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Chemotaxis of Mouse Bone Marrow Neutrophils and Dendritic Cells Is Controlled by ADP-Ribose, the Major Product Generated by the CD38 Enzyme Reaction

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The ectoenzyme CD38 catalyzes the production of cyclic ADP-ribose (cADPR) and ADP-ribose (ADPR) from its substrate, NAD+.

Both products of the CD38 enzyme reaction play important roles in signal transduction, as cADPR regulates calcium release from intracellular stores and ADPR controls cation entry through the plasma membrane channel TRPM2. We previously demonstrated that CD38 and the cADPR generated by CD38 regulate calcium signaling in leukocytes stimulated with some, but not all, chemokines and controls leukocyte migration to inflammatory sites. However, it is not known whether the other CD38 product, ADPR, also regulates leukocyte trafficking. In this study we characterize 8-bromo (8Br)-ADPR, a novel compound that specifically inhibits ADPR-activated cation influx without affecting other key calcium release and entry pathways. Using 8Br-ADPR, we demonstrate that ADPR controls calcium influx and chemotaxis in mouse neutrophils and dendritic cells activated through chemokine receptors that rely on CD38 and cADPR for activity, including mouse FPR1, CXCR4, and CCR7. Furthermore, we show that the calcium and chemotactic responses of leukocytes are not dependent on poly-ADP-ribose polymerase 1 (PARP-1), another potential source of ADPR in some leukocytes. Finally, we demonstrate that NAD+ analogues specifically block calcium influx and migration of chemokine-stimulated neutrophils without affecting PARP-1-dependent calcium responses. Collectively, these data identify ADPR as a new and important second messenger of mouse neutrophil and dendritic cell migration, suggest that CD38, rather than PARP-1, may be an important source of ADPR in these cells, and indicate that inhibitors of ADPR-gated calcium entry, such as 8Br-ADPR, have the potential to be used as anti-inflammatory agents.

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Migration of leukocytes to infected tissues and secondary lymphoid organs is critically important for effective immune responses to most pathogens, yet uncontrolled migration can lead to tissue damage and chronic inflammation. Given the central role that cell trafficking plays in infectious and autoimmune disease, it is important to identify the molecular signals that control this process. It is now clear that a number of phospholipid kinases and phosphatases play important roles in chemokine receptor signal transduction and cell migration, however a role for Ca2+ second messengers in leukocyte migration is less apparent. Indeed, leukocytes deficient in various phospholipase C isozymes, the enzymes responsible for generating the Ca2+-mobilizing second messenger d-myo-inositol 1,4,5-trisphosphate (IP3), are competent to migrate in response to a number of chemokines. However, experiments using intracellular or extracellular Ca2+-chelating agents demonstrate that Ca2+-based signals are necessary for leukocyte migration in response to some, but not all, chemoattractants.

Although IP3 is the best-characterized Ca2+-mobilizing second messenger, two additional Ca2+-mobilizing metabolites, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP+), have been identified. cADPR induces Ca2+ release from intracellular Ca2+ stores by the activation of ryanodine receptors (RyRs; Refs. 7 and 8). Similarly, NAADP+ has been reported to activate RyRs in some cell systems (9–14); however, evidence for a novel NAADP receptor has also been obtained (15–19). Importantly, both of these adenine-based metabolites can be formed upon extracellular stimulation of different cell types (20–23), suggesting that they can act as Ca2+-mobilizing second messengers, similar to IP3.

Although IP3 is produced by the various phospholipase C isozymes, the ectoenzyme CD38 generates cADPR and ADP-ribose (ADPR) from NAD (6). In addition, CD38 can also produce
NAADP⁺, although only under acidic conditions (pH 4 to 5; see Ref. 6). ADPR binds to the TRPM2 cation channel and facilitates Ca²⁺ and Na⁺ influx through this channel (24–26). To address whether any of these less well characterized Ca²⁺-mobilizing metabolites regulate leukocyte migration, we analyzed cell trafficking in CD38-deficient (Cd38⁻/⁻) mice and found that the migration of neutrophils, monocytes, and dendritic cells (DCs) was impaired and that these mice mount poor innate and adaptive immune responses (27–29). We also showed that neutrophil, monocyte, and DC migration to ligands for several chemokine and chemotactant receptors, including CCR1, CCR2, CCR5, CXCR7, CXCR4, N-formyl peptide receptor (FPR)1, and FPR2, is CD38 dependent and that Ca²⁺ signaling triggered by this same subset of chemokine receptors is also dependent on CD38 (27, 28). Finally, we demonstrated that pretreatment of normal mouse and human leukocytes with a cADPR antagonist blocked the Ca²⁺- and chemotactic responses of cells to several different chemotactants and chemokines (27, 28, 30).

Although our data indicated that cADPR, produced by CD38, regulates Ca²⁺ signaling and chemotactant responses in chemokine and chemotactant receptor-stimulated leukocytes, our experiments did not address the possibility that the other two Ca²⁺-mobilizing second messengers produced by CD38 may also be involved in this process. In fact, the most striking defect in the chemokine/chemoattractant-treated Cd38⁻/⁻ cells was a reduction in Ca²⁺ influx from the extracellular space (27, 28). Because one of the metabolites produced by CD38, ADPR, can activate Ca²⁺ influx through TRPM2 cation channels (24–26), we thought it possible that ADPR produced by either CD38 or other NAD⁺-catabolizing enzymes like poly-ADP-ribose polymerase 1 (PARP-1) (31, 32) might also be involved in regulating leukocyte responses to certain chemokines. Unfortunately, this hypothesis could not be experimentally validated because no specific TRPM2 antagonists have been identified and TRPM2-deficient mice are not yet available.

