecules were stable enough to maintain the used concentration without wide variations. Respective carriers were used as negative controls.

To investigate the possible effects of a soluble factor, conditioned medium of either *Swap-70*^{-/-} or *Swap-70*^{+/+} BMDCs was collected at 11 d of medium change, combined, and kept at 4°C until use. Fresh medium of new cultures was supplemented with 50% of conditioned medium at day 0 and at every medium change.

Spleen-derived DCs

Murine spleen-derived dendritic cells (SDCs) were obtained from splenocytes as described elsewhere (23), with small modifications. Spleens taken from *Swap-70*^{+/+} and *Swap-70*^{-/-} mice (6–10 mo of age) were mechanically dissociated using a 100-µm nylon cell strainer and a plunger. After red blood lysis using ACK-lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4) incubation for 3 min, cells were washed once with supplemented medium and seeded at a concentration of 1×10^6 cells per milliliter in supplemented medium containing 10% GM-CSF

supernatant and 1 ng/ml recombinant human TGF-β1 (R&D Systems or PeproTech) into a 24-well plate (1 ml per well). Every 4–5 d, half of the medium was changed. SDCs were used at days 12–16 of culture.

Analysis of spleen-resident classical DCs

Spleens of *Swap-70*^{-/-} and isogenic *Swap-70*^{+/+} C57BL/6 mice were isolated, homogenized, and incubated in 10% FBS RPMI 1640 medium containing 10 U/ml collagenase and 5 µg/ml DNase I. Expression of the maturation marker CD86 in CD11c⁺ MHCII⁺ CD11b⁺ CD8⁻ and CD11c⁺ MHCII⁺ CD11b⁺ CD8⁺ spleen-resident classical dendritic cells (cDCs) was done by FACS using fluorescent-labeled Abs (see Table I).

In vitro S1P chemotaxis assay

Chemotaxis in response to S1P was analyzed by measuring the number of cells migrating through a polycarbonate filter (8-µm pore size) in 24-well Transwell chambers (Costar). The upper chamber included 1×10^5 BMDCs in 100 µl chemotaxis medium (DMEM medium with 0.1% BSA and 10 mM HEPES), and the lower chamber contained 600 µl of the same medium with or without 50 nM S1P. After incubation for 3 h at 37°C, DCs that migrated to the bottom chamber were counted by flow cytometry, using FACSDiva software (BD Biosciences). Results are expressed as percentage of input cells: number of migrated DCs divided by the number of input cells that were incubated in 600 µl chemotaxis medium alone in the lower chamber.

Fluorescence microscopy

Adherent BMDCs were washed once with prewarmed PBS and fixed with 3.6% formaldehyde in PBS at room temperature for 8 min. After permeabilization with 0.1% Triton X-100 for 5 min at room temperature, F-actin was stained with 26.4-nM Alexa Fluor 488 phalloidin (Invitrogen) for 20 min at room temperature. Samples were washed and mounted in Fluoromount-G (SouthernBiotech) containing 1 µg/ml DAPI. Slides were stored at 4°C in the dark until images were taken using a Zeiss Axiophot fluorescence microscope and processed with ImageJ software.

Cell separation by MACS

MACS cell separation was performed according to the manufacturer's recommendation (Miltenyi Biotec). MACS was used for lineage depletion of bone marrow cells. Enriched DC progenitor cells were obtained by negative selection, using either a lineage cell depletion kit or titrated lineage-specific biotin-conjugated Abs as established in the laboratory. For depletion, whole bone marrow cells were labeled with a mixture of biotinylated Abs against a panel of lineage-specific Ags [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7/4, and Ter-119] and magnetically isolated with Anti-Biotin MicroBeads.

MHCI-restricted Ag presentation assay

Presentation of OVA epitope 257–264 in an H-2K^b background was monitored using purified CD8⁺ T cells from OTI mice. OVA (grade VI; Sigma-Aldrich) was added to a day 10 culture of BMDCs at 1 mg/ml (in medium, filtered with 0.45 µm) for 2 h; medium only was used as negative control. The medium containing OVA was carefully removed without disturbing the loosely attached BMDCs in the culture, fresh medium was added, and cells were incubated overnight to allow OVA processing. As a positive control, *Swap-70*^{+/+} BMDCs were matured by cluster disruption using CD11c MicroBeads with the MACS system (Miltenyi Biotec) according to the manufacturer's recommendations, and reseeded with fresh medium for overnight incubation as well. Then the OVA-pulsed BMDCs were washed once with prewarmed PBS and fixed with 1% formaldehyde in PBS for 30 min on ice before washing and addition to CD8⁺ T cells from OTI mouse lymph nodes and spleen. CD8⁺ T cells were isolated using the CD8a⁺ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. A total of 2×10^5 (2:1 ratio) or 1×10^5 (1:1 ratio) fixed BMDCs were incubated with 1×10^5 OTI T cells in RPMI 1640 supplemented with 5% FCS in 96-well plates (U-bottom shape, for cell culture). Supernatants were taken after 24 h and frozen at -80°C. IL-2 concentration was measured by ELISA (BD Biosciences).

Proliferation of OTI cells was evaluated by dilution of the fluorescent dye CFSE in CD8⁺ T cells. OVA-pulsed BMDCs were washed and fixed as above and mixed with CFSE-loaded (1 µM) OTI cells (ratio 2:1). CFSE dilution was assayed by FACS, and cell numbers for each generation were analyzed by FlowJo software (TreeStar).

FACS analysis

To analyze surface expression of markers, DCs were harvested, washed once with ice-cold FACS buffer (2 mM EDTA + 0.1% BSA in PBS), and

stained with different combinations of fluorescently labeled or purified Abs (0.6–0.8 $\mu\text{g/ml}$) for 30 min on ice (Table I). When using an unlabeled primary Ab, cells were washed once with FACS buffer and then incubated with the corresponding secondary Ab for another 20 min on ice. Stained cells were washed once with FACS buffer, resuspended, and analyzed on a BD LSRII (BD Biosciences) using FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). To detect cell death in BMDC cultures, propidium iodide (PI) uptake was measured using FACS. For this BMDCs were harvested at different time points during culture, washed and resuspended in FACS buffer, and PI was added to a final concentration of 10 $\mu\text{g/ml}$. PI uptake was analyzed immediately using FACS.

CCR7 was stained and analyzed using the purified mouse CCL19-Fc staining protocol by eBioscience. Briefly, BMDCs were harvested, washed with FACS buffer, and subsequently incubated with 0.6 $\mu\text{g/ml}$ CCL19-Fc for 30 min on ice. After cells were washed once, they were incubated for another 20 min on ice in 10 $\mu\text{g/ml}$ PE goat anti-human IgG to stain the CCL19-Fc bound to the CCR7 receptor and analyzed with FACS.

Endocytosis assay

Endocytosis of BMDCs was analyzed by FACS, as described (12). Briefly, LPS[−] or LPS⁺ BMDCs were incubated in DMEM with 5% BSA and 10 mM HEPES for 3 h at 37°C. FITC-dextran (Sigma-Aldrich) was added to a concentration of 1 mg/ml, and further incubation took place for 1 h. Endocytosis was stopped by placing the cells on ice and washing with PBS and 0.1% NaN₃. Incubation of BMDCs with FITC-dextran at 4°C was used as negative control. The FITC fluorescent intensity of CD11c⁺ BMDCs was analyzed by FACS.

In vivo CD4⁺ T cell priming by adoptive transfer of BMDCs

At day 11, cultures of BMDCs were loaded with 10 $\mu\text{g/ml}$ E α protein peptide (EAP_{52–68}), specific for MHCII molecules, for 2 h at 37°C and washed extensively. A total of 2×10^6 BMDCs were next injected i.v. at days 0, 2, and 4 into wt mice. Spleen cells of immunized mice were then restimulated with 10 $\mu\text{g/ml}$ EAP_{52–68} at day 7, and the supernatant of these cultures was collected after 72 h. Cytokines IL-10 and IFN- γ were analyzed by ELISA (BD Biosciences).

Retroviral infection of BMDCs

BMDCs were transduced by retroviral infection. Retrovirus was produced by transiently transfecting the Phoenix Eco 293T retroviral packaging cell line, using calcium phosphate/DNA precipitation combined with retroviral vectors (20 μg vector DNA per 10-cm dish of 80% confluent Phoenix cells). For the transduction of BMDCs, the medium was changed to supplemented RPMI 1640 24 h post transfection. The retrovirus-containing supernatant was collected from Phoenix cell cultures at 48 h post transfection and centrifuged once at $500 \times g$ for 5 min at 4°C to remove cell debris before infection of BMDCs. Transfected Phoenix cells were then used to produce retrovirus for a second infection by adding fresh medium for another 24 h. Supernatant containing retrovirus was always used fresh without freezing.

BMDCs were transduced with retroviral vectors coding for C-terminal GFP-tagged SWAP-70 or mutant proteins (Table II). BMDCs were infected with supernatant containing retrovirus at days 6 and 7 of culture by passive incubation for 6–8 h at 37°C and 5% CO₂. The supernatant containing retrovirus produced was supplemented with 10% GM-CSF and 10 $\mu\text{g/ml}$ Polybrene before it was added to the BMDCs. After 6–8 h of incubation, the supernatant containing retrovirus was removed from the BMDCs and replaced with supplemented RPMI 1640 containing 10% GM-CSF. The infection procedure was repeated once more, as described. BMDCs were further cultured as described and analyzed at day 11 of culture, using FACS.

Western blot analysis

Total cell lysates were prepared with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, and protease inhibitor mixture [Roche]). Protein concentration was measured using a Bradford assay (Bio-Rad), and samples were boiled in reducing sample buffer. Total cell lysate samples were subjected to SDS-PAGE, and separated proteins were electrophoretically transferred onto nitrocellulose membranes using a semidry blotting system. Protein transfer was checked by Ponceau S staining, and the membrane was blocked with 5% milk in PBS-T (0.1% Tween-20 in PBS) for 1 h at room temperature. The membrane was washed and incubated with primary Abs in PBS-T overnight at 4°C. After washing, HRP conjugated secondary Abs were added (in PBS-T) for 1 h at room temperature. The blot was developed using HRP substrate solution (Pierce ECL Western Blotting Substrate [Thermo Scientific] or

Immobilon Western [Millipore]). Images were taken using a Kodak Image Station 2000MM (Thermo Scientific), and band densities were analyzed with ImageQuant (GE Life Sciences).

RhoA activity pull-down assay

The activity of RhoA was analyzed using rhotekin–Rho binding domain agarose beads. Beads were subjected to SDS-PAGE and Western blotting with Abs against RhoA. Activity of Rho GTPases was quantified by densitometry analysis using ImageQuant software (GE Life Sciences).

