Dendritic cell maturation and chemotaxis is regulated by TRPM2-mediated lysosomal \( \text{Ca}^{2+} \) release

Adriana Sumoza-Toledo,*,†,‡,§ Ingo Lange,*† Hanna Cortado,†,§ Harivadan Bhagat,†,§ Yasuo Mori,† Andrea Fleig,*† Reinhold Penner,*†,1 and Santiago Partida-Sánchez‡,§,1

*Laboratory of Cell and Molecular Signaling, Center for Biomedical Research, Queen's Medical Center, Honolulu, Hawaii, USA; †John A. Burns School of Medicine, University of Hawaii; Honolulu, Hawaii, USA; ‡Center for Microbial Pathogenesis, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, USA; §The Ohio State University College of Medicine, Columbus, Ohio, USA; and †Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Kyoto, Japan

ABSTRACT  Chemokines induce calcium (\( \text{Ca}^{2+} \)) signaling and chemotaxis in dendritic cells (DCs), but the molecular players involved in shaping intracellular \( \text{Ca}^{2+} \) changes remain to be characterized. Using siRNA and knockout mice, we show that in addition to inositol 1,4,5-trisphosphate (IP₃)-mediated \( \text{Ca}^{2+} \) release and store-operated \( \text{Ca}^{2+} \) entry (SOCE), the transient receptor potential melastatin 2 (TRPM2) channel contributes to \( \text{Ca}^{2+} \) release but not \( \text{Ca}^{2+} \) influx in mouse DCs. Consistent with these findings, TRPM2 expression in DCs is restricted to endolysosomal vesicles, whereas in neutrophils, the channel localizes to the plasma membrane. TRPM2-deficient DCs show impaired maturation and severely compromised chemokine-activated directional migration as well as bacterial-induced DC trafficking to the draining lymph nodes. Defective DC chemotaxis is due to perturbed chemokine-receptor-initiated \( \text{Ca}^{2+} \) signaling mechanisms, which include suppression of TRPM2-mediated \( \text{Ca}^{2+} \) release and secondary modification of SOCE. DCs deficient in both TRPM2 and IP₃ receptor signaling lose their ability to perform chemotaxis entirely. These results highlight TRPM2 as a key player regulating DC chemotaxis through its function as \( \text{Ca}^{2+} \) release channel and confirm ADP-ribose as a novel second messenger for intracellular \( \text{Ca}^{2+} \) mobilization.—Sumoza-Toledo, A., Lange, I., Cortado, H., Bhagat, H., Mori, Y., Fleig, A., Penner, R., Partida-Sánchez, S. Dendritic cell maturation and chemotaxis is regulated by TRPM2-mediated lysosomal \( \text{Ca}^{2+} \) release. FASEBJ. 25, 3529–3542 (2011). www.fasebj.org

Key Words: ADP-ribose · calcium signaling · IP₃R · SOCE

Migration of dendritic cells (DCs) and other phagocytic cells to sites of infection and inflammation is a critical step toward an effective defense against pathogens (1–3). Phagocytes migrate throughout the body by following chemical cues from small molecules that are produced either endogenously (chemokines) or exogenously [microbially derived chemoattractants; e.g., N-formylmethionyl-leucyl-phenylalanine (fMLP)]. DCs sense chemotactic signals via G-protein-coupled receptors (GPCRs) expressed on their plasma membrane (1, 4, 5). Although chemokine receptor stimulation elicits increases in intracellular \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]ᵣ) in DCs (6–9), the ion channels that regulate the \( \text{Ca}^{2+} \) signals associated with chemokine-dependent migration of DCs remain unidentified.

The transient receptor potential melastatin-2 (TRPM2) is a calcium-permeable nonselective cation channel (10, 11) containing a Nudix-like region that binds and hydrolyzes ADP-ribose (ADPR) to ribose 5-phosphate and adenosine monophosphate (AMP) (10). ADPR binding to the Nudix-like domain induces cation currents across the plasma membrane, allowing Na⁺ and \( \text{Ca}^{2+} \) influx (10, 11). TRPM2 gating by ADPR is facilitated further by the presence of nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic ADPR (cADPR), hydrogen peroxide (H₂O₂), and \( \text{Ca}^{2+} \) (12–16), whereas channel activity is regulated negatively by AMP and permeating protons (pH; refs. 10, 17, 18). In addition to its role as a cation channel in the plasma membrane, TRPM2 functions as a lysosomal calcium-release channel in the rat pancreatic cell line INS-1 (19).

TRPM2 is expressed in the plasma membrane of human and mouse polymorphonuclear neutrophils (PMNs), monocytes (19–22), and Jurkat T cells (23). A recent study indicates that TRPM2 represents a key inflammatory mediator in cells of myeloid origin and that TRPM2-deficient mice are more resistant to induced experimental colitis due to defective chemokine production by monocytes and reduction of PMN infil-
tration (21). In addition, earlier studies have shown defects in chemotaxis of phagocytes treated with the ADPR antagonist 8Br-ADPR due to a reduction of [Ca\(^{2+}\)]; on chemotactic stimuli (9), which suggests that ADPR regulates Ca\(^{2+}\) signaling following chemokine receptor stimulation. The relevance of ADPR/TRPM2 signaling pathway for DC biology has not been addressed.

In the present study, we investigated the role of TRPM2 channel during Ca\(^{2+}\) homeostasis and function of DCs. Our results show that TRPM2 function regulates [Ca\(^{2+}\)]; and is required for optimal DC maturation and chemotaxis. DCs express TRPM2 preferentially in endolysosomal compartments, which release Ca\(^{2+}\) on intracellular ADPR or external chemokine stimulation. Depletion of TRPM2 in DCs reduces chemokine-induced release of Ca\(^{2+}\) and secondarily reduces Ca\(^{2+}\) entry through store-operated pathways. Consequently, TRPM2-deficient DCs exhibit impaired chemotaxis in response to chemokines and the absence of both TRPM2 and inositol 1,4,5-trisphosphate receptor (IP\(_3\)) abolishes chemotaxis altogether. Our data reveal a crucial role of TRPM2 in the regulation of Ca\(^{2+}\) signaling of DCs and provide additional support for the emerging concept of TRPM2 as a potential therapeutic target for inflammatory diseases.

MATERIALS AND METHODS

Animals

C57BL/6 wild-type (WT) mice and EGFP-C57BL/6 [C57BL/6-Tg (ACTB-EGFP) 131Osh/LeySop]] mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice deficient in TRPM2, C57BL/6J.129 trpm2\(^{2-/} \), were generated as described previously (21). EGFP-trpm2\(^{2-/} \) mice were produced by crossing EGFP-C57BL/6 mice and C57BL/6J.129 trpm2\(^{2-/} \) mice. All mice used in this study were bred and maintained under specific pathogen-free conditions at the animal facilities of The Research Institute at Nationwide Children’s Hospital (Columbus, OH, USA) and the University of Hawaii (Honolulu, HI, USA). Mice were 8–12 wk old at the time of the experiments. All protocols involving rodents have been reviewed and approved by the institutional animal care and use committees at the Research Institute at Nationwide Children’s Hospital and the University of Hawaii.