Therefore, we decided to take a different approach and designed and tested analogues of ADPR to identify compounds that can specifically block ADPR-gated cation entry without inhibiting Ca²⁺ influx through store-operated channels (SOCs) or hindering intracellular Ca²⁺ release mediated by IP₃, cADPR, or NAADP⁺. In this article we have used one such analog, 8-bromo (8Br)-ADPR, to test whether the ADPR antagonist could be used to block chemokine/chemoattractant receptor signal transduction and chemotaxis in primary mouse and human leukocyte populations. We demonstrate that the ADPR antagonist blocks Ca²⁺ influx in leukocytes activated through chemokine receptors that also require CD38 and cADPR for activity. We further show that whereas PARP-1 plays a critical role in regulating oxidant-induced Ca²⁺ responses in mouse neutrophils, PARP-1 is not required for chemotactant-induced Ca²⁺ signaling or chemotaxis in these cells. In addition, we demonstrate that NAD⁺ analogues can be used to selectively block chemotaxis of mouse neutrophils without affecting PARP-1-dependent responses. Thus, the data strongly suggest that CD38 regulates chemokine/chemoattractant receptor signaling by producing both ADPR and cADPR. In addition, we show for the first time that nucleotide-based compounds that interfere with the Ca²⁺-mobilizing activity of ADPR are very effective at blocking human and mouse leukocyte migration and will likely be useful as anti-inflammatory agents.

Materials and Methods

Cell lines and mice
C57BL/6d (B6 mice), Cd38⁻/⁻ (N-B6.129P2-Cd38 tm1Lnd mice back-crossed 12 generations to B6; Ref. 29), and Parp1⁻/⁻ mice (Ref. 33; obtained from D. Chen at the Lawrence Livermore National Laboratory (Livermore, CA) and subsequently backcrossed 10 generations to B6) were bred and maintained at the Trudeau Institute Animal Breeding Facility (Saranac Lake, NY) in accordance with Trudeau Institute Institutional Animal Care and Use Committee guidelines. Jurkat T lymphocyte cells (clone JUMP) were cultured and maintained as previously described (22).

Reagents

CXCL12 and CCL21 were acquired from R&D Systems. β-NAD⁺ was obtained from Roche Applied Science, trifluoroacetic acid (TFA) was from Pierce Biochemicals, and AG MP-1 resin was from Bio-Rad. Human recombinant CD38 was a gift from Drs. H.C. Lee and R. Graeff (Department of Pharmacology, University of Minnesota, Minneapolis, MN). ADPR, IL-8, MFML, H₂O₂, thapsigargin, ETGA, liquid Br₂, tri-o-ctylamine, 1,1,2-trichlorotrifuoroethane, and Aplysia ADP-ribosyl cyclase were all obtained from Sigma-Aldrich. All reagents were used at the concentrations indicated.

PCR analysis

PCR were performed using TRPM2-specific primers (5′-TGGCTTTTGTGACATCCGTTTTTC-3′ and 5′-GATGGACCCACACCTCCCTTCCCATC-3′) and cDNA was prepared from mouse bone marrow neutrophils and DCs. A 589-bp TRPM2-specific product was detected after 35 cycles of amplification (30 s at 94°C, 30 s at 68°C, and 30 s at 72°C).

Western blot analyses

To detect TRPM2, membrane protein was prepared from Jurkat T cells by differential centrifugation at 100,000 × g and the protein from the pellet was separated on a 7% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane (Hybond ECL; Amersham Biosciences), and Western blotted (22). The primary Ab against TRPM2 was obtained from Novus Biologicals and the secondary goat anti–rabbit HRP conjugate was obtained from Dianova.

To detect phosphorylated ERK1/2, mouse bone marrow neutrophils were purified and resuspended in RPMI 1640 (no serum) in the presence or absence of 8Br-cADPR (100 µM) or cADPR (100 µM) for 30 min at room temperature. The cells were then incubated for 1 h on ice and subsequently stimulated with FMLF (1 µM). Cells (4 × 10⁶ cells/plate) were removed at time 0 (before the addition of FMLF), 1, 5, 10, and 20 min and lysed in 1% Nonidet P-40 buffer in the presence of protease and phosphatase inhibitors. Protein lysates were collected, frozen, and quantitated by Bradford assay (Bio-Rad). Samples (20 µg of protein per sample) were electrophoresed on a 4–12% SDS-polyacrylamide Bio-Tris gel (Invitrogen Life Technologies), transferred to nitrocellulose membranes (NitrOBind), and then probed with anti-phospho-ERK1/2 (Thr-202 and Tyr-204) or anti-ERK-1 Abs (both from Santa Cruz Biotechnology). Blots were developed using goat anti-mouse IgG-HRP (Southern Biotechnology Associates) or goat anti-rabbit IgG-HRP (Zymed Laboratories) Abs.

To detect PARP-1 protein, total cellular proteins were prepared from spleen, mouse bone marrow-derived neutrophils, and DCs. Whole cell lysates (20 µg/lane) were separated on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane (Hybond ECL), and Western blotted. Blots were incubated with primary Abs against PARP-1 obtained from Serotec (MCA-15226) and the secondary goat anti–mouse HRP conjugate was obtained from Zymed Laboratories. All Western blots were developed using Hyperfilm ECL and the ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

Synthesis of brominated compounds

The synthesis and purification of 8Br-NAD⁺ and 8Br-cADPR was performed as previously described (34). 8Br-cADPR was synthesized by incubating 8Br-NAD⁺ with human recombinant CD38 (0.1 µg/ml) for 2 h at 25°C. 8Br-cADPR was then purified on a 1.6 × 11 cm AG MP-1 column. The 8Br-cADPR was eluted at 2.5 ml/min with a concave upward gradient of TFA from 1.5 to 150 mM over 32 min. 8Br-cADPR eluted between 22 and 29 min. To prevent the breakdown of 8Br-cADPR, the TFA was extracted from the purified 8Br-cADPR by treating the pool (17.5 ml) with 12 ml of a 3:1 mixture of 1,1,2-trichlorotrifuoroethane/tri-N-octylamine (35). The remaining acid was neutralized by adding 2M Tris-base and 1M NaOH to 1 and 2 mM, respectively, and the sample was then dialyzed against distilled water. The purity of each of the brominated compounds was confirmed by analyzing 50–100 nmol of purified product on an analytical AG MP-1 column (0.5 × 5 cm). The preparations used were ≥95% pure.