Real-time PCR

Total BMDC RNA was prepared by TRIzol lysis (Invitrogen). RNA was reversed transcribed into cDNA using SuperScript II (Invitrogen). Expression of FZD5, FZD7, CCND2, and GAPDH (control) was analyzed using a Rotor-Gene RG3000 (Qiagen) and the SYBR Green PCR Kit (Qiagen). The relative gene expression was calculated by dividing values to that of GAPDH. Primers: FZD5 forward, 5'-CCATCATCTTCTGTCT-GCGTG-3'; FZD5 reverse, 5'-TAAGAGGAAGACAACCGTGC-3'; FZD7 forward, 5'-CATCCTCTTCATGGTGTCTTAC-3'; FZD7 reverse, 5'-TG-ATTGTCTTGACCGCTG-3'; CCND2 forward, 5'-AACTGGTAGTGTGGGTAAGC-3'; CCND2 reverse, 5'-GTACATGGCAAACCTTGAAGTCG-3'; GAPDH forward, 5'-GAGAAACCTGCCAAGTATGAC-3'; GAPDH reverse, 5'-GGAGTTGCTATGAAGTTGC-3'.

Soluble ICAM-1 binding assay

Binding of ICAM-1 to BMDCs was done according to the method outlined in Ref. 24. Briefly, BMDCs at day 8 were incubated with soluble ICAM-1 Fc-chimeric molecules (20 ng/ml; R&D Systems) in IMDM medium with or without 2 mM Mn²⁺. Ligand binding was terminated by fixing cells with 3.7% paraformaldehyde in PBS. Bound Fc–ICAM-1 was detected with anti-Fc fragment Abs by FACS.

Statistics

Statistical analysis and graphing were carried out with Prism 5 (GraphPad). Paired two-tailed Student *t* test (confidence interval 95%) was used to determine statistical significance.

Results

Spontaneous maturation of SWAP-70-deficient DCs

Without specific stimuli, only ~5% of BMDCs cultured in GM-CSF-containing medium for up to 11 d acquire a mature phenotype, as defined by upregulation of activation markers, including CD86, CD80, B7-DC, MHCII, and CCR7. This percentage does not increase between days 6 and 11 of culture. However, about one-third of *Swap-70*^{−/−} BMDCs spontaneously—that is, without additional stimuli—upregulate such activation markers on their surface (Fig. 1A, Supplemental Fig. 1) at day 11 of culture—meaning that there are up to 6-fold more activated cells than in the corresponding wt BMDC cultures. Analysis of spleen-resident cDCs (Table I) shows that CD11c⁺MHCII⁺CD11b⁺CD8 α [−] DCs, but not CD11c⁺MHCII⁺CD11b[−]CD8 α ⁺ DCs, also have elevated expression levels of the activation marker CD86 (Fig. 1B). Spleens from *Swap-70*^{−/−} mice show a diminished percentage of CD11c⁺MHCII⁺, with a significant reduction in the CD11b[−]CD8 α ⁺ population (Fig. 1B). To compare spleen-resident DCs with cDCs generated from bone marrow precursors, we used Flt3-L in *in vitro* cultures for 9 d to obtain plasmacytoid and cDCs (22). In these cultures, the cell subset CD11c⁺B220[−]CD11b^{lo}CD24^{hi} is homologous to CD8 α ⁺ DCs, and CD11c⁺B220[−]CD11b^{hi}CD24^{lo} is homologous to CD8 α [−] DCs (22). Fig. 1C shows elevated expression of activation marker CD86 in CD8 α [−] and CD8 α ⁺ *Swap-70*^{−/−} DC equivalents. As observed in spleen, *Swap-70*^{−/−} bone marrow produces less of cDCs (CD11c⁺B220[−]) with a significant decrease in the CD8 α [−] and CD8 α ⁺ DC equivalents (Fig. 1C). This result suggests that the deficiency in DC homeostasis is intrinsic to precursor cells and not due to extrinsic factors that may arise in the absence of SWAP-70. This aspect will be subject to further studies. *Swap-70*^{−/−} SDCs differentiated in the presence of GM-CSF and TGF- β also spontaneously mature at an ~3-fold elevated rate (Fig. 1D).

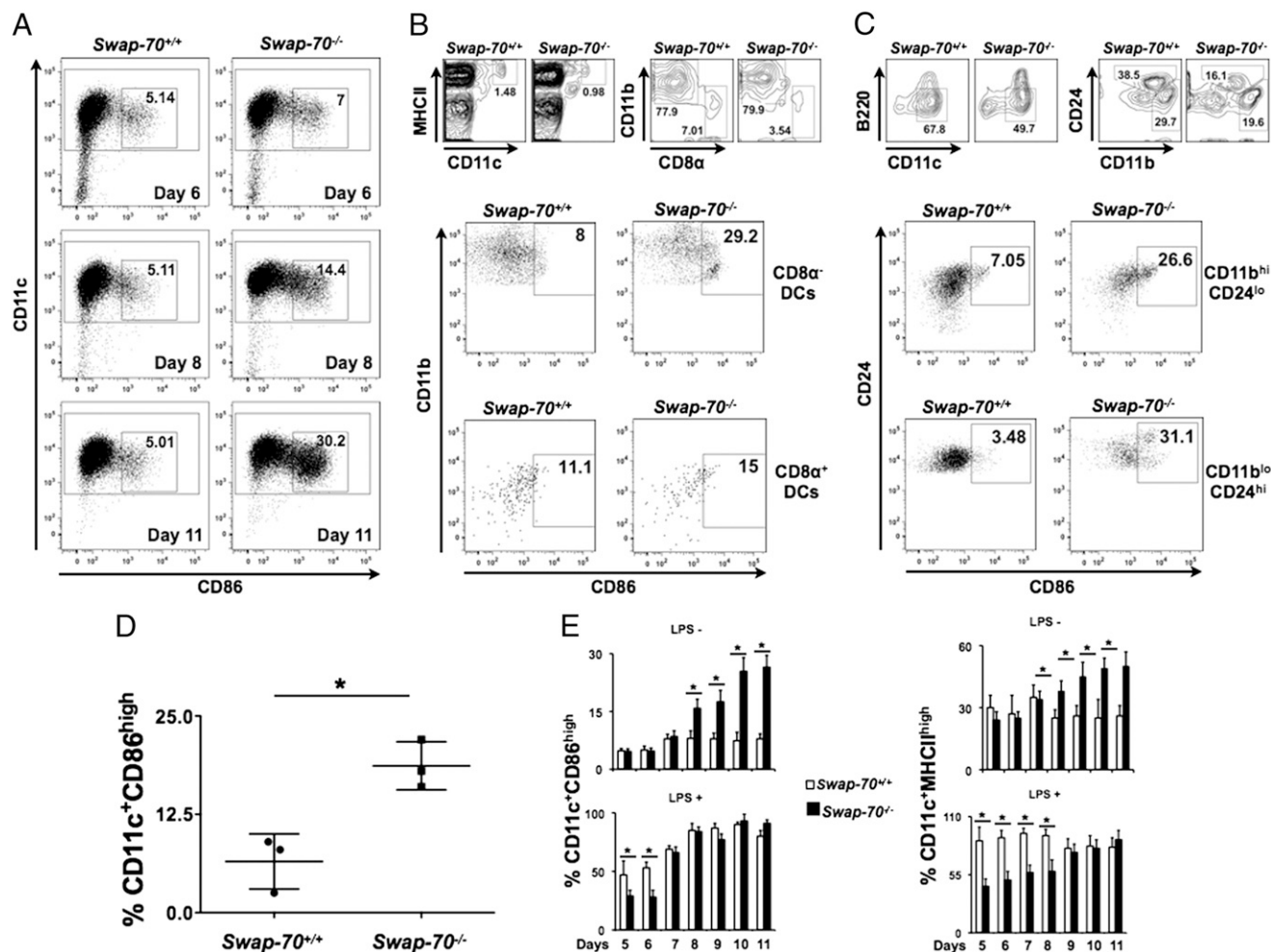


FIGURE 1. SWAP-70 deficiency leads to an increase in spontaneous maturation in DCs. **(A)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were harvested and costained for CD11c and CD86 at the indicated days of culture and then analyzed by FACS. **(B)** *Swap-70*^{+/+} and *Swap-70*^{-/-} spleen-resident cDCs from 129Sv mice were isolated, and expression of CD86 was analyzed by FACS. **(C)** *Swap-70*^{+/+} and *Swap-70*^{-/-} plasmacytoid and cDCs were generated in vitro using Flt3-L, and cell subsets homologous to CD8⁺ (CD11c⁺B220⁻CD24^{hi}CD11b^{lo}) and CD8⁻ (CD11c⁺B220⁻CD24^{lo}CD11b^{hi}) DCs were analyzed for expression of CD86. **(D)** *Swap-70*^{+/+} and *Swap-70*^{-/-} SDCs were harvested at day 14 of culture, costained for CD11c and CD86, and analyzed by FACS. Data are shown as quantification of percentage of CD11c⁺ and CD86^{hi} SDCs. Mean values with error bars indicating \pm SD are shown. **(E)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were activated or not with LPS and then harvested and costained for CD11c, CD86 (left), and MHCII (right) at the indicated days of culture and then analyzed by FACS. Data are shown as quantification of percentage of CD11c⁺ and CD86^{hi} or MHCII^{hi} BMDCs. Mean values with error bars indicating \pm SD are shown. * $p < 0.05$ by t test.

Time course analysis of expression of CD86 and MHCII revealed that the spontaneous maturation phenotype of *Swap-70*^{-/-} BMDCs appears at day 8 and continues to increase up to day 11 (Fig. 1E). We also activated BMDCs with the TLR4 ligand LPS for 16 h. With the exception of days 5 and 6, *Swap-70*^{-/-} and wt BMDCs showed equal expression of CD86 after LPS treatment (LPS⁺) (Fig. 1E). These experiments also confirmed the failure of *Swap-70*^{-/-} BMDCs to upregulate MHCII on the cell surface at days 5–8 after LPS activation for 16 h, as previously reported (13) (Fig. 1E). The increased percentage of spontaneously matured cells in *Swap-70*^{-/-} BMDC cultures was not caused by a difference in proliferation or vitality between wt and *Swap-70*^{-/-} cultures (Supplemental Fig. 2A).