Reagents and antibodies

CXCL12, CCL19, and mGM-CSF were acquired from R&D Systems (Minneapolis, MN, USA). The anti-mouse CD11c biotin, CD11c allophycocyanin (APC), CD11b eFluor 605, Gr-1 biotin, CD16/32, CXCR4 phycoerythrin (PE), CCR7 PE, CCR5, CD80 PE, CD86 PE, MHC II PE, and CD83 purified rat anti-mouse I-A/I-E antibodies, were purchased from eBioscience (San Diego, CA, USA). Monoclonal mouse anti-RAb5 (clone Rab5–65), mouse anti-RAb7 (clone Rab7–117) antibodies, ADPR, AMP, bafilomycin A, and concanamycin A were from Sigma (St. Louis, MO, USA). EEA1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-human TRPM2 was acquired from Bethyl Laboratories (Montgomery, TX, USA), and the rabbit anti-mouse TRPM2 was produced in our laboratory as described previously (24). Mouse monoclonal antibody (mAb) to lysosome-associated membrane protein 1 (LAMP; Ly1c6), mouse mAb to 58K Golgi protein, and mouse antibody anti-PDI protein were purchased from Abcam (Boston, MA, USA). Alexa Fluor 488-goat anti-rabbit IgG, Alexa Fluor 568-goat anti-mouse IgG, Alexa Fluor 568-goat anti-rat IgG, and MitoTracker red were purchased from Invitrogen (Carlsbad, CA, USA). DC-LAMP antibody was from Beckman Coulter (Fullerton, CA, USA). 8-Br-ADPR was a generous gift from Tim Walseth (University of Minnesota, Minneapolis, MN, USA).

Phagocytic cell isolation

Mouse bone marrow (BM) was prepared from femurs and tibiae. PMNs were purified by positive selection using biotinylated anti-Gr-1 antibody and MACS streptavidin microbeads (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer instructions. PMN purity was ≥95% as assessed by flow cytometry. BM-derived immature DCs (BMDCs) were generated in vitro by culture of 0.5 × 10\(^6\) BM cells/ml in RPMI medium (ATCC, Manassas, VA, USA) supplemented with 7% FBS (v/v; Hyclone, South Logan, UT, USA), 50 μM 2-β-macaptoethanol (Sigma-Aldrich), 10 μg/ml penicillin/streptomycin, and 20 ng/ml mGM-CSF for 5–6 d. BMDCs were matured by stimulating the culture with TNF-α (10 ng/ml; Invitrogen) overnight at d 5. Cells were used for experiments 24 h later. Where indicated, CD11c\(^{+}\) DCs were selected by magnetic sorting using biotinylated anti-CD11c antibody (eBioescence) and MACS streptavidin microbeads (Miltenyi Biotec). BMDC purity was ≥95% as assessed by flow cytometry. Splenic DCs were obtained by pretreatment of spleen tissues with collagenase D (1 mg/ml) before mechanical disaggregation.

Immunofluorescence

For TRPM2 staining, BMDCs and PMNs were attached to coverslips, pretreated with poly-L-lysine, for 20 min at room temperature. Cells were then fixed with 2% paraformaldehyde for 15 min at room temperature. Samples were permeabilized with 0.2% Triton X-100 for 5 min. Cells were then rinsed with PBS and blocked with 10% goat serum or 2% BSA for 30 min at 37°C. For TRPM2 detection, cells were incubated with rabbit preimmune serum as a negative control, or rabbit anti-mouse TRPM2 serum, or TRPM2 immune serum plus blocking peptide or polyclonal rabbit anti-human TRPM2 antibody, for 1 h at 37°C. Alexa Fluor 488 anti-rabbit IgG was used as secondary antibody. For detection of subcellular organelles, TRPM2-stained cells were further incubated with mouse anti-LAMP1, mouse anti-58K Golgi protein, mouse anti-PDI protein, mouse anti-human DC-LAMP, purified rat anti-mouse I-A/I-E, mouse anti-RAb5 or anti-RAb7, or the mitochondrion-selective dye MitoTracker red. Alexa Fluor 568 anti-mouse IgG was used as secondary antibody. Samples were mounted using Prolong Gold antifade reagent (Invitrogen). Samples were analyzed using the Zeiss 510 LSM Meta confocal laser scanning microscope and Zeiss LSM Image Browser program (Carl Zeiss, Hudson, OH, USA).

Flow cytometry

BMDCs were cultured in the presence of 20 ng/ml GM-CSF and then split into halves at d 5. Half of the culture was induced to mature by adding 10 ng/ml TNF-α (d 5); the other half was left untouched. On d 6, immature and mature BMDCs were stained with fluorescent anti-CD11c, CD11b, MHC class-II, CD80, CD83, CD86, CXCR4, CCR7, and CCR5

PE and analyzed using an LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA). Splenic DCs were stained with fluorescent anti-CD11c, CD11b, MHC class-II, CD80, CD83, and CD86 PE and analyzed using an LSR-II flow cytometer.

**TRPM2 mRNA detection**

Total RNA was extracted from fresh isolated PMNs, immature DCs (5–6 d of culture and CD11c+ selected) using TRIzol reagent (Invitrogen). cDNA was prepared by reverse transcription using SuperScript III RNase H-Reverse Transcriptase (RT) and oligo (dT). For TRPM2, PCR reactions were performed using the specific primer pair mTRPM2 3380 sense 5‘-CAGATCCCAATCATGACCAG-3’ and mTRPM2 3394, antisense 5‘-GAAGGTGCTGTTGAACATGGCCA-3’. A 215-bp TRPM2-PCR product was detected after 30 cycles of amplification: 30 s at 94°C, annealing for 30 s at 46°C, and extension for 40 s at 72°C, followed by a final extension for 10 min. For GADPH, PCR reactions used the specific primer pair mGADPH 563 sense 5’-ACCACATCTGACCGCCATC-3’ and mGADPH 1014 antisense 5’-TCCACCCGCTTGTGCTGTA-3’. A 451-bp GADPH-PCR product was detected after 30 cycles of amplification: 30 s at 94°C, annealing for 30 s at 53°C, and extension for 40 s at 72°C, followed by a final extension for 10 min. For real-time PCR, total RNA extraction was performed using the Qiagen RNEasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA from 1 μg of RNA was synthesized using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). The CDNA was then subjected to quantitative real-time PCR reaction using a TaqMan qTR-PCR assay with predesigned probe and primers for mouse TRPM2 (Mm00606098_m1), GADPH (Mm9999915_q1) and CD86 (Mm00444543_m1) (Applied Biosystems).

**Chemotaxis assays**

Chemotaxis assays were performed using 24- or 96-transwell plates (Costar, Corning, NY, USA) with 5-μm-pore polycarbonate filter. For standard chemotaxis assay (25), chemotacticants were diluted in HBSS and placed in the lower chamber, whereas 5 × 10^5 immature or mature DCs were added to the upper chamber of the transwell. The checkerboard chemotaxis assay was performed by placing chemoattractants in the lower chamber, whereas chemoattractants were diluted in HBSS and placed in the lower chamber in 5-μm-pore polycarbonate transwell plates (Costar, Corning, NY, USA) with 5-μm-pore polycarbonate filter. For control chemotaxis, cells were incubated in the lower chamber only (chemokinesis), or both (chemokine + chemotaxis), or using predesigned probe and primers for mouse TRPM2 (Mm00606098_m1), GADPH (Mm9999915_q1) and CD86 (Mm00444543_m1) (Applied Biosystems).