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Purification of neutrophils and DCs

Bone marrow neutrophils were purified by positive selection using biotinylated GR-1 (BD Pharmingen) and MACS streptavidin microbeads (Miltenyi Biotec). Neutrophil purity was ≥95% as assessed by FACS. To isolate immature DCs, mouse bone marrow cells were cultured in complete medium containing GM-CSF (20 ng/ml) for 6–8 days and the CD11c+ class-IIhi cells were sort-purified using a FACSVantage SE with DiVa option (BD Biosciences). To induce DC maturation, TNF-α (10 ng/ml) was added to the cultures on day 6 and the mature CD11c+ class-IIhi cells were sort purified 48 h later.

Ca2+ mobilization assays

Bone marrow neutrophils (1 × 10⁶/ml) and DCs (1 × 10⁶/ml) were loaded with a mixture of Fluo-3 AM and Fura Red AM as previously described (27, 28). The cells were preincubated in medium, 8Br-cADPR, 8Br-ADPR, or 8Br-NAD+ (100 μM each) for 15 min and then stimulated. The accumulation of cytosolic free Ca2+ was assessed by flow cytometry by measuring the fluorescence emission of Fluo-3 in the FL-1 channel and Fura Red in the FL-3 channel over time. Data were analyzed using FlowJo 4.0 software (Tree Star). Relative intracellular free Ca2+ levels are expressed as the ratio between Fluo-3 and Fura Red mean fluorescence intensity.

Chemotaxis assays

Chemotaxis assays were performed as previously described (27, 28). Briefly, cells were pretreated for 15 min with medium, 8Br-cADPR, 8Br-ADPR, or 8Br-NAD+ (100 μM each). Treated cells (1 × 10⁶ neutrophils or 1 × 10⁵ DCs) were added to the upper chamber of the Transwell unit (Costar). After incubating the chambers for 60 min (neutrophils) or 90 min (DCs) at 37°C, the transmigration cells were collected from the lower chamber, fixed, and counted on a flow cytometer. The results are expressed as the mean ± SD of the chemotactic index (CI) for triplicate wells. The CI represents the fold-change in the number of untreated or inhibitor-pretreated cells that migrated in response to the chemoattractant divided by the basal migration of untreated or antagonist pretreated cells that migrated in response to control medium.

Electrophysiology

Membrane currents were recorded in the whole cell configuration of the patch clamp technique (36). An EPC9 patch clamp amplifier was used in conjunction with the PULSE stimulation and data acquisition software (HEKA Elektronik). The patch electrodes were made from 1.5-mm diameter borosilicate glass capillaries and filled with intracellular solution. Data were low pass-filtered at 1 kHz and compensated for both fast and slow capacity transients. Series resistance was compensated by 70–99%. All experiments were performed at room temperature with Jurkat T lymphocytes attached to tissue culture dishes before the experiment. The pipette solution contained 140 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 2.5 mM EGTA, and 10 mM HEPES adjusted to pH 7.4 with KOH. In some experiments, the pipette solution additionally contained ADPR (0.3 mM, Sigma Aldrich) or ADPR (0.3 mM) plus either 8Br-cADPR (0.9 mM, 8Br-cADPR) or 8Br-NAD+ (0.9 mM, 8Br-NAD+) (27, 28). The transmembrane potential was held at −60 mV and current-voltage relations were obtained every 20 s using 200-ms voltage ramps from −85 to +65 mV.

Ca2+ imaging and microinjection

Intact Jurkat T lymphocytes were loaded with Fura2/AM as described (37). Confocal calcium imaging of T cells was conducted on thin glass coverslips. The cells were double labeled with BSA (5 mg/ml) and poly-l-lysine (0.1 mg/ml). Silicon grease was used to seal small chambers consisting of a rubber O-ring on the glass coverslips. Then, 60 μl of buffer A (140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM NaH2PO4, 5.5 mM glucose, and 20 mM HEPES (pH 7.4)) and 40 μl of cells (2 × 10⁶ cells/ml suspended in buffer A) were added to the small chamber (38). The coverslip with cells slightly attached to the BSA/poly-l-lysine coating was mounted on the stage of a fluorescence microscope (Leica DM IRE2). Ratiorometric Ca2+ imaging was performed as described recently (38–40). In brief, an Immersion imaging system built around the Leica microscope at 100-fold magnification was used. The sample was illuminated at 340 and 380 nm using a monochromator system (Polychromat IV; TILL Photonics). Images were obtained with a gray scale charge-coupled device camera (type C4744-95-12ER from Hamamatsu; operated in 8-bit mode). The spatial magnification was used. The sample was illuminated at 340 and 380 nm using a monochromator system (Polychromator IV; TILL Photonics). Imaging was performed as described recently (38–40). Briefly, we used an Eppendorf system (transector type 5246, micromanipulator type 5171 from Eppendorf-Netheler-Hinz) with Femtotips I as pipettes. IP3, cADPR, and NAADP were diluted to their final concentrations in intracellular buffer (20 mM HEPES, 110 mM KCl, 2 mM MgCl2, 5 mM KH2PO4, and 10 mM NaCl (pH 7.2)) and filtered (0.2 μm) before use. In some experiments, 900 μM 8Br-ADPR was also included in the pipette solution. For injection, the pipette was connected to the semiautomated mode of the system with the following instrumental settings was used: injection pressure 60 hectaropascals, compensatory pressure 30 hectaropascals, injection time 0.5 s, and velocity of the pipette 700 μm/s. Under such conditions the injection volume was 1–1.5% of the cell volume (41).