The hypermaturation phenotype was observed with BMDCs derived from SWAP-70-deficient mice either of the 129SvEMS genetic background, mostly used in this study, or of the C57BL/6 genetic background (Supplemental Fig. 2B). The use of recombinant GM-CSF instead of a GM-CSF-containing cell culture supernatant (Supplemental Fig. 2C) or of a bone marrow lineage-depleted population (depleted for CD11b, CD3e, CD19, CD45R,

Gr-1, Ter119, and NK 1.1 positive cells) did not affect the spontaneous activation of *Swap-70*^{-/-} BMDCs (Supplemental Fig. 2D). Taken together, these findings indicate that SWAP-70 restricts spontaneous maturation of BMDCs.

Increased cross-presentation and activation of CD8⁺ T cells by *Swap-70*^{-/-} BMDCs

When BMDCs are induced to mature through mechanical disruption of cell–cell interactions, they upregulate their capacity to process exogenous proteins and present MHCII-restricted Ags to activate CD8⁺ T cells (7, 25). We tested whether the spontaneous maturation observed in *Swap-70*^{-/-} DCs correlates with increased cross-presentation and activation of CD8⁺ T cells. OVA protein was added to the BMDCs to allow processing of the protein and loading of fragments onto their MHCII. The cells were then fixed and mixed with OTI CD8⁺ T cells specific for H-2Kb/OVA_{257–264} complexes. Activation of OTI cells was determined by detection of IL-2 in the incubation medium. This in vitro assay determines differences in the CD8⁺ T cell stimulatory capacity of BMDCs owing to their capacity to cross-present Ag derived from exoge-

Table I. Abs for FACS, Western blotting, and functional assays used in this study

| Ag | Conjugate | Clone/Origin | Company |
|--------------------------|-------------------|-------------------|--------------------------|
| FACS | | | |
| CD11c | eFluor 450, APC | N418 | eBioscience |
| CD11c | FITC | HL3 | BD Biosciences |
| CD45.1 | APC | A20 | BioLegend |
| CD45.2 | PerCP-Cy5.5 | 104 | eBioscience |
| CD86 (B7-2) | APC | GL1 | eBioscience |
| CD4 | PE | L3T4 | BD Biosciences |
| CD8a | APC | 53-6.7 | eBioscience |
| CD24 | PE | M1/69 | BD Biosciences |
| CD11b | PE-Cy7 | M1/70 | eBioscience |
| MHCII | eFluor 450 | M5/114.15.2 | eBioscience |
| MHCI | FITC | AF6-88.5 | BD Biosciences |
| ICAM-1 | Unlabeled | Human Fc-chimeric | R&D Systems |
| Anti human Fc | FITC | rabbit | The Jackson Laboratory |
| Western blotting | | | |
| GAPDH | Unlabeled | 6C5 | Santa Cruz Biotechnology |
| RhoA | Unlabeled | 119 | Santa Cruz Biotechnology |
| Active- β -catenin | Unlabeled | 8E7 | Millipore |
| β -Catenin | Unlabeled | Rabbit | Sigma-Aldrich |
| E-cadherin | Unlabeled, AF 488 | DECMA-1, rat | eBioscience |
| SMC3 | Unlabeled | Rabbit | Lab self-made |
| Anti-mouse | HRP | Donkey | Jackson ImmunoResearch |
| Anti-rabbit | HRP | Goat | Jackson ImmunoResearch |
| Functional assays | | | |
| CD11b (α_M) | Unlabeled | M1/70 | eBioscience |
| CD18 (β_2) | Unlabeled | M18/2 | eBioscience |
| VCAM | Unlabeled | 429 | eBioscience |

nous proteins. *Swap-70^{-/-}* BMDCs matured through mechanical cluster disruption were significantly more potent in activating CD8⁺ T cells than cluster-disrupted wt cells used as positive control (25) (Fig. 2A). Without mechanical cluster disruption, *Swap-70^{-/-}* BMDCs show a markedly increased capacity to activate CD8⁺ T cells when compared with wt BMDCs (Fig. 2A). The capacity of spontaneously matured BMDCs to activate CD8⁺ T cells is enhanced when LPS is added to these cells (25). Accordingly, although LPS addition has no significant effect on wt BMDCs, *Swap-70^{-/-}* BMDCs markedly increase their capacity to cross-present Ags to CD8⁺ T cells (Fig. 2A). Activation of CD8⁺ T cells by *Swap-70^{-/-}* BMDCs was also tested by proliferation assays in which OVA-pulsed BMDCs were incubated with CFSE-loaded OTI cells for 3 days and cell division was measured by FACS as dilution of the dye CFSE. Fig. 2B shows that *Swap-70^{-/-}* BMDCs induce significantly higher numbers of proliferating CD8⁺ T cells than do wt BMDCs. The difference in the activation capacity is not due to higher expression of MHCI molecules, as *Swap-70^{-/-}* BMDCs and their wt counterparts do not differ significantly in the expression of MHCI molecules before and after LPS activation (Fig. 2C). These results demonstrate that spontaneous maturation of *Swap-70^{-/-}* BMDCs enhances their cross-presentation and activation of CD8⁺ T cells.

Changes in endocytosis in wt and *Swap-70^{-/-}* BMDCs after incubation with SIP suggested that *Swap-70^{-/-}* BMDCs have a deficiency in uptake of soluble substrates (12). Cross-presentation is directly affected by the endocytosis capacity of DCs (25). After a maturation stimulus with TLR ligands, such as LPS, DCs downregulate their capacity to endocytose soluble proteins (26). We tested whether the difference in cross-presentation observed in this study could be due to changes in the endocytosis capacity of wt and *Swap-70^{-/-}* BMDCs. Fig. 2D shows that non-LPS-treated wt BMDCs endocytose more of soluble dextran than do *Swap-70^{-/-}* BMDCs after 1 h of incubation, although they have a diminished cross-presentation capacity (Fig. 2A, 2B). The capacity to endocytose dextran is downregulated in LPS-treated wt

BMDCs, but not in *Swap-70^{-/-}* BMDCs (Fig. 2D). This result agrees with the higher spontaneous maturation rate of *Swap-70^{-/-}* BMDCs and suggests that further activation with TLR ligands does not have an effect on their endocytosis capacity. This finding also indicates that the increased cross-presentation of *Swap-70^{-/-}* BMDCs is due to factors different from the uptake of proteins by endocytosis, such as rate of spontaneous maturation.

Swap-70^{-/-} BMDCs induce tolerogenic immune responses

Spontaneous maturation of DCs by disruption of clusters induces a tolerogenic T cell response, as observed in several studies (7, 9, 25). Immunization with BMDCs previously loaded with peptides and activated by mechanical disruption of clusters led to high production of IL-10 and less increased secretion of IFN- γ by T cells compared with non-mechanically disrupted cells (7, 9). We investigated the type of immune response elicited by immunizing wt mice three times with day 11 cultures of *Swap-70^{+/+}* or *Swap-70^{-/-}* BMDCs previously loaded with the peptide covering aa 52–68 of the E α protein specific for MHCII molecules (EAP_{52–68}:I-A^b) (27). After the last immunization, splenocytes of immunized animals were incubated ex vivo with EAP_{52–68} peptide, and the production of IL-10 and IFN- γ was detected in the supernatant 3 d afterward. Fig. 2E shows that *Swap-70^{-/-}* BMDCs induced more IL-10 production than did wt cells. This statistically significant difference of 22% of the mean values may reflect the higher level of spontaneously matured *Swap-70^{-/-}* cells. Manipulation of BMDCs prior to immunization involves mechanical disruption of cell clusters that will result in induction of a tolerogenic response by both wt and *Swap-70^{-/-}* BMDCs. There was no difference in the expected lower production of IFN- γ (Fig. 2E).

Consistent with their capacity to induce tolerogenic immune responses, BMDCs previously matured by cluster disruption produce fewer inflammatory cytokines, such as IL-1 α , IL-6, and TNF- α , after stimulus with LPS than do BMDCs that did not undergo cluster disruption (7). Inflammatory cytokine production