**Dendritic cell migration in vivo**

Immature BMDCs (2×10^5/50 μl) cultured for 5 d from EGF-ftrp2^−/− mice or control mice (EGF-F-C57BL/6) were injected in the right footpad of C57BL/6 mice 2 h before injection of *Escherichia coli* (1×10^6 bacteria/footpad). Single-cell suspensions from the right side popliteal and inguinal draining lymph nodes and nondraining lymph nodes (left side controls) were incubated with fluorescent anti-CD11c, and anti-MHC class-II, CD11b and analyzed by flow cytometry.

**TRPM2 depletion by siRNA**

Specific siRNA sequences for TRPM2 (Entrez Gene ID 28240) were selected from the murine HP GenomeWide siRNA oligos available from Qiagen; 3’ Cy5-labeled siRNA oligos were synthesized and annealed by the manufacturer. Scrambled siRNA sequences were used as negative controls. siRNA transfection was carried out using the GeneSilencer siRNA transfection reagent (Gene Therapy Systems, San Diego, CA, USA). BMDCs (5) were washed and plated in 6-well plates at 2.5×10^5 in 1 ml of serum-free RPMI 1640. Annealed siRNA (≤ 20 mM) was incubated with GeneSilencer reagent following the manufacturer’s protocol. Transfection mixture was then added to the DC culture. Mock controls were transfected with GeneSilencer siRNA transfection reagent alone.

**Electrophysiology**

For patch-clamp experiments, cells were kept in standard Ringer’s solution: 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.2 adjusted with NaOH). Standard pipette-filling solutions contained 140 mM Cs-glutamate, 8 mM NaCl, 1 mM MgCl2, and 10 mM HEPES (pH 7.2 adjusted with CsOH). Experiments were performed under Ca^{2+}-buffered or unbuffered conditions (as indicated). ADPR was added to its final concentrations as appropriate. DCs were rinsed once with extracellular solution and transferred to coverslips. Patch-clamp experiments were performed in the whole-cell configuration at 21–25°C. All data were acquired with Pulse software controlling an EPC-9 amplifier (Heka, Lambrecht, Germany) and analyzed using FitMaster (Heka) and Igor Pro (WaveMetrics, Lake Oswego, OR, USA). Voltage ramps of 50 ms spanning the voltage range from −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 200–500 s. Voltages were corrected for liquid junction potentials. Currents were filtered at 2.9 kHz and digitized at 100-μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp. The ramp current amplitudes at −80 mV (inward current) were extracted from individual ramp data and displayed as current development over time. Some individual ramps were displayed as representative current-voltage (I-V) relationships.

**Ca^{2+} measurements**

For Ca^{2+} measurements, cells were loaded with 5 μM Fura-2-AM (acetoxymethylester; Molecular Probes, Eugene, OR, USA) for 30 min in medium at 37°C. Balanced Ca^{2+} experiments were then performed with subsequent patch-clamp experiments where 200 μM Fura-2 (pentapotassium salt; Molecular Probes) was added to the standard internal solution to ensure continuous Fura-2 signals. The cytosolic Ca^{2+} concentration at a rate of 5 Hz with a dual excitation fluorometric system using a Zeiss Axiovert 200 fluorescence microscope equipped with a ×40 LD Achromat objective. The monochromatic light source (monochromator B; TILL Photonics, Lambrecht, Germany) was tuned to excite Fura-2 fluorescence at 360 and 390 nm for 20 ms each. Emission was detected at 450–550 nm with a photomultiplier, whose analog signals were sampled and processed by X-Chart software (Heka). Fluorescence ratios (F_{390}/F_{360}) were translated into free intracellular Ca^{2+} concentration based on calibration parameters derived from patch-clamp experiments with cali-
brated Ca\(^{2+}\) concentrations. For IP\(_3\) experiments, cells were permeabilized with 4 \(\mu\)M digitonin and exposed to 100 \(\mu\)M IP\(_3\).

For Ca\(^{2+}\) measurements in intact cells, Fura-2-AM pre-loaded-CDC11c\(^{+}\) DGs were stimulated with 300 ng/ml CXCL12 (immature DCs) or 75 ng/ml CCL19 (mature DCs) in the presence or absence of 1 mM CaCl\(_2\) at 30 s. To obtain Ca\(^{2+}\)-free conditions, external Ca\(^{2+}\) was washed out with zero Ca\(^{2+}\) external Na\(^{+}\)-Ringer for 60 s prior to the stimulation with the specific chemoattractant. Ca\(^{2+}\) responses were monitored using a CCD camera-based Ca\(^{2+}\) imaging system (TILL Photonics). Ca\(^{2+}\) signal patterns were defined as follows: a Ca\(^{2+}\) spike or Ca\(^{2+}\) transient was defined as a rapidly rising transient increase in \([\text{Ca}^{2+}]_i\) of \(\geq 10\) mM above baseline \([\text{Ca}^{2+}]_i\) in resting conditions; multiple \([\text{Ca}^{2+}]_i\) transients arising periodically from a stable baseline were considered Ca\(^{2+}\) oscillations; and the slower rising wave-like phase of sustained Ca\(^{2+}\) entry was considered a Ca\(^{2+}\) plateau phase. Experiments were analyzed using Igor Pro software; areas under individual Ca\(^{2+}\) transients (integral in \(\mu\)M/s), and the initiation times (s) of the Ca\(^{2+}\) plateau phase were calculated using the multipeak fit analysis function in Igor Pro. Intracellular Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\) was determined following the formula \(f(x) = K_D \left(\frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}\right)\), where \(K_D\) is the \(K_D\) for BAPTA, \(R_{\text{min}}\) is the ratiometric measurement of the internal Ca\(^{2+}\) clamped to 0 (10 mM BAPTA), \(R_{\text{max}}\) is the ratiometric measurement of internal Ca\(^{2+}\) clamped at saturating Ca\(^{2+}\) (1 mM), and \(r\) is the ratiometric measurement of intracellular Ca\(^{2+}\) on chemokine stimulation.

**Ca\(^{2+}\) measurement by flow cytometry**

For Fluo-3 flow cytometry assays, immature BMDCs were resuspended in cell-loading medium (HBSS with 1 mM Ca\(^{2+}\), 1 mM Mg\(^{2+}\), 1% FBS, and 4 mM probenecid) at 5 \(\times\) 10\(^6\) cells/ml and loaded with the calcium-sensitive dye Fluo-3 AM (4 \(\mu\)g/ml; Invitrogen). Cells were stimulated with CXCL12 chemokine, and the accumulation of intracellular free Ca\(^{2+}\) was assessed by FACS over the next 200 s by measuring the fluorescence emission of Fluo-3 in the FL-1 channel. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA) using the kinetic platform.