HPLC analysis of catabolites produced by CD38-expressing cells

Neutrophils were incubated with 8Br-NAD+ (500 μM), 8Br-cADPR (100 μM), or 8Br-ADPR (100 μM) for 0 (no cells in reaction) to 15 min at 37°C. The supernatants were collected after centrifugation, concentrated, and flash frozen. Aliquots were analyzed by reversed-phase HPLC (Kontron Instruments) using a Multilyp BDS C18 column (250 mm × 4.6 mm, particle size 5 μm; Chromatographic Service) as previously described (22). Absorbance was measured at 270 nm using a UV detector (Kontron 432) and data were processed by the MT2 data acquisition system from Kontron Instruments. Peaks were identified by comparison to known standards, and the area under each peak was quantified to determine the relative amounts of each metabolite.

Statistical analysis

Data sets were analyzed using GraphPad Prism version 4.0 for Macintosh (GraphPad Software). Student’s t tests analyses were applied to the data sets and differences were considered significant when p ≤ 0.05.

Results

An ADPR analog specifically blocks ADPR-gated cation entry

Our previous data indicated that cADPR not only modulates intracellular Ca2+ release but also controls Ca2+ influx in both mouse and human leukocytes stimulated with a discrete subset of chemokines (27, 28, 30, 42). However, we could not understand how CDPRP, which activates intracellular Ca2+ release through RyR-gated channels in the endoplasmic reticulum (7), was controlling Ca2+ influx across the plasma membrane (43, 44). Interestingly, recent reports revealed that CDPRP can synergize with ADPR to activate the TRPM2 plasma membrane calcium channel (45–47). Thus, we hypothesized that CD38-generated cADPR might facilitate ADPR-mediated activation of TRPM2 in chemo- kine-stimulated neutrophils and DCs. To test this hypothesis, we first used PCR to determine whether TRPM2 transcripts were expressed by freshly isolated mouse bone marrow neutrophils and bone marrow-derived immature DCs. Similar to previous reports using human neutrophils (48) and a human monocytic cell line (26), we found that mouse bone marrow neutrophils as well as mouse myeloid-derived DCs express TRPM2 mRNA (Fig. 1A), suggesting that these cells may be responsive to agonists of TRPM2, like ADPR.

Next, we wished to test whether ADPR-mediated activation of TRPM2 was required for leukocyte chemotaxis. However, no highly specific TRPM2 inhibitors have been identified (49), and TRPM2-deficient mice are not yet available. Therefore, we took a different approach and asked whether we could make analogues of ADPR that would specifically antagonize ADPR-mediated chemotaxis. To this end, we synthesized (Fig. 1B) and purified (Fig. 1C) 8Br-ADPR. HPLC analysis indicated that the compound was very pure and remained stable even after incubation with leukocytes, as...
Inward current induced by ADPR was characterized by a linear current-voltage relationship, typical for currents carried by TRPM2-like channels (data not shown). In contrast, when the pipette contained ADPR and only a 3-fold excess of 8Br-ADPR, the ADPR-induced cation entry was abrogated in the Jurkat cells (Fig. 2, B and C). Importantly, the infusion of equivalently high concentrations of other brominated nucleotides including 8Br-cADPR and 8Br-NAD⁺ had no effect on the cation influx induced by the addition of ADPR (Fig. 2, B and C).

The data indicated that our novel compound, 8-BrADPR, could block ADPR-induced cation entry in Jurkat cells. To further test the specificity of this compound, we first performed additional experiments combining Ca²⁺ imaging and microinjection of the other known Ca²⁺-mobilizing second messengers, IP₃, cADPR, and NAAD⁺. As expected based on our previous reports, Ca²⁺ release was easily detected in Jurkat cells injected with cADPR (Fig. 3A), NAAD⁺ (Fig. 3B), or IP₃ (Fig. 3C). However, co-injecting 8Br-ADPR in great excess (9- to 9000-fold) had no significant inhibitory effect on either the peak or plateau Ca²⁺ response induced by either cADPR (Fig. 3A) or IP₃ (Fig. 3C) and, if anything, slightly enhanced (not significantly; p > 0.1) the Ca²⁺ response induced by NAAD⁺ (Fig. 3B).
Finally, we assessed the impact of 8Br-ADPR on Ca\(^{2+}\)/H\(_{11001}\) influx through SOCs that are activated in response to intracellular Ca\(^{2+}\)/H\(_{11001}\) store depletion (50). We therefore incubated mouse neutrophils (Fig. 3D, left panel) or DCs (Fig. 3D, middle and right panel) in the presence or absence of 8Br-ADPR and then stimulated the cells with thapsigargin, a drug that causes intracellular Ca\(^{2+}\) store depletion and subsequent Ca\(^{2+}\) entry through SOCs (51). Interestingly, 8Br-ADPR pretreatment of mouse bone marrow neutrophils and DCs had absolutely no effect on capacitative Ca\(^{2+}\) influx induced by thapsigargin (Fig. 3D). Taken altogether, the data show that 8Br-ADPR can block cation entry in cells that express TRPM2 but does not obviously affect Ca\(^{2+}\) entry through SOCs. Furthermore, 8Br-ADPR appears to specifically block cation entry through a plasma membrane channel sensitive to ADPR and does not inhibit intracellular Ca\(^{2+}\) release through any of the known Ca\(^{2+}\) second messengers. Thus, 8Br-ADPR can be used to specifically study the effect of ADPR-induced Ca\(^{2+}\) entry in cells.