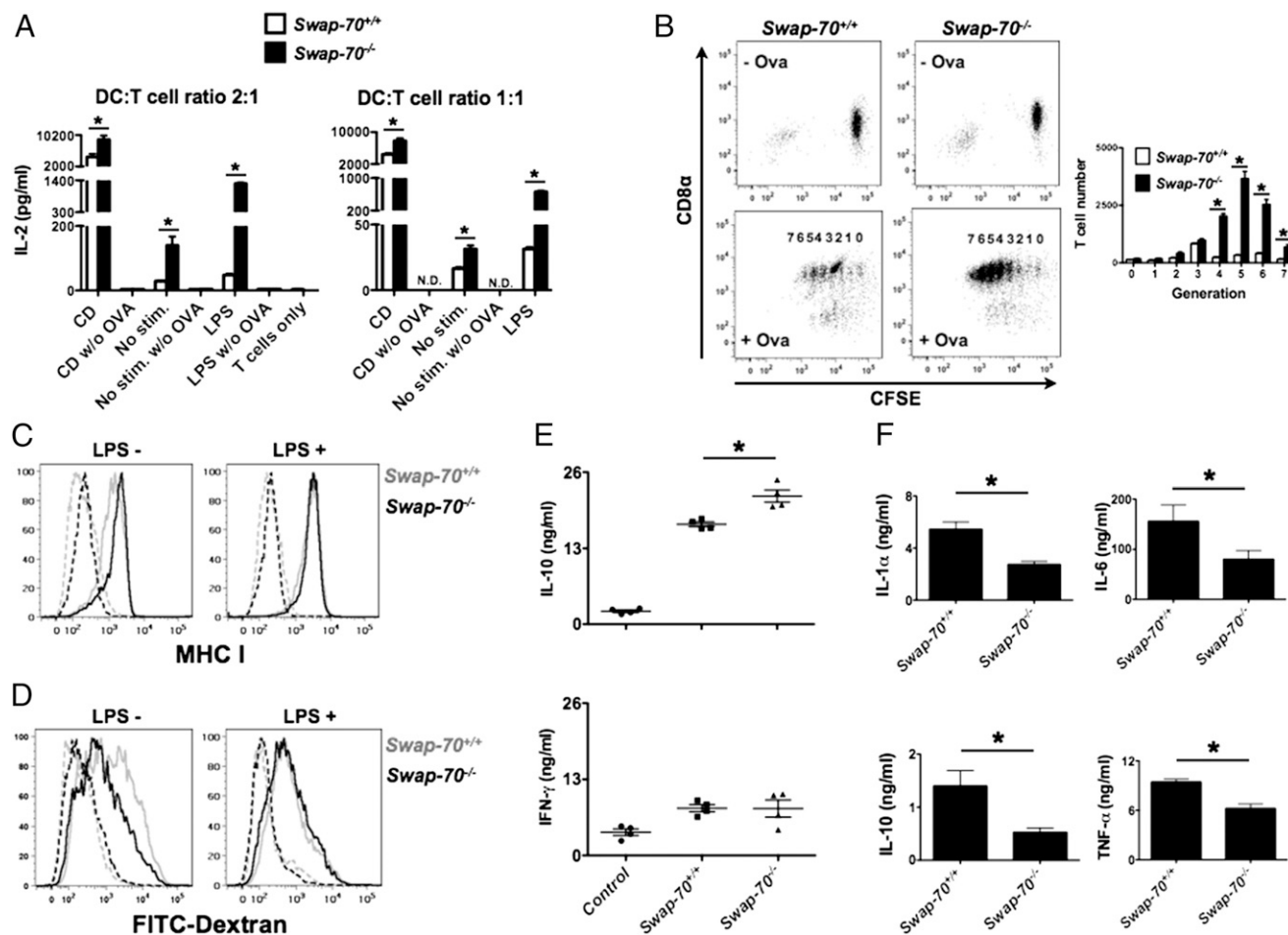


FIGURE 2. *Swap-70*^{-/-} BMDCs show increased activation of CD8⁺ T cells and generate tolerogenic immune response. (A) Day 11 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were pulsed or not with OVA protein for 2 h and then washed thoroughly, followed by activation or not with LPS. Some cells were activated by cluster disruption (CD) after incubation with OVA as positive control. After an overnight chase, the BMDCs were fixed and cultured with OTI CD8⁺ T cells in a DC/T cell ratio of 2:1 or 1:1. T cell responses were monitored after 24 h by measuring IL-2 secretion by ELISA. A representative of three independent experiments is shown. Mean values with error bars indicating \pm SD are shown. N.D., not detectable. **p* < 0.05 by *t* test. (B) Day 11 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were incubated with OVA, washed and fixed as in (A), and mixed with CFSE-loaded OTI cells in a DC/T cell ratio of 2:1. After 3 d, dilution of CFSE was evaluated by FACS. Cell number per generation was also analyzed (right). **p* < 0.05 by *t* test. (C) BMDC *Swap-70*^{+/+} and *Swap-70*^{-/-} cultures were harvested and costained for CD11c and MHC I at day 11 of culture and analyzed by FACS. Cells were pregated in the CD11c⁺ population. A representative of three independent experiments is shown. Staining with isotype Abs was used as control (dotted lines). (D) Endocytosis was assayed by FACS by incubating day 11 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs with FITC-dextran for 1 h. Cells were pregated in the CD11c⁺ population. A representative of three independent experiments is shown. Incubation at 4°C was used as negative control (dotted lines). (E) Day 11 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were incubated with EAP₅₂₋₆₈ peptide for 2 h, followed by extensive washing. A total of 2×10^6 cells were i.v. injected into wt recipient mice at days 0, 2, and 4. Spleen cells of recipient mice were challenged ex vivo with EAP₅₂₋₆₈ peptide at day 7 for 3 d. IL-10 and IFN-γ in supernatants were analyzed by ELISA. Mock immunized mice (only PBS) were used as control. Mean values with error bars indicating \pm SD are shown. (F) Levels of inflammatory cytokines were analyzed in supernatants of day 11 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs after 18 h of LPS stimulus. Mean values with error bars indicating \pm SD are shown. **p* < 0.05 by *t* test.

in wt and *Swap-70*^{-/-} BMDCs without LPS activation was below detectable levels (data not shown). We then determined the production of inflammatory cytokines by wt and *Swap-70*^{-/-} BMDCs after LPS activation at day 11 of culture. In agreement with the increased rate of spontaneous maturation observed in *Swap-70*^{-/-} BMDCs, they produced significantly fewer proinflammatory cytokines, such as IL-1α, IL-6, IL-10, and TNF-α, than did wt cells (Fig. 2F). Taken together, these results indicate that enhanced spontaneous maturation levels in *Swap-70*^{-/-} BMDCs lead to a tolerogenic type of immune response.

Cell-cell interactions independent of E-cadherin regulate spontaneous maturation of *Swap-70*^{-/-} BMDCs

It has been reported that mechanical disruption of homotypic E-cadherin (CDH1) interactions between BMDCs initiates β-catenin

signaling and adhesion-related spontaneous maturation (7). Signaling through β-catenin became recently known as an important component of tolerogenic DC function in peripheral immune tolerance (28) and can be suppressed by TGF-β (9). Thus, β-catenin signaling appears to be an important pathway for maturation of DCs triggered by cluster disruption. To determine whether activation of the β-catenin pathway contributes to the hypermaturation phenotype of *Swap-70*^{-/-} BMDCs, we performed immunoblotting of total cell lysates of BMDCs at days 6, 8, and 11 of culture and analyzed expression of E-cadherin, total β-catenin, and the active form of β-catenin. Fig. 3A shows no significant difference in total and active β-catenin between wt and *Swap-70*^{-/-} BMDCs. *Swap-70*^{-/-} BMDCs show decreased expression of E-cadherin at day 11 (Fig. 3A). This decrease can be explained by the higher number of spontaneously matured cells in

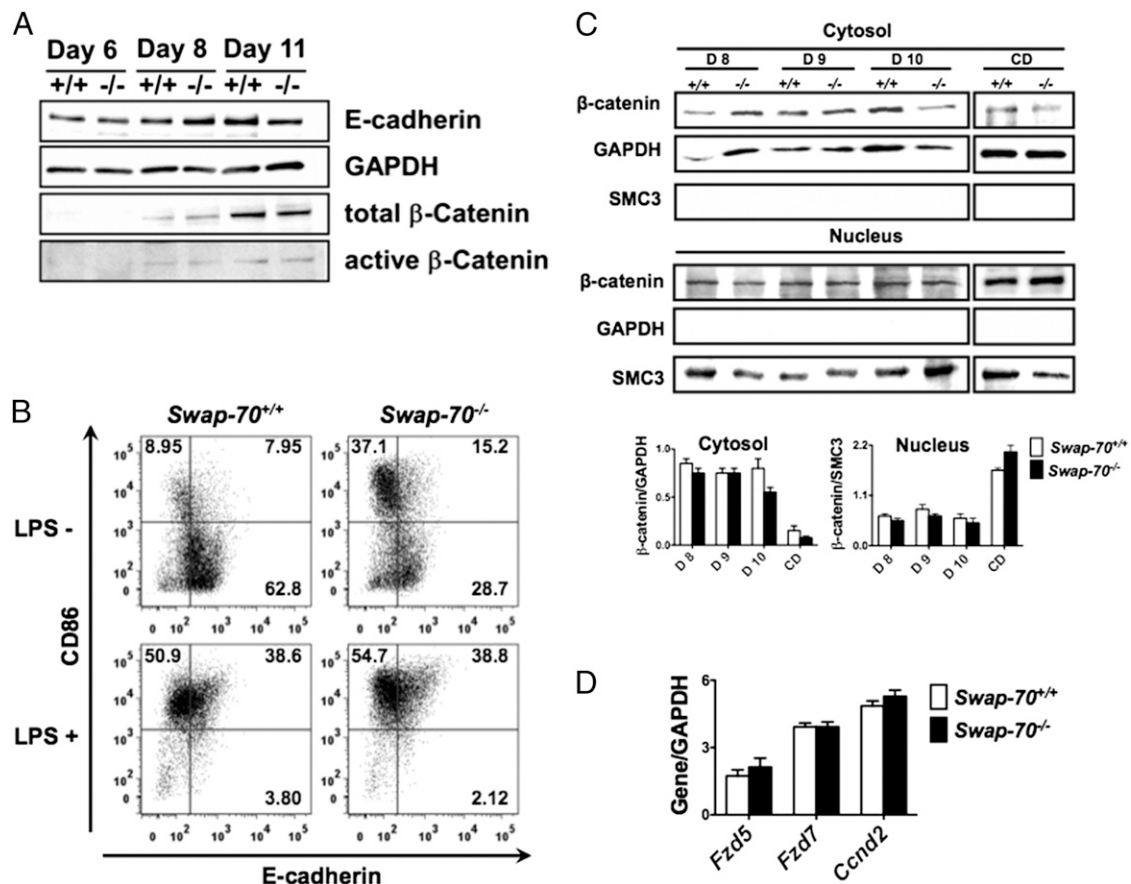


FIGURE 3. Spontaneous maturation of *Swap-70*^{-/-} BMDCs is not mediated by β -catenin. **(A)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDC cultures were harvested at indicated days of culture, and expression of E-cadherin, total β -catenin, and active β -catenin was analyzed by SDS-PAGE and Western blotting. A representative of three independent experiments is shown. **(B)** BMDC *Swap-70*^{+/+} and *Swap-70*^{-/-} cultures LPS-matured (LPS +) or without LPS treatment (LPS -) were harvested and costained for CD11c, CD86, and E-cadherin at day 11 of culture and analyzed by FACS. Cells were pregated in the CD11c⁺ population. Representative of three independent experiments is shown. **(C)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDC cultures were harvested at indicated days of culture, and cytosolic and nuclear fractions were prepared as described in *Materials and Methods*. Expression of β -catenin was analyzed by SDS-PAGE and Western blotting. GAPDH and SMC3 were used as cytosolic and nuclear markers, respectively. Activation by cluster disruption (CD) was used as positive control. Densitometry analysis of two independent experiments is shown (*lower panels*). **(D)** The mRNA expression of FZD5, FZD7, and CCND2 in day 10 *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs. Mean values with error bars indicating \pm SD are shown.

Swap-70^{-/-} BMDCs cultures because the expression of E-cadherin in DCs is downregulated by activation signals (11, 29). Analysis of E-cadherin surface expression by FACS confirmed these results, showing a lower mean fluorescence intensity of E-cadherin staining on spontaneously matured or LPS-matured BMDCs (Fig. 3B). After disruption of E-cadherin interactions, β -catenin is translocated to the nucleus and acts as transcriptional activator (30, 31). We also analyzed the presence of β -catenin in nuclear fractions of wt and *Swap-70*^{-/-} BMDCs at days 8, 9, and 10 of culture. The translocation of β -catenin to the nucleus of wt and *Swap-70*^{-/-} BMDCs did not differ (Fig. 3C). Translocation of β -catenin to the nucleus of wt and *Swap-70*^{-/-} BMDCs is less than the translocation seen in the cells matured by mechanical cluster disruption that were used as positive control (Fig. 3C). Furthermore, we did not detect an increase in the expression of the β -catenin target genes FZD5, FZD7, and cyclin D2 (7) by RT-PCR in *Swap-70*^{-/-} BMDCs (Fig. 3D).