**Statistical analysis**

Data are expressed as mean \(\pm\) se values. Statistical evaluation was determined by unpaired Student’s t test or 1-way ANOVA test where appropriate by using GraphPad Prism software (GraphPad, San Diego, CA, USA). The algorithms Kolmogorov-Smirnov (K-S) and probability binning \([\chi^2(T)\) or PB\] and FlowJo software were used for statistical comparison of FACS data. A value of \(P < 0.05\) was considered to be statistically significant.

**RESULTS**

**Differential cellular compartmentalization of TRPM2 in phagocytes**

To assess the functionality of TRPM2 channels in dendritic cells, we first examined the expression of TRPM2 transcripts in various tissues and cell types, including PMNs, spleen T cells, and bone marrow-derived DCs (BMDCs) from C57BL/6 mice. Figure 1A shows that TRPM2 mRNA was highly expressed in PMNs and BMDCs when compared to the DC cell line DC 2.4. TRPM2 transcripts were nearly undetectable in T cells and 3T3 fibroblasts (Fig. 1A). Consistent with previous reports (27, 28), TRPM2 was greatly expressed in the brain (Fig. 1A). Since TRPM2 expression and activation is well documented in human PMNs (20, 22, 29), we next examined the cellular distribution of TRPM2 in PMNs and BMDCs by immunofluorescence. We stained PMNs and BMDCs with an anti-human TRPM2 antibody (Fig. 1B) or an anti-mouse TRPM2 antibody (Supplemental Fig. S1), while the nucleus was visualized with either DAPI (Fig. 1B) or propidium iodine (Supplemental Fig. S1). As evident in Fig. 1B, we observed plasma membrane expression of TRPM2 in mouse PMNs, similar to what has been reported for human PMNs (22, 29). Interestingly, TRPM2 exhibited a punctate cytoplasmic distribution in immature BMDCs (Fig. 1B). Moreover, we did not detect any TRPM2 signal in 3T3 cells or when a specific blocking peptide was used together with the anti-human TRPM2 antibody (data not shown) or when we used mouse preimmune serum (Supplemental Fig. S1). A similar subcellular pattern of TRPM2 expression was observed using either anti-human or anti-mouse TRPM2 antibodies.

**ADPR does not induce TRPM2 plasma membrane currents in DCs**

To confirm whether TRPM2 was present in the plasma membrane of PMN but not BMDCs, we assessed TRPM2 channel expression directly using whole-cell patch-clamp recordings. Intracellular perfusion of mouse PMN with 10 \(\mu\)M ADPR elicited a large current that rapidly developed after establishment of the whole cell configuration (Fig. 1D, black circles). The currents induced by ADPR had identical \(I-V\) relationships (Fig. 1E) to those of heterologously expressed TRPM2 channels (10). Figure 1C illustrates the ADPR-TRPM2 dose-response in mouse PMNs, with an EC\(_{50}\) of \(\sim 540\) nM, slightly more sensitive than in PMNs isolated from human blood (EC\(_{50}\) \(\sim 1\) \(\mu\)M; refs. 22, 29). To distinguish TRPM2 currents from leak currents, we replaced extracellular Na\(^+\) with \(\beta\)-methyl-D-glucamine (NMDG\(^+\); an impermeable cation), resulting in complete suppression of inward current (Fig. 1D). Figure 1E (gray trace) shows the TRPM2 \(I-V\) relationship under NMDG\(^+\) conditions. In contrast to PMNs, ADPR at concentrations of up to 1 mM failed to induce any currents in immature BMDCs (Fig. 1D, blue circles) and mature BMDCs (Supplemental Fig. S2A), demonstrating the absence of functional TRPM2 channels in the plasma membrane of BMDCs. Furthermore, the quantitative analysis of TRPM2 mRNA expression in TNF-\(\alpha\)-stimulated-BMDCs shows that TRPM2 is not up- or down-regulated on TNF-\(\alpha\) treatment, whereas up-regulation of the costimulatory molecule CD86 indicates that maturation of BMDCs occurred (Supplemental Fig. S2B).

**ADPR causes Ca\(^{2+}\) release in mouse DCs by gating TRPM2 channels**

Because of the predominant intracellular expression of TRPM2 in BMDCs, we reasoned that it might function...
sufficient to elicit significant changes in intracellular Ca$^{2+}$ signals. Figure 2 shows that 1 mM ADPR failed to induce Ca$^{2+}$ release in BMDCs perfused with 1 mM ADPR in the internal solution (n=5; red circles). WT BMDCs did not develop any TRPM2 currents in response to perfusion with 1 mM ADPR in the internal solution (n=6; blue circles). E) Representative I-V relationships of ADPR-induced currents in WT PMNs (black trace; extracted at 44 s), WT PMNs plus NMDG$^+$ (black trace; extracted at 110 s), TRPM2$^{-/-}$ PMNs (red trace; extracted at 50 s) and WT BMDCs (blue trace; extracted at 100 s).

**Figure 1.** Differential expression and function of TRPM2 in mouse phagocytic cells. A) TRPM2 mRNA detection in DC, 2.4 cells, BMDCs, PMNs, 3T3 cells, brain cells, and T cells by RT-PCR. Amplification without retrotranscriptase was performed to exclude DNA contamination. GADPH housekeeping gene was used as control. B) Cellular localization of TRPM2 in PMNs and BMDCs. TRPM2 localization was assessed using rabbit anti-human TRPM2 antibody and Alexa Fluor 488-anti-rabbit IgG as secondary antibody. Images are representative of >3 independent experiments. C) Dose-response curve of TRPM2 currents in response to increasing concentrations of intracellular ADPR in mouse PMNs. Cells were kept in standard external solution supplemented with 1 mM Ca$^{2+}$. Cells were perfused with standard Cs-glutamate-based internal solution supplemented with ADPR as indicated and in the absence of Ca$^{2+}$ buffers (unbuffered conditions). Data were acquired using a 50-ms voltage ramp from −100 to +100 mV given at 0.5 Hz. Current amplitudes were extracted at −80 mV, normalized to cell size (in pF), averaged, and plotted vs. the respective ADPR concentration (n=5–6 for each data point). The half-maximal excitatory concentration (EC$_{50}$ 540 nM) and Hill coefficient (Hill 2) was estimated using a dose-response fit. Error bars = sem. D) Average TRPM2 currents in WT PMNs with 10 µM ADPR added to the internal solution (n=11; black circles). NMDG$^+$ was applied to distinguish TRPM2 currents from leak (black bar). In PMNs isolated from TRPM2$^{-/-}$ mice, no currents developed with 1 mM ADPR (n=10; red circles). WT BMDCs did not develop any TRPM2 currents in response to perfusion with 1 mM ADPR in the internal solution (n=6; blue circles). E) Representative I-V relationships of ADPR-induced currents in WT PMNs (black trace; extracted at 44 s), WT PMNs plus NMDG$^+$ (black trace; extracted at 110 s), TRPM2$^{-/-}$ PMNs (red trace; extracted at 50 s) and WT BMDCs (blue trace; extracted at 100 s).