Ca\(^{2+}\) influx in chemoattractant-stimulated neutrophils and DCs is blocked by 8Br-ADPR

Because we now had a selective inhibitor of ADPR-gated Ca\(^{2+}\) influx, we next addressed whether Ca\(^{2+}\) mobilization in chemokine- and chemoattractant-stimulated neutrophils and DCs is dependent on ADPR-gated Ca\(^{2+}\) influx. We therefore loaded bone marrow neutrophils with Ca\(^{2+}\)-sensitive fluorescent dyes and pretreated the cells for 15 min with 8Br-ADPR or the cADPR antagonist 8Br-cADPR. We
then measured intracellular free Ca\(^{2+}\) concentrations in cells stimulated with fMLF, a ligand for mouse formyl peptide receptor 1, or with IL-8, a ligand for CXCR1 and CXCR2. To analyze the effect of 8Br-cADPR and 8Br-ADPR on Ca\(^{2+}\) mobilization from intracellular Ca\(^{2+}\) stores, we first performed experiments in Ca\(^{2+}\)-free buffers. Consistent with our published results using Cd38\(^{-}\) neutrophils (27), we found that 8Br-cADPR pretreatment decreased intracellular Ca\(^{2+}\) release in the fMLF-stimulated neutrophils by ~25% (Fig. 4A) but had no effect on IL-8-induced intracellular Ca\(^{2+}\) release (Fig. 4B). In contrast, 8Br-ADPR treatment had no effect on intracellular Ca\(^{2+}\) release after either fMLF (Fig. 4A) or IL-8 stimulation (Fig. 4B).

To assess the potential role of ADPR in regulating extracellular Ca\(^{2+}\) influx in chemokine-stimulated leukocytes, we performed the same experiments in Ca\(^{2+}\)-containing medium. As expected, we observed a biphasic Ca\(^{2+}\) response in wild-type (WT) neutrophils stimulated with fMLF (Fig. 4C) that included a prolonged influx of extracellular Ca\(^{2+}\). Identical with our previously published data (27), we found that the calcium response was significantly decreased in WT neutrophils that were pretreated with 8Br-cADPR (Fig. 4C). Interestingly, 8Br-ADPR pretreatment also caused a significant reduction in the calcium response of the fMLF-stimulated neutrophils (Fig. 4C). Again neither compound had any effect on the IL-8 induced Ca\(^{2+}\) response (Fig. 4D). Importantly, the inhibition of Ca\(^{2+}\) influx observed in the 8Br-ADPR-treated, fMLF-stimulated neutrophils was specific, because pretreatment of the neutrophils with 8Br-AMP, the only other nucleotide present in our preparation (<1.5% of total; see Fig. 1C), had absolutely no effect on fMLF-induced Ca\(^{2+}\) influx (Fig. 4E). Taken together, the data indicate that 8Br-ADPR primarily affects calcium influx rather than intracellular calcium release in the fMLF-stimulated neutrophils.

To determine whether the effect of 8Br-ADPR on Ca\(^{2+}\) influx was limited to a single chemoattractant receptor or cell type, we analyzed the effect of 8Br-ADPR on the Ca\(^{2+}\) response of mouse DCs that were stimulated with the CXCR4 ligand CXCL12, as we previously showed that this response is dependent on CD38, cADPR, and Ca\(^{2+}\) influx (28). Therefore, we sort purified immature DCs from day 8 GM-CSF-cultured bone marrow cells, loaded the cells with Ca\(^{2+}\)-sensitive dyes, preincubated them for 15 min with medium, 8Br-cADPR, or 8Br-ADPR, and then stimulated the cells with CXCL12. As expected (28), pretreatment of the DCs with 8Br-cADPR blocked the Ca\(^{2+}\) response of the CXCL12-stimulated DCs (Fig. 4F). Interestingly, 8Br-ADPR pretreatment also blocked CXCL12-induced Ca\(^{2+}\) responses (Fig. 4F). Similar results were observed when we treated purified mature splenic DCs with 8Br-ADPR and measured the Ca\(^{2+}\) response to the CCR7 ligand CCL19 or CCL21 (data not shown). Together, these data indicate that ADPR regulates extracellular Ca\(^{2+}\) influx in at least two distinct cell types activated with different chemoattractants and chemokines.

![Figure 4](http://www.jimmunol.org/) 8Br-ADPR inhibits Ca\(^{2+}\) influx in chemoattractant-activated leukocytes. A–D, 8Br-ADPR blocks Ca\(^{2+}\) influx in fMLF-stimulated mouse bone marrow neutrophils. Mouse bone marrow neutrophils were loaded with Fluo-3 and Fura Red and preincubated for 15 min in medium (black traces), 8Br-cADPR (100 μM; blue traces), or 8Br-ADPR (100 μM; green traces). Cells were stimulated with fMLF (1 μM; A and C) or IL-8 (100 nM; B and D), and cytosolic free Ca\(^{2+}\) levels were measured by flow cytometry. In A and B the extracellular Ca\(^{2+}\) was chelated with EGTA (2 mM) immediately before stimulation. E, 8Br-AMP does not block Ca\(^{2+}\) influx in chemokine-stimulated neutrophils. Fluo-3 and Fura Red-loaded neutrophils were preincubated in medium (black traces) or 8Br-AMP (100 μM; red traces) for 15 min and stimulated with fMLF (1 μM), and the accumulation of free cytosolic Ca\(^{2+}\) was measured by flow cytometry. F, 8Br-ADPR blocks Ca\(^{2+}\) influx in CXCL12-stimulated mouse bone marrow DCs. Immature bone-marrow derived DCs were sort purified, loaded with Fluo-3 and Fura Red, and pretreated for 15 min in medium (black traces), 8Br-cADPR (100 μM; blue traces), or 8Br-ADPR (100 μM; green traces). The cells were stimulated with CXCL12 (50 ng/ml) and cytosolic free Ca\(^{2+}\) levels were measured by flow cytometry. All data shown are representative of three or more independent experiments.
8Br-ADPR blocks chemotaxis of mouse leukocytes to multiple chemoattractants and chemokines