Direct or indirect cell-cell interactions may affect maturation of cultured BMDCs through activation of adhesion molecules. Therefore, we tested whether interactions between wt BMDCs and *Swap-70*^{-/-} BMDCs influence the hypermaturation phenotype of *Swap-70*^{-/-} BMDCs or vice versa. We used BMDCs derived from CD45.2 (wt) or CD45.1 (*Swap-70*^{-/-}) mice. In isolated cultures used as control, CD45.1 (*Swap-70*^{-/-} BMDCs) showed higher

levels of spontaneous maturation than did CD45.2 (wt BMDCs) (Fig. 4A, 4C). In cocultures of BMDCs of both genotypes, the percentage of spontaneously matured wt BMDCs at day 11 was increased and the percentage of spontaneously matured *Swap70*^{-/-} BMDCs was reduced (Fig. 4B, 4C). We did not observe an effect of secreted activating cues, as cultivation of BMDCs with conditioned medium from either *Swap-70*^{-/-} or wt BMDCs at day 11 did not affect spontaneous maturation (Fig. 4D). Taken together, these results suggest a role for direct cell-cell interactions in promotion of BMDC activation independent of E-cadherin and β -catenin.

The PH domain of SWAP-70 is essential for spontaneous maturation of BMDCs

To reveal the mechanism involved in preventing spontaneous maturation of BMDCs by SWAP-70, we expressed full-length and mutant variants of SWAP-70 in BMDCs and performed complementation experiments to reduce the spontaneous maturation phenotype of *Swap-70*^{-/-} BMDCs. With use of a retroviral infection system (12, 13), wt or mutant SWAP-70 was expressed as GFP fusion protein to facilitate the analysis of GFP⁺ cells expressing SWAP-70 by flow cytometry (Fig. 5A, Table II). Expression of GFP alone was used for control. We validated the function of the SWAP-70-GFP fusion protein, comparing it with

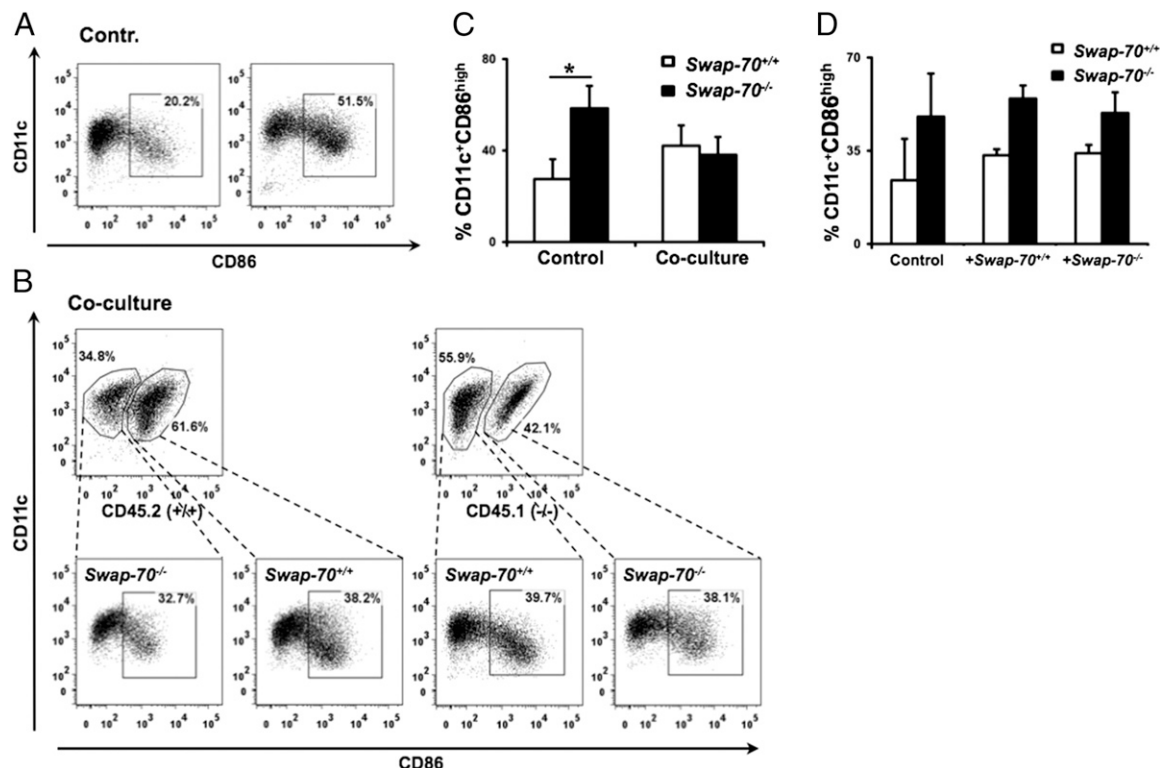


FIGURE 4. Spontaneous maturation of *Swap-70*^{-/-} BMDCs is mediated by cell-cell contact. **(A)** Day 11 C57BL/6N *Swap-70*^{+/+} (CD45.2⁺) and *Swap-70*^{-/-} (CD45.1⁺) BMDCs expressing congenital markers were harvested and costained for CD11c and CD86 and analyzed by FACS as control. **(B)** C57BL/6N *Swap-70*^{+/+} (CD45.2⁺) and *Swap-70*^{-/-} (CD45.1⁺) bone marrow was mixed 1:1 and differentiated into BMDCs for 11 d. BMDCs were harvested and costained for CD11c, CD86, and either CD45.1 or CD45.2 and analyzed by FACS. **(C)** Mean values with error bars indicating \pm SD are shown. * $p < 0.01$ by *t* test. **(D)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were cultivated with 50% of conditioned medium of either *Swap-70*^{+/+} (+*Swap-70*^{+/+}) or *Swap-70*^{-/-} (+*Swap-70*^{-/-}) BMDC cultures at day 11. Quantification of percentage of CD86^{hi} BMDCs at day 11 of culture, mean values with error bars indicating SD, is shown.

a previously used SWAP-70-internal ribosome entry site-GFP construct (12, 13). The expression of both forms of SWAP-70 significantly improved the otherwise impaired migratory capacity to S1P of *Swap-70*^{-/-} BMDCs (12) (Supplemental Fig. 3). This finding shows that the SWAP-70-GFP fusion protein is functional and can be used for complementation experiments.

Expression of full-length SWAP-70 decreases spontaneous maturation of *Swap-70*^{-/-} BMDCs to wt cell levels (Fig. 5B). Expression of an N-terminal deletion mutant (SWAP-70 ^{Δ 1-198}) or a smaller or larger C-terminal deletion mutant (SWAP-70 ^{Δ 527-585} or SWAP-70 ^{Δ 353-585}, respectively) complemented the hypermaturation phenotype as well (Fig. 5B). These protein fragments lack the putative N-terminal EF hand domain (SWAP-70 ^{Δ 1-198}) or the C-terminal F-actin binding domain (SWAP-70 ^{Δ 527-585}) or the C-terminal F-actin binding domain and the Dbl-homology/coiled-coil domain (SWAP-70 ^{Δ 353-585}) of SWAP-70. All these proteins still contain the PH domain. However, an N-terminal 197 aa fragment did not complement (Fig. 5B). This observation indicated a role for the central PH domain in spontaneous maturation of BMDCs. Like other PH domains (32), the PH domain of SWAP-70 consists of seven β -sheets and one α -helix (33, 34). Previous studies showed that binding of SWAP-70 to phosphoinositides [phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 3,4,5-triphosphate (PIP₃)] depends on positively charged amino acids in the loop between β 1 and β 2 strands and on the β 2 strand of the PH domain and that binding to PIP₃ contributes to localization of SWAP-70 to the cell membrane (34-36). A deletion of six amino acids in the loop between the β 1 and β 2 strands (SWAP-70 ^{Δ 220-225}) of the PH domain did not complement the

phenotype (Fig. 5C). In contrast, a deletion of six amino acids within the β 2 strand (SWAP-70 ^{Δ 229-234}) decreased spontaneous maturation of *Swap-70*^{-/-} BMDCs to wt levels (Fig. 5C). This result indicates that the loop between β 1 and β 2 strands is critical for this function. Expression of the SWAP-70^{Y517F} mutant that prevents phosphorylation of SWAP-70 by Syk (37) also decreases spontaneous maturation in *Swap-70*^{-/-} BMDCs to wt levels (Fig. 5C).

Full-length GFP-tagged SWAP-70 in BMDCs localizes close to the cytoplasmic membrane, whereas the SWAP-70 ^{Δ 220-225} and SWAP-70 ^{Δ 229-234} mutants localize mostly to the interior of the cell (Fig. 5D). Both mutant proteins accumulate in one major site, but this accumulation per se does not render them nonfunctional, as the SWAP-70 ^{Δ 229-234} protein still complements. These results indicate that SWAP-70 controls spontaneous maturation in BMDCs through a function of the loop between the β 1 and β 2 strands of the PH domain. These results further suggest that PIP₃ binding and membrane localization of SWAP-70 are dispensable for restriction of spontaneous maturation of BMDCs.

Distinct effects of CD11b or CD18 activation on spontaneous maturation of BMDCs

It has been proposed that integrins can initiate maturation in BMDCs, as direct activation of CD11b (α_M) with Abs leads to spontaneous maturation similar to that observed upon mechanical disruption (9). The anti-CD11b mAb M1/70 was reported to increase the level of spontaneous maturation in BMDCs (9). We added this Ab to the BMDC cultures from day 4 onward, and the expected increase in spontaneous maturation was observed in