488-anti-rabbit IgG as secondary antibody. Images are representative of >3 independent experiments. C) Dose-response curve of TRPM2 currents in response to increasing concentrations of intracellular ADPR in mouse PMNs. Cells were kept in standard external solution supplemented with 1 mM Ca$^{2+}$. Cells were perfused with standard Cs-glutamate-based internal solution supplemented with ADPR as indicated and in the absence of Ca$^{2+}$ buffers (unbuffered conditions). Data were acquired using a 50-ms voltage ramp from −100 to +100 mV given at 0.5 Hz. Current amplitudes were extracted at −80 mV, normalized to cell size (in pF), averaged, and plotted vs. the respective ADPR concentration (n=5–6 for each data point). The half-maximal excitatory concentration (EC$_{50}$ 540 nM) and Hill coefficient (Hill 2) was estimated using a dose-response fit. Error bars = sem. D) Average TRPM2 currents in WT PMNs with 10 µM ADPR added to the internal solution (n=11; black circles). NMDG$^+$ was applied to distinguish TRPM2 currents from leak (black bar). In PMNs isolated from TRPM2$^{-/-}$ mice, no currents developed with 1 mM ADPR (n=10; red circles). WT BMDCs did not develop any TRPM2 currents in response to perfusion with 1 mM ADPR in the internal solution (n=6; blue circles). E) Representative I-V relationships of ADPR-induced currents in WT PMNs (black trace; extracted at 44 s), WT PMNs plus NMDG$^+$ (black trace; extracted at 110 s), TRPM2$^{-/-}$ PMNs (red trace; extracted at 50 s) and WT BMDCs (blue trace; extracted at 100 s).

as a Ca$^{2+}$ release channel. To test this hypothesis, we performed balanced Fura-2 experiments, measuring changes in intracellular Ca$^{2+}$ in immature BMDCs preloaded with Fura-2-AM. Cells were then perfused intracellularly with 0.1–1000 µM ADPR in the presence of 200 µM Fura-2. Na$^+$-based solution without Ca$^{2+}$ was applied externally before whole-cell break-in to avoid contribution of Ca$^{2+}$ influx to the measured Ca$^{2+}$ signals. Figure 2A shows that 1 µM of ADPR was sufficient to elicit significant [Ca$^{2+}$]$_i$ increases in BMDCs (blue line). The Ca$^{2+}$ elevation was abrogated when BMDCs were perfused with ADPR along with 8-Br-ADPR (Fig. 2B, black line) or 100 µM AMP (Fig. 2B, blue line), which are both known TRPM2 channel inhibitors. Similar results were obtained in mature BMDCs perfused with 10 µM ADPR (n=5, Fig. S2C). These responses were mediated by TRPM2, since even 1 mM ADPR failed to induce Ca$^{2+}$ release in BMDCs generated from TRPM2$^{-/-}$ mice (Fig. 2B, red line). We also investigated whether TRPM2 might function as a Ca$^{2+}$ release channel in PMN isolated from BM of WT mice. PMNs were loaded with Fura-2-AM and subsequently perfused with standard internal solution supplemented with 100 µM ADPR and 200 µM Fura-2. Figure 2C shows that ADPR did not elicit measurable intracellular Ca$^{2+}$ increase in mouse PMNs. These results identify TRPM2 exclusively as a Ca$^{2+}$ release channel in DCs but not in PMNs.

**DCs express TRPM2 in late endosomal and lysosomal compartments**

To determine the TRPM2 localization within BMDCs, we performed costaining of TRPM2 and subcellular organelles. Confocal microscopy analysis revealed well-defined intracellular structures containing TRPM2 (Fig. 3). TRPM2 did not significantly colocalize with structures expressing the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI; Fig. 3A, top panels[b]) or the Golgi apparatus expressing the 58K Golgi protein (Fig. 3A, middle panels), or with the mitochondrial marker MitoTracker (Fig. 3A, bottom panels[b]). Instead, TRPM2 showed a punctate cyto-
Chemokines produce increases of \([\text{Ca}^{2+}]_i\) in DCs (9, 33); however, the calcium channels that control \([\text{Ca}^{2+}]_i\) signals associated with the activation of chemokine receptors are not well known in these cells. To determine whether TRPM2-mediated \([\text{Ca}^{2+}]_i\) release is activated during chemokine receptor signaling, we examined \([\text{Ca}^{2+}]_i\) responses following chemokine stimulation (CXCL12 and CCL19; CXCR4 and CCR7 ligands in immature and mature DCs, respectively) in intact CD11c\(^+\) immature and mature WT and TRPM2\(^{-/-}\) BMDCs. We examined \([\text{Ca}^{2+}]_i\) patterns defined as \([\text{Ca}^{2+}]_i\) spikes (single \([\text{Ca}^{2+}]_i\) transients), \([\text{Ca}^{2+}]_i\) oscillations (multiple \([\text{Ca}^{2+}]_i\) transients) and plateau phases (sustained, wave-like \([\text{Ca}^{2+}]_i\) increases) as described in Materials and Methods. The integral of \([\text{Ca}^{2+}]_i\) spikes and beginning of the plateau phases were determined using the multi-peak fit analysis function in Igor Pro software.

In the presence of 1 mM extracellular \([\text{Ca}^{2+}]_i\) (Fig. 4A-E), 54% of WT BMDCs (30/56 cells) responded with an average of 2.5 \pm 0.4 \([\text{Ca}^{2+}]_i\) oscillations within the first 300 s after stimulation with CXCL12 (n=56). This condition was followed by a secondary \([\text{Ca}^{2+}]_i\) plateau phase at 327 \pm 27 s after stimulation (Fig. 4B, black circles). Of the cells, only 46% (26/56 cells) developed the \([\text{Ca}^{2+}]_i\) plateau phase at 311 \pm 31 s without initial oscillations. Examples of these two types of \([\text{Ca}^{2+}]_i\) responses of WT BMDC stimulated with CXCL12 are shown in Fig. 4A. In contrast, the majority of TRPM2\(^{-/-}\) BMDCs (81% or 63/78 cells) showed no initial oscillations, and the remainder of cells had strongly reduced numbers of \([\text{Ca}^{2+}]_i\) oscillations (1.5 \pm 0.2 oscillations; Fig. 4A, red trace; Fig. 4B inset, red circles; P<0.05). This condition was also evident from analysis of the \([\text{Ca}^{2+}]_i\) integral of \([\text{Ca}^{2+}]_i\) oscillations (Fig. 4C; P<0.05) as well as during the rising phase of the \([\text{Ca}^{2+}]_i\) plateau (P<0.05). The \([\text{Ca}^{2+}]_i\) plateau phase began at a similar time point as in WT BMDCs (313 \pm 35 s in cells with oscillation, n=15; and 300 \pm 15 s in cells with no initial oscillation, n=63) but reached slightly lower levels of \([\text{Ca}^{2+}]_i\) (P<0.05; Fig. 4B).