We previously demonstrated that chemotaxis of mouse bone marrow neutrophils and DCs to mouse formyl peptide receptor 1, CXCR4, and CCR7 ligands is dependent on Ca\[^{2+}\] influx (27, 28). Because 8Br-ADPR blocked Ca\[^{2+}\] influx in the chemokine-stimulated mouse DCs and neutrophils, we predicted that 8Br-ADPR would also inhibit the chemotaxis of mouse neutrophils and DCs to these chemoattractants. To test this hypothesis, we pretreated bone marrow-derived immature or TNF-\(\alpha\)-matured DCs with 8Br-cADPR or 8Br-ADPR and then measured the chemotactic response of the cells to CXCL12 (immature DCs) or CCL21 (mature DCs). As expected, we observed very robust chemotactic responses from the untreated immature (Fig. 5A) and untreated mature DCs (Fig. 5B) to CXCL12 or CCL21, respectively. As we previously reported (28), neither the immature nor the mature DCs migrated efficiently to CXCL12 or CCL21 when they were pretreated with 8Br-cADPR (Fig. 5, A and B). Similarly, the 8Br-ADPR-treated immature and mature DCs made poor chemotactic responses to CXCL12 and CCL21 (Fig. 5, A and B), indicating that ADPR-gated Ca\[^{2+}\] influx is required for the chemotaxis of DCs to CXCR4 and CCR7 ligands.

Next, we pretreated mouse bone marrow neutrophils with 8Br-cADPR or 8Br-ADPR and then measured the chemotactic response of these cells to fMLF or IL-8. Similar to our results measuring Ca\[^{2+}\] responses (Fig. 4), neither 8Br-ADPR nor 8Br-cADPR blocked the chemotactic response of the mouse neutrophils to IL-8 (Fig. 5C). However, as we previously published (27), pretreating neutrophils with 8Br-cADPR effectively blocked chemotaxis to fMLF (Fig. 5D). Likewise, the chemotactic response of the neutrophils to fMLF was efficiently inhibited by pretreatment with 8Br-ADPR (Fig. 5D). The inhibitory effect of 8Br-ADPR on neutrophil chemotaxis was very potent, as the treatment of cells with low micromolar concentrations of 8Br-ADPR (~2.5 \(\mu\)M) was sufficient to inhibit cell migration by at least 50% (Fig. 5E). Taken altogether, these data demonstrate that ADPR-gated Ca\[^{2+}\] influx is required for the chemotaxis of mouse neutrophils and DCs to multiple, although not all, chemoattractants.

![FIGURE 5. 8Br-ADPR inhibits chemotaxis but not MAPK activation. A and B, Mouse bone marrow-derived immature DCs (A) and TNF-\(\alpha\)-matured DCs (B) were sort purified, preincubated for 15 min in medium (filled bars), 8Br-cADPR (open bars; 100 \(\mu\)M), or 8Br-ADPR (gray bars; 100 \(\mu\)M) and then placed in Transwell chambers containing CXCL12 (A) or CCL21 (B) in the bottom chamber. The cells that migrated to the bottom chamber in response to the chemotactic gradient were collected and enumerated by FACS. The results are expressed as the mean \(\pm\) SEM of the chemotaxis index (CI; see Materials and Methods for description) of triplicate cultures. *, \(p \leq 0.001\) or **, \(p \leq 0.015\) between untreated DCs and all other groups. C and D, Mouse bone marrow neutrophils were preincubated with medium (filled bars), 8Br-cADPR (100 \(\mu\)M, open bars), or 8Br-ADPR (100 \(\mu\)M; gray bars) and then placed in Transwell chambers containing 100 nM IL-8 (C) or 1 \(\mu\)M fMLF (D) in the bottom chamber. The cells that migrated in response to the chemotactic gradient were collected at 45 min and enumerated by flow cytometry. The data are reported as the mean \(\pm\) SEM of the CI of triplicate cultures. *, \(p \leq 0.001\) between untreated neutrophils and all other groups. E, 8Br-ADPR blocks neutrophil chemotaxis in a dose-dependent fashion. Mouse bone marrow neutrophils were incubated in the presence of increasing amounts of 8Br-ADPR (0–100 \(\mu\)M) for 15 min. The chemotactic response of the cells to fMLF (1 \(\mu\)M) was then determined as described above. The data are reported as the mean \(\pm\) SEM of the CI of triplicate cultures. *, \(p \leq 0.003\) between untreated neutrophils and all other groups. F, fMLF-induced MAP kinase activation is not inhibited by 8Br-ADPR treatment. Mouse bone marrow neutrophils were preincubated in the presence of medium, 8Br-ADPR (100 \(\mu\)M), or 8Br-cADPR (100 \(\mu\)M) and then stimulated with fMLF (1 \(\mu\)M) for 0 to 20 min. Whole cell lysates were prepared, separated by SDS-PAGE, Western blotted, and then probed with anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1.](http://www.jimmunol.org/ Downloaded from)
8Br-ADPR does not block MAPK activation in fMLF-activated neutrophils

Our data showed that 8Br-ADPR treatment of chemokine/chemoattractant-activated mouse neutrophils and DCs blocked both Ca\(^{2+}\) influx and chemotaxis, at least in response to a subset of chemotactic stimuli. To address whether other receptor-induced signaling pathways such as the MAPK cascade were affected by 8Br-ADPR treatment, we prepared mouse bone marrow neutrophils and stimulated them with fMLF in the presence or absence of 8Br-ADPR or 8Br-cADPR. We then analyzed protein lysates from these cells by Western blotting using Abs specific for activated (phosphorylated) ERK1/2. As expected, the stimulation of neutrophils with fMLF induced rapid phosphorylation of ERK1/2 that peaked within 5 min and began to decline within 20 min (Fig. 5F). Interestingly, pretreatment of the cells with either 8Br-ADPR or 8Br-cADPR did not significantly affect the kinetics or levels of ERK1/2 phosphorylation in the fMLF-stimulated neutrophils (Fig. 5F). Similar results were found when the phosphorylation of JNK was analyzed (data not shown). Together, these data show that 8Br-ADPR treatment does not simply shut down chemokine receptor signal transduction but appears to specifically regulate the Ca\(^{2+}\)-dependent signaling pathways induced upon chemotactic receptor engagement by a ligand.