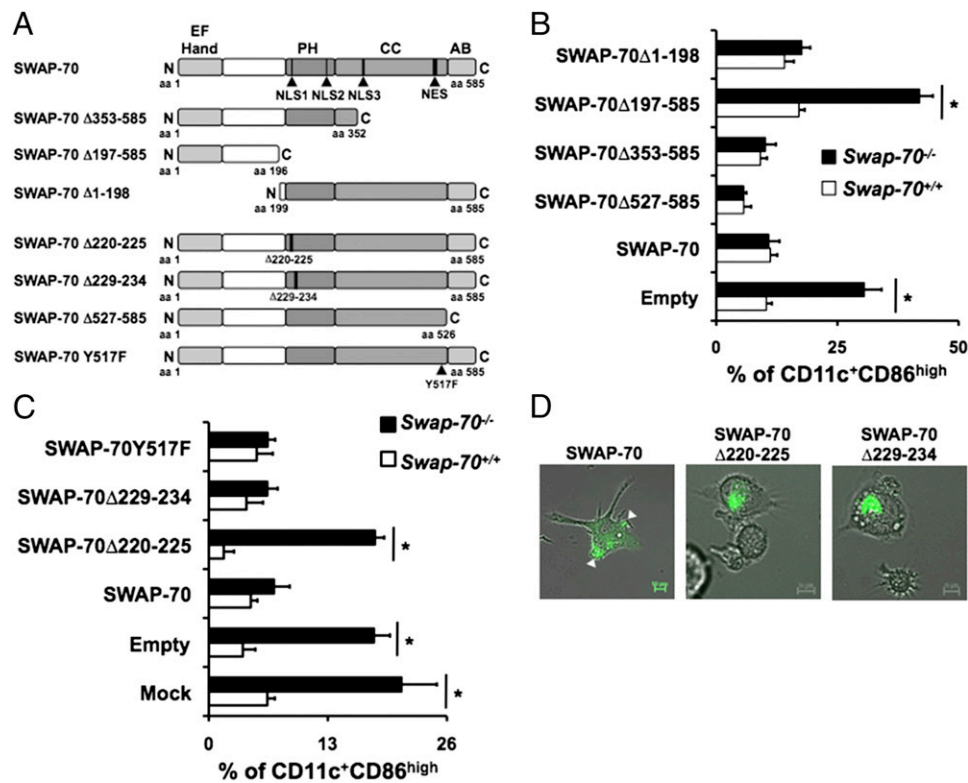


FIGURE 5. PH domain of SWAP-70 is essential for spontaneous activation of BMDCs. **(A)** Schematic representation of SWAP-70 wt and mutants used. GFP-tag is not shown. AB, actin binding; CC, coiled coil; NES, nuclear export signal; NLS, nuclear localization signal. **(B and C)** Quantification of percentage of CD11c⁺CD86^{hi}GFP⁺ BMDCs. *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were transduced with vectors coding for GFP-tagged SWAP-70 and SWAP-70 mutants at days 6 and 7 of culture. Transduced BMDCs were costained with CD11c and CD86, and GFP⁺ cells were analyzed by FACS at day 11. **(B)** SWAP-70 mutants with big N-terminal and C-terminal deletions were analyzed. **(C)** SWAP-70 mutants with small deletions in the PH domain (Δ220–225, Δ229–234), deletion of the F-actin binding site (Δ527–585), and point mutation Y517F were analyzed. Mean values with error bars indicating ±SD are shown. **p* < 0.01 by *t* test. **(D)** *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were transduced with vectors coding for GFP-tagged SWAP-70 and SWAP-70 mutants at days 6 and 7 of culture. Transduced BMDCs were analyzed by fluorescent microscopy and ImageJ. SWAP-70 full-length (SWAP 70) and SWAP-70 mutants with small deletions in the PH domain (Δ220–225, Δ229–234) were analyzed. Arrowheads show localization of GFP-tagged SWAP-70 to membrane extensions only in full-length SWAP-70, but not in PH mutants. A representative of two experiments is shown.

wt BMDCs. However, spontaneous maturation in *Swap-70*^{-/-} BMDCs did not change significantly (Fig. 6A, 6B). The mAb M1/70 has been reported to stabilize the active integrin conformation, leading to spontaneous maturation of DCs (9). This finding indicates that in the absence of SWAP-70, DCs already have an integrin conformation that favors spontaneous maturation and would not respond to an additional stimulus with Abs.

As DCs express high levels of CD18 (β₂) integrins (38), we tested whether activation of these integrins with Abs (39) also causes spontaneous maturation of BMDCs. Anti-CD18 treatment of BMDCs suppresses spontaneous maturation of both wt and *Swap-70*^{-/-} BMDCs to about half the level of untreated cells (Fig. 6A, 6B). Although this result suggests that CD18 does not play a role in the spontaneous maturation of *Swap-70*^{-/-} BMDCs, it will be of interest to investigate the role of integrin p150,95 (CD11c/CD18) in spontaneous maturation using activating Abs against CD11c, as DCs express high levels of p150,95 (38). The reduction of spontaneous maturation was not due to cell death or the capacity to become activated, as the treated and nontreated cells responded equally to LPS treatment (data not shown). No effect on spontaneous maturation was noted when we used an Ab against VCAM as negative control (Fig. 6A, 6B). Because a soluble CD18 integrin ligand (ICAM-1-Fc) was bound with approximately the same efficiency, CD18 integrins on *Swap-70*^{-/-} BMDCs do not exist in a hyperactivated conformation when compared with wt cells (Fig. 6C). Thus, SWAP-70 does not restrict the acquisition of an active conformation by β₂.

Table II. Retroviral vectors used for transduction of BMDCs in this study

| Name | Protein |
|-----------------------------|--|
| pGfus-SWAP70-GFP | SWAP-70 GFP fusion |
| pGfus-SWAP-70- Δ527–585-GFP | SWAP-70 aa 1–526 C-term GFP fusion |
| pGfus-SWAP-70- Δ353–585-GFP | SWAP-70 aa 1–352 C-term GFP fusion |
| pGfus-SWAP-70- Δ197–585-GFP | SWAP-70 aa 1–196 C-term GFP fusion |
| pGfus-SWAP-70- Δ1–198-GFP | SWAP-70 aa 199–585 C-term GFP fusion |
| pGfus-SWAP-70- Δ220–225-GFP | SWAP-70 aa 220–225 deleted C-term GFP fusion |
| pGfus-SWAP-70- Δ229–234-GFP | SWAP-70 aa 229–234 deleted C-term GFP fusion |
| pGfus-SWAP-70-Y517F-GFP | SWAP-70 Y517F GFP fusion |

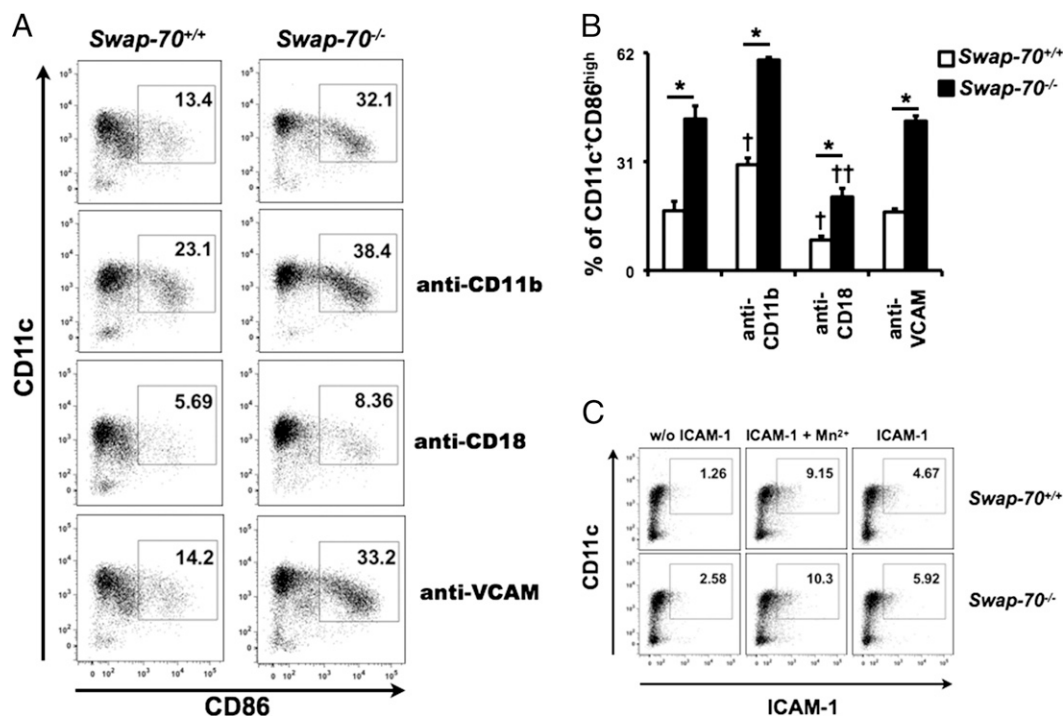


FIGURE 6. Distinct effects of CD11b and CD18 activation on spontaneous maturation of Swap-70^{-/-} BMDCs. **(A)** Quantification of percentage of CD11c⁺CD86^{hi} BMDCs. Swap-70^{+/+} and Swap-70^{-/-} BMDCs were incubated with 1 μ g/ml of the indicated Ab from day 4 of culture onward. BMDCs were costained with CD11c and CD86 and analyzed by FACS at day 11. **(B)** Mean values with error bars indicating \pm SD are shown. * $p < 0.05$ by t test, †significantly different from untreated Swap-70^{+/+} ($p < 0.05$ by t test), ‡significantly different from untreated Swap-70^{-/-} ($p < 0.05$ by t test). **(C)** Swap-70^{+/+} and Swap-70^{-/-} BMDCs were incubated with soluble ICAM-1 Fc-chimeric molecules at day 10 in the absence or presence of Mn²⁺ (positive control). Cells were then fixed, and bound ICAM-1 was detected with anti-Fc fragment Abs by FACS.

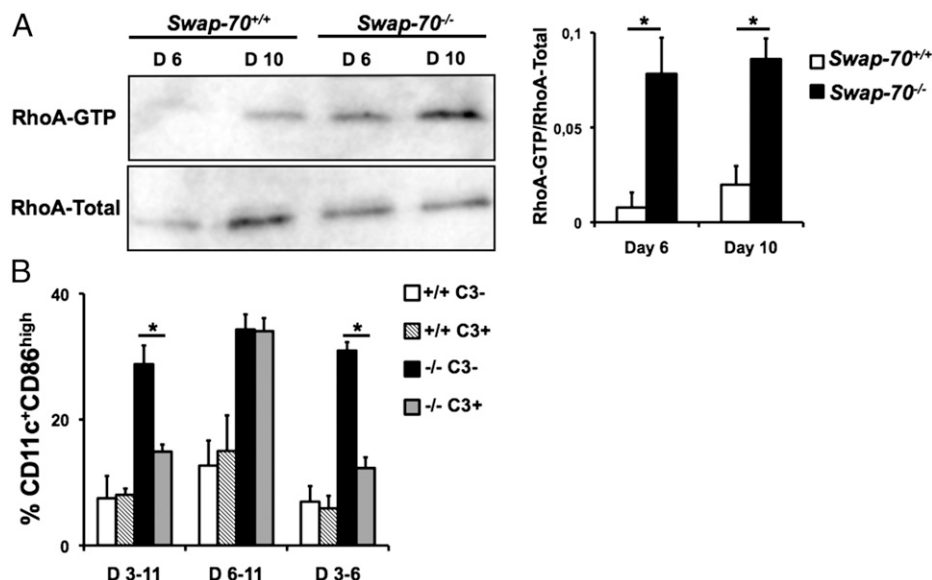
These results underscore the importance of the CD11b integrin in regulation of spontaneous maturation and suggest that SWAP-70 restricts CD11b to acquire an active conformation. Thus, SWAP-70 limits outside-in signaling of this integrin.