Mature WT BMDCs rarely showed oscillations of intracellular \([\text{Ca}^{2+}]_i\) in response to CCL19 (Fig. 4D, E), and the \([\text{Ca}^{2+}]_i\) plateau phase developed with a delay of 651 \pm 23 s (n=30) after stimulation (Fig. 4E, black circles; F, black bar). Mature TRPM2\(^{-/-}\) BMDCs responded slightly earlier (Fig. 4F, red bar) to chemokine stimulation (486 \pm 39 s; n=11; P<0.01); however, the \([\text{Ca}^{2+}]_i\) signal tapered off faster than in WT and to a lower overall level within the measured time frame (Fig. 4E, red circles). Thus, although TRPM2 itself does not directly contribute to \([\text{Ca}^{2+}]_i\) entry as a plasma membrane

Figure 2. TRPM2 functions as \([\text{Ca}^{2+}]_i\) release channel in mouse DCs but not PMNs. BMDCs and PMNs isolated from WT and TRPM2\(^{-/-}\) mice were loaded with 5 \(\mu\text{M}\) Fura-2-AM. Calcium mobilization was analyzed using the whole-cell patch-clamp configuration where the standard internal solution was supplemented with various concentrations of agonist/antagonist and 200 \(\mu\text{M}\) Fura-2. Red arrow indicates whole-cell break-in. Just before break-in, the standard extracellular solution was switched from 1 mM \([\text{Ca}^{2+}]_i\) to 0 \([\text{Ca}^{2+}]_i\) to isolate release events. A) Average \([\text{Ca}^{2+}]_i\) release in BMDCs induced by perfusion of cells with increasing concentrations of ADPR (n=6–8). B) Average inhibition of ADPR-induced (10 \(\mu\text{M}\)) \([\text{Ca}^{2+}]_i\) release in WT BMDCs by 100 \(\mu\text{M}\) 8-Br-ADPR (black trace, n=10) and 100 \(\mu\text{M}\) AMP (blue trace, n=11). Absence of ADPR-induced (1 \(\mu\text{M}\)) \([\text{Ca}^{2+}]_i\) release in BMDCs isolated from TRPM2\(^{-/-}\) mice (red trace, n=7). C) Absence of ADPR-induced (100 \(\mu\text{M}\)) \([\text{Ca}^{2+}]_i\) release in PMNs isolated from WT mice (C57BL/6; n=4).
channel, its absence does appear to affect Ca\(^{2+}\) influx through chemokine signaling in DCs.

**TRPM2 deficiency differentially affects Ca\(^{2+}\) release depending on DC maturity**

Since TRPM2 acts as a Ca\(^{2+}\) release channel in DCs (Fig. 2), we examined Ca\(^{2+}\) signaling in the absence of external Ca\(^{2+}\) (Fig. 5). Two patterns of Ca\(^{2+}\) signals were observed in Ca\(^{2+}\)-free extracellular solution: cells responded with either a Ca\(^{2+}\) spike or with Ca\(^{2+}\) oscillations. These patterns were seen in both immature BMDCs stimulated with CXCL12 and mature BMDCs stimulated with CCL19. The relative amount of cells responding with a Ca\(^{2+}\) spike (~25% of immature cells and ~10% of mature BMDCs) or Ca\(^{2+}\) oscillations was comparable in WT and TRPM2\(^{-/-}\) cells. As expected, the secondary phase never developed in the absence of extracellular Ca\(^{2+}\), indicating that it was mainly carried by Ca\(^{2+}\) influx. No difference was observed in the delay of Ca\(^{2+}\) oscillations evoked by CXCL12 in WT and TRPM2\(^{-/-}\) BMDCs (~200 s; Supplemental Fig. S3A). However, immature TRPM2\(^{-/-}\) BMDCs that responded with a Ca\(^{2+}\) spike to a CXCL12 challenge had significantly smaller Ca\(^{2+}\) spikes compared to WT (Fig. 5A), as assessed by the integral of individual Ca\(^{2+}\) spikes (Fig. 5B, \(P<0.05\)). The inset in Fig. 5A represents a typical release transient measured in a WT cell over the course of 300 s of experimental time.

In contrast to immature cells, the amplitudes and delays of Ca\(^{2+}\) spikes in mature BMDCs stimulated with CCL19 were identical in WT and TRPM2\(^{-/-}\) BMDCs (Fig. S3C). Mature cells with Ca\(^{2+}\) oscillations also had indistinguishable initial responses to the chemokine...
except for the first transient, Ca\textsuperscript{2+} amplitude, resulting in an overall suppressed Ca\textsuperscript{2+} TRPM2-mediated Ca\textsuperscript{2+} observed in WT (9.5 ± 0.6 transients) did not differ. However, for the first transient, Ca\textsuperscript{2+} oscillations measured in TRPM2\textsuperscript{−/−} cells were strongly reduced in amplitude, resulting in an overall suppressed Ca\textsuperscript{2+} signal compared to WT (Fig. 5C). This was also evident in the integral of all Ca\textsuperscript{2+} oscillations between 0 and 500 s (including the first transient) in individual WT and TRPM2-deficient cells (Fig. 5D). The inset in Fig. 5C presents a typical release transient measured in a WT cell. Similar to experiments performed with CCL19 in the presence of extracellular Ca\textsuperscript{2+} (Fig. 4E), mature BMDCs responding to chemokine stimulation with Ca\textsuperscript{2+} oscillations developed Ca\textsuperscript{2+} transients earlier in TRPM2\textsuperscript{−/−} BMDCs than in WT BMDCs (223 ± 16 s in WT vs. 174 ± 15 s in TRPM2\textsuperscript{−/−} cells; \(P < 0.02\)).

TRPM2 deficiency affects Ca\textsuperscript{2+} release in DCs, we next examined whether TRPM2-dependent Ca\textsuperscript{2+} signals are required during DC differentiation, maturation and migration \textit{in vitro} as well as \textit{in vivo}. BMDCs were prepared from WT and TRPM2\textsuperscript{−/−} mice and the expression of DC lineage (CD11c\textsuperscript{+}) and maturation markers (MHC-II, CD80, CD86, and CD83) were analyzed by flow cytometry. Both BMDC cultures showed no significant difference in the proportion of CD11c\textsuperscript{+} cells at d 6 (Fig. 6A), and cell proliferation rates were also similar (Fig. 6B). However, when TNF-\alpha or CpG DNA (TLR9 activator) were used as maturation stimulus, a smaller fraction of CD11c\textsuperscript{+} TRPM2\textsuperscript{−/−} BMDCs up-regulated Class-II, CD86, CD80, and CD83 molecules (Fig. 6C, red line) compared to TRPM2\textsuperscript{+/+} BMDCs (Fig. 6C, black line). Furthermore, we quantitatively evaluated differences in the expression of each activation marker by applying the \(\chi^2\) (T) or PB algorithm to determine whether two histograms are statistically significantly different (ref. 35 and Supplemental Table S1). The analysis revealed that stimulated TRPM2\textsuperscript{−/−} BMDCs not only failed to reach optimal levels of maturation molecules but also showed that a greater
TRPM2 controls DC migration and homing to lymph nodes on inflammation

Previous work has shown that TRPM2−/− mice exhibit impaired PMN infiltration during experimentally induced colitis (21). Therefore, we investigated whether TRPM2-mediated Ca2+ release may also play a role in DC chemotaxis and trafficking on chemokine or inflammatory stimuli. We isolated BMDCs from WT and TRPM2−/− mice and initially performed standard transwell chemotaxis assays using CXCL12 and CCL19 chemokines as stimuli. First, we examined whether TRPM2 deficiency affected chemokine-induced random migration in a chemotaxis assay by checkerboard analysis (25). We did not observe differences between immature WT and TRPM2−/− DCs in random migration toward CXCL12, as BMDCs were placed on the top chamber and the chemoattractant on both sides of the transwell membrane (Fig. 7A). Next, we analyzed chemokine-induced directional migration by placing the chemokine only on the bottom chamber of the transwell (26). Figure 7B shows that both immature and mature TRPM2−/− BMDCs exhibited a significantly reduced chemotactic response to CXCL12 and CCL19, respectively.