Chemotaxis and Ca\(^{2+}\) responses in chemoattractant-stimulated leukocytes is dependent on both cADPR and ADPR

Although cADPR was first identified as a Ca\(^{2+}\)-signaling second messenger that mobilizes intracellular Ca\(^{2+}\) release (7), our data indicated that 8Br-cADPR regulates both intracellular Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) influx. Given that cADPR can be hydrolyzed, albeit very inefficiently, to ADPR by CD38 (52), it was important to assess the stability of our 8Br-cADPR preparation to ensure that it was not being degraded to 8Br-ADPR. We therefore incubated purified 8Br-cADPR alone or with CD38-expressing bone marrow neutrophils for 15 min and analyzed the supernatant by HPLC to determine the relative proportions of the different brominated catabolites. The purity of the 8Br-cADPR used in these studies was very high (Fig. 6A, 98–99%). Furthermore, although we did detect a very small amount of 8Br-ADPR in the preparation (<2% of the total compound), no significant additional hydrolysis was observed after a 15-min incubation with CD38-expressing bone marrow neutrophils (Fig. 6A). To address whether the small amount of contaminating 8Br-ADPR in our 8Br-cADPR preparation was responsible for the inhibition of chemotaxis that we observed after exposing cells to the 8Br-cADPR, we incubated mouse neutrophils with decreasing amounts of 8Br-cADPR and then measured the chemotaxis of the treated cells to fMLF. Similar to what we previously showed with FPRL1-activated human neutrophils (30), we determined that the IC\(_{50}\) of 8Br-cADPR on fMLF-stimulated mouse bone marrow neutrophils was in the low micromolar range (Fig. 6B, IC\(_{50}\) ~ 1–5 \(\mu\)M). Because the amount of contaminating 8Br-ADPR present in a 1 \(\mu\)M solution of 8Br-cADPR was <20 nM, a value well below the IC\(_{50}\) of 8Br-ADPR (see Fig. 5), the data support the conclusion that both cADPR and ADPR are required to activate Ca\(^{2+}\) influx in chemokine-stimulated, TRPM2-expressing leukocytes.

PARP-1 and CD38 differentially regulate control of chemoattractant- and oxidant-induced Ca\(^{2+}\) responses

Our data show that cADPR and ADPR are each necessary for the activation of Ca\(^{2+}\) influx in chemokine- and chemoattractant-stimulated mouse neutrophils and DCs. Although CD38 appears to be the major or even sole source of cADPR in bone marrow neutrophils and DCs (27, 28), it is unclear which enzyme or enzymes are responsible for generating the ADPR that regulates Ca\(^{2+}\) signaling (53). One favored candidate is the NAD\(^{+}\)-using enzyme PARP-1.

![FIGURE 6. Leukocyte chemotaxis is dependent on both ADPR and cADPR. A, Purity of 8Br-cADPR before and after incubation with leukocytes. Purified 8Br-cADPR (100 \(\mu\)M) was incubated alone or in the presence of WT neutrophils for 15 min. The supernatants from the samples were collected and analyzed by HPLC to identify the brominated metabolites present in the cultures. The HPLC chromatograms of standards for each of the compounds are included for comparison and the relative proportion of each catabolite is indicated. B, 8Br-cADPR blocks mouse neutrophil chemotaxis in a dose-dependent fashion. Mouse bone marrow neutrophils were incubated in the presence of increasing amounts of 8Br-cADPR (0–100 \(\mu\)M) for 15 min. The chemotactic response of the neutrophils was then determined as described for Fig. 5. The data are reported as the mean ± SD of the CI of triplicate cultures. *p < 0.04 between untreated neutrophils and indicated groups. The data are representative of two or more independent experiments.](http://www.jimmunol.org/Downloadedfrom)
(49, 53). Unlike CD38, PARP-1 does not produce free ADPR but instead covalently attaches ADPR polymers to proteins in a reaction referred to as ADP ribosylation (32). The ADPR polymers produced by PARP-1 can then be hydrolyzed into free ADPR monomers by the enzyme poly-ADP-ribose glycohydrolase (PARG) (54). Because PARP-1 is expressed by many cell types, it is conceivable that the Ca\(^{2+}\)-mobilizing ADPR monomers could be generated by either CD38 or the PARP-1/PARG metabolic pathway. Although PARP-1 is reported to be absent in human neutrophils (55), it is expressed, albeit at low levels, by mouse bone marrow neutrophils and DCs (Fig. 7A). To test whether ADPR generated by the PARP-1/PARG pathway is necessary for chemoattractant-induced Ca\(^{2+}\) influx and chemotaxis, we measured the Ca\(^{2+}\) responses of fMLF-stimulated WT and Parp1\(^{-/-}\) bone marrow neutrophils. As shown in Fig. 7, B and C, the Ca\(^{2+}\) and chemotactic response of the fMLF-stimulated Parp1\(^{-/-}\) neutrophils was equivalent to that seen for WT neutrophils. In contrast, Ca\(^{2+}\) influx (Fig. 7B) and chemotaxis (Fig. 7C) were significantly inhibited when WT or Parp1\(^{-/-}\) neutrophils were first pretreated for 15 min with 8Br-ADPR. As expected, 8Br-ADPR treatment of Cd38\(^{-/-}\) neutrophils did not further inhibit the already reduced chemotactic response (Fig. 7D). Together, these data demonstrate that the ADPR required for chemokine/chemoattractant receptor signaling is not generated via the PARP-1/PARG metabolic pathway and may in fact be generated by CD38.