Early inhibition of activated RhoA in Swap-70^{-/-} BMDCs suppresses spontaneous maturation

Important DC functions like upregulation of MHCII after a TLR stimulus or the chemotactic response to S1P are impaired in Swap-70^{-/-} DCs (12, 13). Abnormal constitutive activation of RhoA

even in nonstimulated Swap-70^{-/-} DCs is responsible for these diminished functions, because inhibition of RhoA activation through the exoenzyme C3 ADP-ribosyltransferase restores the capacity of Swap-70^{-/-} DCs to upregulate MHCII molecules after TLR activation and to migrate toward S1P (12, 13). SWAP-70 interacts with RhoA in BMDC lysates, and through this interaction it might control the activation state of RhoA (13). We therefore investigated whether RhoA activation continues to be upregulated in Swap-70^{-/-} BMDCs undergoing spontaneous hypermaturation. Fig. 7A shows that RhoA activation is upregu-

FIGURE 7. Inhibition of activated RhoA in SWAP-70^{-/-} BMDCs suppresses spontaneous activation. **(A)** Activation of RhoA in Swap-70^{+/+} and Swap-70^{-/-} BMDCs at days 6 and 10 was analyzed using rhotekin-Rho binding domain agarose beads. Beads were then subjected to SDS-PAGE and Western blotting (left panel). Activity of RhoA was quantified by densitometry analysis and expressed as the ratio of activated to total RhoA (right panel). **(B)** Percentage of spontaneously matured BMDCs. Swap-70^{+/+} (+/+) and Swap-70^{-/-} (-/-) BMDCs were cultivated (C3⁺) or not (C3⁻) with 0.1 μ g/ml of exoenzyme C3 ADP-ribosyltransferase (C3) from days 3 to 11 (D 3–11), from days 6 to 11 (D 6–11), or from days 3 to 6 (D 3–6). Mean values of at least two independent experiments, with error bars indicating \pm SD, are shown. * $p < 0.05$ by t test.



lated in *Swap-70*^{-/-} BMDCs at day 6 and day 10 when compared with wt cells. This also confirms the previous observation that RhoA is constitutively active in *Swap-70*^{-/-} BMDCs in day 6 cultures (12, 13). The modest upregulation of RhoA activation in wt BMDCs at day 10 compared with day 6 may be explained by the increase of cell–cell contacts owing to greater numbers of cells in the culture, which would trigger signaling pathways leading to mild activation of RhoA.

To test whether constitutively active RhoA plays a role at the onset of spontaneous maturation observed in *Swap-70*^{-/-} BMDCs, we added the exoenzyme C3 ADP-ribosyltransferase at the low concentration of 0.1 μg/ml to the BMDCs at day 3 or 6 of the cultures. The exoenzyme was maintained in the cultures until day 11. This treatment reduced the spontaneous maturation of *Swap-70*^{-/-} BMDCs only when C3 was added at day 3, but did not reduce maturation if added starting at day 6 (Fig. 7B). There was no significant effect on spontaneous maturation of wt. The addition of C3 restricted to the time interval between days 3 and 6 was sufficient to reduce the spontaneous maturation of *Swap-70*^{-/-} BMDCs (Fig. 7B). C3-treated cells showed characteristics of RhoA inactivation like loss of stress fibers and increase of dendritic extensions (40) (Supplemental Fig. 4). These findings indicate that the failure to control RhoA activation in *Swap-70*^{-/-} BMDCs at an early stage leads to high levels of spontaneous maturation later in BMDC cultures.

Constitutively activated RhoA in *Swap-70*^{-/-} BMDCs significantly affects integrin-mediated spontaneous maturation

In the absence of SWAP-70, RhoA is hyperactive and activity of CD11b is not properly restricted. In human peripheral mononuclear cells and neutrophils, activation of CD11b or CD18 through Abs

causes RhoA activation (41, 42). We therefore asked if treatment of BMDCs with the mAbs against CD11b and CD18 used in this study, as described above, also leads to RhoA activation. Fig. 8A shows significant increase in RhoA activation in wt BMDCs when they are treated with anti-CD11b or anti-CD18. This increase in active RhoA correlates with the increase in spontaneous maturation in the case of anti-CD11b treatment, but not with the decrease in spontaneous maturation in the case of anti-CD18 treatment. In *Swap-70*^{-/-} BMDCs, anti-CD11b treatment, but not anti-CD18 treatment, significantly reduced the levels of active RhoA (Fig. 8A). This finding is in contrast to the unchanged activation status of anti-CD11b-treated *Swap-70*^{-/-} BMDCs as well as to the much reduced activation status of anti-CD18-treated *Swap-70*^{-/-} BMDCs (Fig. 6A, 6B). Thus, no obvious correlation is seen between anti-integrin-driven spontaneous maturation and RhoA activation, although, as shown above, the early phase of spontaneous maturation requires active RhoA.

Would the increase of active RhoA in wt cells be responsible for the CD11b integrin-mediated increase in spontaneous maturation in wt cells? To answer this, we tested the effect of the exoenzyme C3 in integrin-mediated spontaneous maturation. C3 treatment of anti-CD11b-incubated wt BMDCs did not abolish anti-CD11b-mediated induction of spontaneous maturation (Fig. 8B). C3 treatment of wt cells that were not treated with anti-integrin Ab did not decrease their low level of spontaneous maturation, but anti-CD18 does (Fig. 8B). Adding C3 and anti-CD18 to the cells yielded the same very low levels of spontaneous maturation as treatment with anti-CD18 alone. Thus, the regulation of spontaneous maturation through anti-CD11b or anti-CD18 does not depend on active RhoA. The increase of active RhoA by anti-CD11b or anti-CD18 treatment, as shown in Fig. 8A, is a consequence of

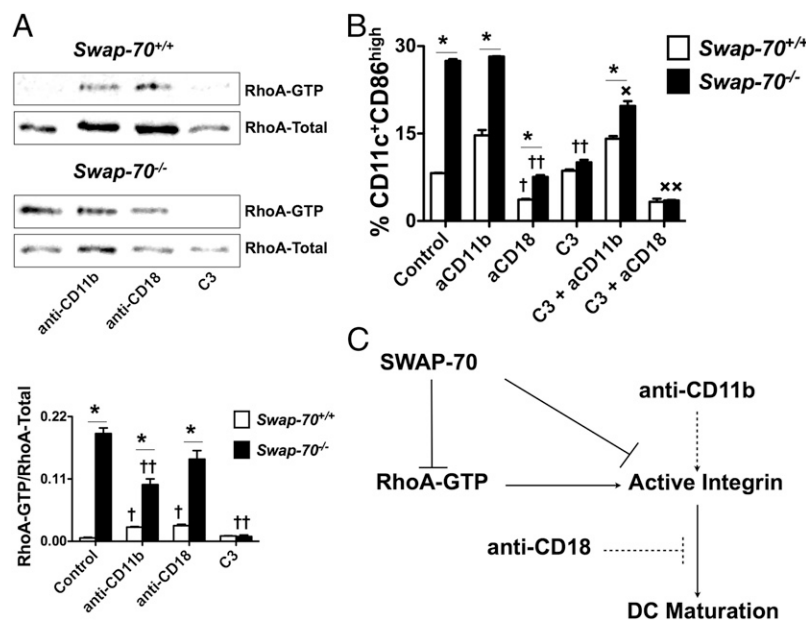


FIGURE 8. Constitutively activated RhoA in *Swap-70*^{-/-} BMDCs significantly affects integrin-mediated spontaneous maturation. **(A)** Activation of RhoA in *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs at day 8 in the presence or not of anti-CD11b, anti-CD18 (both 1 μg/ml), or exoenzyme C3 ADP-ribosyltransferase (C3) (0.1 μg/ml) from day 3 was analyzed as in Fig. 6A. Mean values of at least two independent experiments, with error bars indicating ± SD, are shown. **p* < 0.05 by *t* test. †Significantly different from untreated *Swap-70*^{+/+} (*p* < 0.05 by *t* test). ††Significantly different from untreated *Swap-70*^{-/-} (*p* < 0.05 by *t* test). **(B)** Percentage of spontaneously matured BMDCs. *Swap-70*^{+/+} and *Swap-70*^{-/-} BMDCs were cultivated with 0.1 μg/ml of exoenzyme C3 ADP-ribosyltransferase (C3) from day 3, and from day 4 anti-CD11b or anti-CD18 (1 μg/ml) was added and left until day 10 for CD11c⁺CD86^{hi} analysis by FACS. Mean values of at least two independent experiments, with error bars indicating ± SD, are shown. **p* < 0.05 by *t* test. †Significantly different from untreated *Swap-70*^{+/+} (*p* < 0.05 by *t* test). ††Significantly different from untreated *Swap-70*^{-/-} (*p* < 0.05 by *t* test). *Significantly different from anti-CD11b-treated *Swap-70*^{-/-} (*p* < 0.05 by *t* test). **Significantly different from anti-CD18-treated *Swap-70*^{-/-} (*p* < 0.05 by *t* test). **(C)** Model depicting how spontaneous maturation of DCs is regulated by SWAP-70. SWAP-70 inhibits spontaneous maturation triggered by signaling through RhoA and CD11b integrin. The effects of anti-CD11b and anti-CD18 are depicted with dotted lines.

outside-in signaling triggered by both Abs, but is not required for spontaneous maturation.

In contrast, in *Swap-70*^{-/-} BMDCs the early (day 3 to day 6) preactive state of RhoA is required for spontaneous maturation, as shown in Fig. 7B, but anti-CD11b treatment can alleviate the inhibitory effect of C3 and enhances spontaneous maturation of *Swap-70*^{-/-} BMDCs (Fig. 8B). The combined inhibitory effect of C3 and anti-CD18 is needed in *Swap-70*^{-/-} BMDCs—but not in wt, as described above—to reduce spontaneous maturation to the same low levels seen in anti-CD18-inhibited wt cells (Fig. 8B). Together, these findings suggest that integrin-mediated spontaneous maturation of wt BMDCs does not depend on active RhoA, whereas the increase in spontaneous maturation of *Swap-70*^{-/-} BMDCs is supported by integrin CD11b and RhoA, thus revealing two pathways contributing to spontaneous maturation of BMDCs.

Discussion

Although the functional pathways leading to inflammatory maturation of DCs triggered by TLR ligands, microbial products, inflammatory cytokines, or T cell surface proteins have been extensively investigated and described (for a review, see Ref. 43), the functional and regulatory mechanisms governing distinct pathways of DC maturation are incompletely understood (44, 45). We describe SWAP-70 as a novel factor restricting spontaneous maturation of DCs. *Swap-70*^{-/-} DCs spontaneously hypermature when compared with wt DCs, as determined by the increased surface expression of costimulatory and migratory molecules. Spontaneous maturation in *Swap-70*^{-/-} DCs depends on cell–cell interactions and in BMDC cultures phenotypically appears from day 8 onward, although the initiating events occur earlier, about day 3, as our RhoA inactivation experiments showed.