To rule out the possibility that genetic TRPM2 depletion could result in disruption of additional signaling pathways that might affect DC chemotaxis, we performed TRPM2 protein depletion by siRNA methodology. We depleted TRPM2 channels by treating WT CD11c+ BMDCs with a specific TRPM2 siRNA. At 48 h after transfection of siRNA, TRPM2 transcripts or proteins were undetectable by standard RT-PCR and immunofluorescence (Fig. 7C). CD11c+ BMDCs treated with scrambled siRNA showed ability to migrate directionally in response to CXCL12 and CCL19, whereas TRPM2-siRNA-treated WT CD11c+ cells failed to migrate toward CXCL12 or CCL19, respectively (Fig. 7D). We confirmed that TRPM2 knockdown did not affect the plasma membrane expression of chemokine receptors in immature BMDCs, since flow cytometry revealed

Figure 5. TRPM2 deficiency differentially affects Ca2+ release depending on DC maturity. BMDCs were isolated and treated as described in Fig. 4. Cells were maintained in standard external solution containing 1 mM Ca2+. Extracellular Ca2+ was removed 1 min before the addition of chemokines (300 ng/ml of CXCL12 or 75 ng/ml of CCL19). Error bars = sem. Peak of the first Ca2+ transient measured in each cell was aligned to the earliest peak recorded in each group (immature and mature BMDCs). No difference was found in response time to chemokine between WT and TRPM2−/− immature BMDCs (~200 s). Ca2+ spikes appeared earlier in TRPM2−/− (~170 s) than in WT (~200 s) mature BMDCs. A) Average Ca2+ release of immature WT (black circles; n = 51) and TRPM2−/− BMDCs (red circles; n = 81) responding to CCL19 stimulation (black bar). Inset: data trace from an example WT cell. Arrow indicates chemokine application. B) Averaged Ca2+ integral of the Ca2+ spike in WT BMDCs (black bar, n = 51) and TRPM2−/− BMDCs (red bar, n = 81) taken from the data in A. C) Average Ca2+ responses of mature WT (black circles; n = 96) and TRPM2−/− (red circles; n = 96) BMDCs with Ca2+ oscillations induced by CCL19 stimulation (black bar). Inset: representative Ca2+ oscillation pattern measured in a WT cell. D) Averaged Ca2+ integral of Ca2+ oscillations measured in individual WT BMDCs (black bar, n = 916 spikes) and TRPM2−/− BMDCs (red bar, n = 798 spikes) taken from the data in C. A Ca2+ transient was defined as a peak if it showed an increase and decrease in Ca2+ of ≥10 nM following a normal Gaussian distribution. Graphs represent means of 3 independent experiments. Traces were lined up to the cell with lowest basal calcium.
similar levels of CXCR4, CCR5, and CCR7 in WT and TRPM2-knockout cells (Supplemental Fig. S4A). However, TRPM2-deficient DCs did not up-regulate the chemokine receptors CCR5, CXCR4, and CCR7 on TNF-a stimulation (Supplemental Fig. S4A). Because TRPM2-/- BMDCs showed impaired chemotaxis in vitro, we reasoned that the maturation and antigen-induced trafficking of immature DCs from the periphery to the secondary lymphoid organs could be also affected in the TRPM2-/- mice. To test this hypothesis, immature BMDCs (2x10^6/50 µl) from EGFP-TRPM2-/- mice or control mice (EGFP-C57BL/6) were injected into the right footpad of C57BL/6 mice 2 h before injection of E. coli (1x10^8 bacteria/footpad) to induce maturation and migration (33). Migratory DCs into the draining lymph nodes and nondraining lymph nodes were then analyzed by flow cytometry. Similarly to the in vitro experiments, the migration of EGFP-TRPM2-/- BMDCs to the lymph node was significantly impaired at 18 h after subcutaneous injection (Fig. 7E).

**IP3 receptors and lysosomal TRPM2 regulate Ca^{2+} signaling and chemotaxis in DCs**

While defective Ca^{2+} responses to chemokines were apparent in individual cells of the TRPM2-/- BMDC populations, the average [Ca^{2+}]_i in response to CXCL12 was also found to be significantly reduced in
large TRPM2−/− BMDC populations in the absence of external Ca2+ or when WT BMDCs were treated with bafilomycin A (Fig. 8A) in 1 mM external Ca2+. These results are depicted as the mean fluorescence intensity of Flu-3 on chemokine stimulation (Fig. 8). Treatment of WT DCs with bafilomycin A or concanamycin A (37, 38) significantly reduced CXCL12-induced chemotaxis (Fig. 8B), suggesting that lysosomal TRPM2-mediated Ca2+ release is important for DC chemotaxis.

Although the absence of TRPM2 clearly affected the Ca2+ responses of DCs, TRPM2−/− BMDCs can mobilize a sizeable amount of [Ca2+]i, likely through IP3-mediated signaling downstream of chemokine receptors (39). To confirm that IP3 also induces Ca2+ release from DC intracellular stores (39, 40), we permeabilized WT BMDCs using a 4 μM digitonin-containing external solution and coapplied 100 μM IP3. Supplemental Fig. S3E shows that IP3 indeed evokes Ca2+ release in DCs. To determine the relative contribution of TRPM2 and IP3, we analyzed Ca2+ increases in WT and TRPM2−/− BMDCs. Ablation of TRPM2 reduced the CXCL12-induced Ca2+ transient by ~50% (Fig. 8C). [Ca2+]i was suppressed further in TRPM2−/− BMDCs by 10 μM of the IP3R inhibitor xestospongin C (XeC; refs. 41, 42). Additional XeC treatment of TRPM2−/− BMDCs suppressed CXCL12-induced chemotaxis (Fig. 8D) completely, indicating that both IP3R and TRPM2 participate in chemokine-induced Ca2+ signaling and migration of DCs.

**DISCUSSION**

Migration of DCs from site of infections to lymphoid organs is central for the immune response. DC directional movement (chemotaxis) is initiated on activation of chemokine receptors in the plasma membrane and is regulated by the cytosolic Ca2+-concentration ([Ca2+]i) (39–42). However, little is known about the proteins, including calcium channels that regulate Ca2+ signals during DC chemotaxis. We here demonstrate the functional expression of the ADP-activated Ca2+-permeable channel TRPM2 in mouse BMDCs. Our data show that TRPM2 expresses preferentially in lysosomes of immature and mature BMDCs but not in the plasma membrane, as confirmed by immunoﬂuorescence, patch-clamp, and Ca2+ imaging experiments. In addition, our ﬁndings show that TRPM2 proteins are not accumulated within the cellular trafﬁcking system, since TRPM2 staining is not observed in ER, Golgi, or mitochondria. Instead, TRPM2 staining overlaps with that of LAMP1 in immature DCs or DGLAMP in mature DCs and may play a role in MHC class-II processing and antigen presentation by DCs.