Immunization with DCs spontaneously matured by cluster disruption leads to production of tolerogenic IL-10 by activated CD4⁺ T cells, not of inflammatory IFN- γ (7, 9, 11). Investigators have proposed that this maturation mechanism is responsible for induction of peripheral tolerance in vivo (7, 9). In the immunization protocol used in this study, we detected higher levels of tolerogenic IL-10 than of IFN- γ induced by *Swap-70*^{-/-} BMDCs, an observation in agreement with their decreased production of inflammatory cytokines after TLR stimulus. In our previous study, we have demonstrated that *Swap-70*^{-/-} BMDCs without LPS stimulus were less potent in stimulating CD4⁺ T cells in vitro compared with wt BMDCs (13). Although this observation was associated with failure to upregulate MHCII molecules, it can also be partly due to the tolerogenic response induced by the high level of spontaneous maturation in *Swap-70*^{-/-} DCs. In vivo, steady-state intestinal DCs induce a tolerogenic state through the stimulation of regulatory T cell induction (28). The frequency of regulatory T cells in the spleen and lymph nodes in *Swap-70*^{-/-} mice does not differ from that in wt animals (data not shown). Spontaneous maturation mediated by cluster disruption of DCs is a potent stimulus for an efficient cross-presentation of Ags necessary to stimulate CD8⁺ T cells (7, 25). The physiological importance of this function is still unclear, but it has been observed that targeting steady-state DEC-205⁺ DCs in vivo without further DC activation is sufficient to induce CD8⁺ T cell tolerance, possibly owing to the disappearance of Ag-specific T cells (46). DCs thus targeted were able to cross-present Ags in the absence of inflammatory cytokines (46). This activity resembles the processes observed during spontaneous maturation of DCs. Steady-state *Swap-70*^{-/-} BMDCs excel in their capacity to cross-present and activate CD8⁺ T cells in vitro. Further studies will be needed to address the physiological significance of SWAP-70 in the induction of CD8⁺ T cell tolerance in vivo.

Disruption of E-cadherin interactions between DCs triggers activation of the β -catenin pathway, leading to spontaneous maturation in vitro (7, 9, 11, 25). However, spontaneous maturation can also be initiated in the absence of cell–cell interactions or through other adhesion molecules such as integrins (9). Although we found that cell–cell interaction plays a role in the spontaneous maturation of *Swap-70*^{-/-} BMDCs, we excluded a significant role of activation of the E-cadherin/ β -catenin pathway. Use of blocking Abs against E-cadherin in these cultures had no effect on spontaneous maturation (not shown), and the presence of β -catenin in the nucleus of *Swap-70*^{-/-} BMDCs is no different from that of wt cells. Furthermore, TGF- β inhibits spontaneous maturation induced by β -catenin signaling (9). Thus, addition of TGF- β to *Swap-70*^{-/-} DCs would be expected to result in low levels of spontaneous maturation, similar to those in wt. However, *Swap-70*^{-/-} SDCs generated in the presence of TGF- β still showed significantly increased spontaneous maturation when compared with wt, further arguing against a role for β -catenin signaling. This finding strongly suggested an alternative pathway of spontaneous maturation of DCs, independent of β -catenin. A β -catenin-independent pathway was also observed in experiments using isolated spleen-resident DCs, in which no difference was seen in spontaneous maturation between DCs lacking expression of β -catenin and their wt counterparts (10). In those experiments, spontaneous maturation was induced during the isolation of DCs from the spleen, and it was dependent on DC–DC contact and a yet undefined soluble factor (10). In our studies, although the spontaneous maturation was dependent on cell–cell contact, we did not observe that soluble factors secreted by *Swap-70*^{-/-} BMDCs had an effect on the maturation state of wt or *Swap-70*^{-/-} BMDCs.

Expression of GFP-tagged wt or mutant SWAP-70 allowed us to gain insights into the mechanisms involved in spontaneous maturation of BMDCs. The PH domain of SWAP-70, specifically the loop between the β 1 and β 2 strands, proved to be essential in the control of spontaneous maturation of BMDCs. The β 2 strand in the pleckstrin domain of SWAP-70 necessary for PIP₃ binding and membrane localization (34, 35) was not required, suggesting that binding to PIP₃ and membrane localization are not essential in the control of spontaneous maturation by SWAP-70. This idea is consistent with the observed localization of mutant SWAP-70 protein: a mutant that still complements localizes not to the membrane but to the nucleus or its periphery. There are three nuclear localization signals in SWAP-70 sequence—two “pat4” (HRRK; KKKK) in the PH domain and one “bipartite” signal (KKLEEAASRAAEEKKR) at position 360–376 (33). The loop between the β 1 and β 2 strands of the PH domain of SWAP-70 contains one nuclear localization signal (33). Although SWAP-70 has been detected in nuclear fractions of BMDCs (C. Ocaña-Morgner and R. Jessberger, unpublished observations), it is not yet known whether SWAP-70 has a function in the nucleus of DCs. Thus a possible new regulatory role for SWAP-70 is suggested for future study.

In DCs, SWAP-70 controls upregulation of MHCII and migration to S1P (12, 13). The mechanism of control of these functions involves negative regulation of RhoA activation by SWAP-70 (12, 13). Nonstimulated *Swap-70*^{-/-} BMDCs show significantly increased active RhoA at days 6 and 10 of culture. Our previous studies show interaction of SWAP-70 and active RhoA, suggesting control of RhoA activation by binding to SWAP-70 (13). The kinetics and the mechanism responsible for the precocious activation of RhoA in *Swap-70*^{-/-} BMDCs are not yet fully understood and will need further research, but from our previous studies it appears as if SWAP-70, dependent on the

maturation status of cells, on the cell type, and on the specific Rho GTPase, may either positively or negatively control the activation status of Rho GTPases. As we show in this article, in *Swap-70*^{-/-} BMDCs, inhibition of active RhoA with the exoenzyme C3 transferase significantly decreased spontaneous maturation of *Swap-70*^{-/-} BMDCs. The rather low level of matured wt cells was not affected by C3 treatment. In *Swap-70*^{-/-} BMDCs, RhoA needs to be inhibited early in these cultures, that is, from day 3 onward. Later inhibition is not effective. Differentiation of DCs from bone marrow progenitors in GM-CSF cultures starts between days 2 and 4 after bone marrow isolation (47). Thus, RhoA-dependent signaling at the initiation of BMDC differentiation is controlled by SWAP-70. This important relation between active RhoA and maturation of BMDCs is revealed in *Swap-70*^{-/-} BMDCs.

Spontaneous maturation of *Swap-70*^{-/-} BMDCs is mediated by cell–cell interaction and not by secreted factors. Activation of CD11b with Abs (mAb M1/70) indicated in an earlier study that activation of this integrin chain, α_M , causes spontaneous maturation in BMDCs (9). The activation effect of mAb M1/70 was dependent on β -catenin expression (9). We confirmed the stimulatory property of mAb M1/70 on wt BMDCs, but *Swap-70*^{-/-} BMDCs could not be significantly further stimulated. They appear to carry this integrin in its activated conformation already, indicating that SWAP-70 restricts integrin-mediated signaling, albeit through a β -catenin-independent mechanism. This idea is in agreement with regulation of integrin signaling by SWAP-70 in other cells, such as B cells, mast cells, and myeloid–erythroid precursors (24, 48, 49). In addition, we demonstrated that, contrary to CD11b, activation of CD18 integrin (β_2) with mAb M18/2 inhibits spontaneous maturation. Both wt and *Swap-70*^{-/-} BMDCs are inhibited to about the same extent, suggesting that there is no specific effect of SWAP-70 on β_2 integrin-mediated inhibition of spontaneous maturation in BMDCs. In a different setting, it has been shown that SWAP-70 regulates CD18 in S1P-triggered transendothelial migration of activated DCs (12). This finding indicates that in distinct biological processes and activation states of cells, SWAP-70 regulates different integrins in different ways. The difference in the outcome of targeting the CD11b or CD18 integrins can be attributed to the distinct functional effects of each mAb. It was previously reported that mAb M18/2 against CD18 significantly increased binding of macrophages to C3b-sensitized erythrocytes and C3bi-coated beads, whereas anti-CD11b (mAb M1/70) had the opposite effect (39, 50). Consistent with these observations and the effects on spontaneous maturation induced by anti-CD11b or anti-CD18, the activation of integrin CD11b/CD18 has been shown to be involved in the maintenance of tolerance and the control of inflammation by DCs (51–53).

The experiments on RhoA activation and on the effects of integrins, and the data on combined treatment of BMDCs with C3 and/or anti-integrin Ab, revealed that integrin-mediated spontaneous maturation of wt BMDCs does not depend on active RhoA in the initial stages of BMDC development. In contrast, the increase in spontaneous maturation of *Swap-70*^{-/-} BMDCs is supported by integrin CD11b and by early-phase activation of RhoA. The *Swap-70* mutant therefore reveals two pathways that contribute to spontaneous maturation of BMDCs. SWAP-70 itself controls both RhoA activation and integrin activity (Fig. 8C).

The above results may suggest a hypothetical mechanism based on cell–cell interaction, through integrins, that leads to spontaneous maturation. Activation of the Src family kinases through integrins induces Syk-mediated activation of Rho GTPases (54). It has been shown that phosphorylation of SWAP-70 by Syk at position 517 regulates B cell migration (37); thus Syk may also have

a role in SWAP-70-controlled spontaneous maturation of BMDCs. However, a mutant of SWAP-70 lacking the tyrosine at position 517 still complements spontaneous maturation of *Swap-70*^{-/-} BMDCs. Therefore, Syk-mediated phosphorylation of SWAP-70 is not required for its role in restricting spontaneous activation of BMDCs.

This study indicates that SWAP-70 is an important molecule in the pathways controlling spontaneous maturation of DCs. SWAP-70 integrates and limits signals from integrins and prevents hyperactivation of RhoA, thereby restricting DC maturation, as schematically depicted in Fig. 8C. The role of RhoA in spontaneous maturation is revealed only in the *Swap-70*^{-/-} background. Comprehending this novel pathway for DC maturation may not only promote understanding of a vital aspect of DC biology, particularly the generation of tolerance, but may also contribute to developing new strategies for DC-based immunotherapies in which manipulation of the activation status of DCs is involved.

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Disclosures

The authors have no financial conflicts of interests.

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