Our studies demonstrate that TRPM2 represents a...
membrane and in intracellular structures of mono-
dicate localization of TRPM2 both at the plasma
ingly, although immunofluorescent imaging data in-
organelles and the plasma membrane (19). Interest-
store-operated Ca\(^{2+}\) function as Ca\(^{2+}\)
pancreatic
PMNs is restricted to the plasma membrane. Rat
TRPM2 expression in human and mouse
expression of TRPM2 is not an exclusive property of
channel, as other members of the TRP family
expression of chemotaxis in both mature and immature BMDCs (9) and suggested a TRPM2-mediated
reliant on TRPM2-dependent Ca\(^{2+}\) release channels that are localized in lysosomes when exposed in HEK293 cells (47).
Optimal DC maturation in vitro and in vivo was reliant on TRPM2-dependent Ca\(^{2+}\) signaling, as demonstrated by reduced surface expression levels of co-
stimulatory molecules CD80, CD86, MHC class-II, and CD83 (Fig. 4). A suboptimal mature population of DCs, CD11b\(^{+}\)CD11c\(^{int}\), was also found in the spleen of TRPM2\(^{-/-}\) mice, and this phenotype might predict additional DC-related functional defects in these mice, as DC maturation is critical for effective generation of immune responses. The molecular mechanism by which TRPM2 controls DC maturation remains unclear, although our results point to a regulatory role of Cd\(^{2+}\) signaling, in agreement with previous studies that showed a requirement for Ca\(^{2+}\) during DC maturation (6, 7, 48).

Chemotaxis of leukocytes is known to operate via chemokine receptors in the plasma membrane. These are GPCRs that activate phospholipase C (PLC), which subsequently produces IP\(_3\) and diacylglycerol (DAG). IP\(_3\) releases Ca\(^{2+}\) from the ER, leading to store depletion and a rise in [Ca\(^{2+}\)\(_i\)] (1, 4, 5). Our results show that responses to chemokine receptors CXCR4 and CCR7 are impaired in TRPM2\(^{-/-}\) BMDCs, and this correlates with diminished intracellular Ca\(^{2+}\) release in these cells. Interestingly, while siRNA treatment or genetic deletion of TRPM2 leads to a comparable suppression of chemotaxis in both mature and immature BMDCs in vitro, and defective migration of mature DCs in vivo, the
underlying intracellular Ca\(^{2+}\) signaling events exhibit some complexity. Immature TRPM2\(^{−/−}\) BMDCs display strongly suppressed initial oscillatory activity and slightly suppressed secondary Ca\(^{2+}\) plateaus in the presence of external Ca\(^{2+}\). Mature BMDCs, however, react faster to chemokine stimulation but then taper off in their Ca\(^{2+}\) response compared to WT. Removal of external Ca\(^{2+}\) reveals that only cells responding with one individual Ca\(^{2+}\)-release transient display suppressed Ca\(^{2+}\) release in immature TRPM2\(^{−/−}\) BMDCs, while cells with Ca\(^{2+}\) oscillations are unaffected. In contrast, mature TRPM2\(^{−/−}\) BMDCs show no difference in the Ca\(^{2+}\)-spike responders but produce fewer and reduced oscillatory events subsequent to the initial transient. These differential Ca\(^{2+}\) responses may reflect the change in chemokine receptor expression DGs undergo during the maturation process. Furthermore, the additional use of the IP\(_3\)R inhibitor XeC (43) reveals that suppression of both TRPM2 and IP\(_3\)R-mediated Ca\(^{2+}\) release abolishes chemokine-induced rises in [Ca\(^{2+}\)]\(_i\), and chemotaxis. This finding indicates that TRPM2 and IP\(_3\)R are both critical for Ca\(^{2+}\) release in mouse DCs.

A recent study demonstrated impaired chemotaxis of SYT7-deficient neutrophils and splenocytes, a Ca\(^{2+}\)-dependent regulator of lysosomal fusion (49). It also showed that the small GTPases Rab27a and Rab3a, proteins known to regulate lysosome exocytosis, play a role in chemotaxis. These findings identify a molecular pathway required for chemotaxis that links chemoattractant-induced Ca\(^{2+}\) signals to lysosomal-dependent exocytosis and uropod release, in good agreement with our observations that Ca\(^{2+}\)-dependent lysosomal function is essential for cell migration of DCs.

Since Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (CRACs) are present in mouse DCs (34, 50), it is likely that SOCE plays a significant role in the chemoattractant-Ca\(^{2+}\) entry response of DCs. Indeed, we detected TRPM2-dependent Ca\(^{2+}\) entry in TRPM2\(^{−/−}\) DCs in the presence of external Ca\(^{2+}\) that followed the initial Ca\(^{2+}\) release transient, which could be explained by the activation of CRAC channels. One of the most intriguing observations of the present report was that TRPM2, although absent from the plasma membrane of DCs, still affected Ca\(^{2+}\) entry. Knockdown of TRPM2 actually accelerated the kinetics of Ca\(^{2+}\) entry, suggesting that TRPM2-mediated Ca\(^{2+}\) release can delay the activation of Ca\(^{2+}\) entry. Possible explanations for this phenomenon could be that lysosomal Ca\(^{2+}\) release occurring concomitantly with IP\(_3\)R-induced release of Ca\(^{2+}\) from the ER causes Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels, and/or the lysosomal Ca\(^{2+}\) release is taken up by the ER, resulting in delayed activation of SOCE. Finally, another TRP channel from the melastatin family member, TRPM4, may further affect Ca\(^{2+}\) entry in DCs through regulation of the driving force for Ca\(^{2+}\) entry (33). In summary, our findings demonstrate a novel role of TRPM2 in the regulation of Ca\(^{2+}\) signaling of DGs, and suggest that inhibitors of TRPM2 function could potentially be used to block undesired DC-mediated responses.

The authors thank S. Johne for technical support. This work was supported by the Research Institute at Nationwide Children’s Hospital (S.P.-S. and A.S.-T.), U.S. National Institutes of Health grants GM063954 (R.P.) and GM078195 (A.F.), U.S. National Institute of Allergy and Infectious Diseases grant R01AI092117 (S.P.-S.), and The Queen Emma Research Foundation (A.S.-T.). The John A. Burns School of Medicine (JABSOM) Histology and Imaging Core Facility is supported by both Research Centers in Minority Institutions and Centers of Biomedical Research Excellence grants (G12-RR03061 and P20-RR016453). The JABSOM Molecular and Cellular Immunology Core facility is supported by COBRE grant 2P20RR018727-06. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflicting financial interests.

REFERENCES

transient receptor potential channel 2 (LTRPC2) by hydrogen peroxide. A splice variant reveals a mode of activation independent of ADP-ribose. J. Biol. Chem. 277, 23150–23156

Received for publication December 28, 2010. Accepted for publication July 1, 2011.