Although PARP-1 is not required for fMLF-activated Ca\(^{2+}\) influx in neutrophils, it is reported that PARP-1 does regulate Ca\(^{2+}\) influx in an ADPR-dependent fashion in at least some oxidant-stimulated cell types (56–58). To test whether the Cd38/ADPR pathway is also required for this response, we exposed bone marrow neutrophils (WT, Parp1\(^{-/-}\), and Cd38\(^{-/-}\) cells and WT cells pretreated with 8Br-ADPR) to H2O2 and measured the calcium response. As expected, we observed a robust Ca\(^{2+}\) response in WT neutrophils treated with H2O2 (Fig. 7E). This response was due to the influx of extracellular Ca\(^{2+}\) because it was not observed when the extracellular Ca\(^{2+}\) was chelated with EGTA (not shown). As expected, based on analysis of PARP-1 activity in other cell types (56–58), the Ca\(^{2+}\) response in H2O2-treated Parp1\(^{-/-}\) neutrophils was significantly decreased (Fig. 7E), indicating that PARP-1 regulates oxidant-induced responses in mouse neutrophils. In contrast, the H2O2-induced calcium response was not affected in the Cd38\(^{-/-}\) neutrophils or in the 8Br-ADPR treated WT neutrophils (Fig. 7E). Thus, oxidant-induced Ca\(^{2+}\) influx in mouse bone marrow neutrophils is regulated by PARP-1 (and not Cd38), whereas chemoattractant-induced Ca\(^{2+}\) influx is regulated by Cd38 (and not PARP-1). Furthermore, 8Br-ADPR specifically blocks the chemoattractant-induced Ca\(^{2+}\) response without affecting oxidant-induced Ca\(^{2+}\) responses in the mouse neutrophils.

\textit{NAD}\(^{+}\)-analogues block chemotaxis but do not affect oxidant-induced responses

Together, our data indicated cADPR and ADPR are each needed to activate Ca\(^{2+}\) influx in chemoattractant- and chemokine-stimulated neutrophils and DCs and that Cd38, and not PARP-1, is required for chemokine receptor signaling. We previously showed that the treatment of either human or mouse neutrophils and DCs with a NAD\(^{+}\) analog, 8Br-NAD\(^{+}\), blocked the chemotactic responses of these cells to several chemokines and proposed that this compound could be used to specifically target the Cd38 signaling pathway. Indeed, we proposed that 8Br-NAD\(^{+}\) was catabolized by Cd38-expressing cells into the cADPR antagonist 8Br-cADPR. However, given that we now know that 8Br-ADPR can also block chemotaxis, we needed to consider whether the 8Br-NAD\(^{+}\) was...
neutrophils with 8Br-NAD\(^+\), 8Br-ADPR was easily detected in the culture medium (Fig. 8A). Furthermore, in agreement with studies using recombinant CD38 (6, 52), 8Br-ADPR was the predominant product present in the supernatant and represented \(\sim 98\%\) of the total reaction products (not shown). Finally, no production of 8Br-ADPR was observed in the \(Cd38^{+/−}\) cell cultures (Fig. 8A), strongly suggesting that CD38 is responsible for producing the extracellular 8Br-ADPR from 8Br-NAD\(^+\). However, this did not exclude the possibility that intracellular 8Br-ADPR was also generated by the PARP-1/PARG pathway. In fact, it has been previously reported that extracellularly applied NAD\(^+\) can be transported to the cytosol of cells through connexin 43 hemichannels (59). Therefore, to determine whether 8Br-NAD\(^+\) could be catalyzed into 8Br-ADPR in a PARP-1-dependent manner, we incubated WT and \(Parp1^{+/−}\) neutrophils with 8Br-NAD\(^+\) and measured Ca\(^{2+}\) responses after oxidant or chemotactant (fMLF) stimulation. As shown in Fig. 8B, 8Br-NAD\(^+\) treatment had no effect on oxidant-induced Ca\(^{2+}\) influx in WT neutrophils, indicating that it does not block this PARP-1 dependent pathway. In contrast, Ca\(^{2+}\) entry in response to fMLF stimulation was significantly reduced in the 8Br-NAD\(^+\)-treated WT neutrophils as well as in the 8Br-NAD\(^+\)-treated \(Parp1^{+/−}\) cells (Fig. 8C). Likewise, the chemotactic response of both WT and \(Parp1^{+/−}\) neutrophils to fMLF was inhibited in the presence of 8Br-NAD\(^+\) (Fig. 8D), Taken collectively, the data show that nucleotide analogues such as 8Br-ADPR and 8Br-NAD\(^+\) can be used to selectively target chemokine and chemotactant receptor signaling without affecting oxidant-induced responses. The implications of these findings for the treatment of inflammation-based disease are discussed.

Discussion

In this article we describe and characterize 8Br-ADPR, a novel inhibitor of mouse neutrophil and DC chemotaxis. Although this analog very efficiently inhibited chemotaxis in neutrophils and DCs, it did not block Ca\(^{2+}\) release mediated by any of the other known Ca\(^{2+}\)-mobilizing second messengers including IP\(_3\), NAADP\(^+\), and cADPR. Likewise, 8Br-ADPR had no effect on capacitative Ca\(^{2+}\) entry in thapsigargin-stimulated neutrophils and DCs, indicating that it is not an inhibitor of SOCs. Instead, 8Br-ADPR specifically blocked ADPR-gated cation entry in TRPM2-expressing cells, suggesting that it functions as a competitive antagonist to block the binding of ADPR to TRPM2 or another unknown ADPR-gated plasma membrane channel. Although significant amounts of 8Br-ADPR (900 \(\mu\)M) were needed to block ADPR-gated Ca\(^{2+}\) entry in the patch clamp experiments, this was due to the fact that large quantities of ADPR (300 \(\mu\)M) are necessary to activate the TRPM2 cation channel using the patch clamp method (24, 45). In fact, the presence of 8Br-ADPR in only 3-fold excess was sufficient to entirely block ADPR-gated Ca\(^{2+}\) influx in the patch-clamped Jurkat cells. Furthermore, much lower concentrations of 8Br-ADPR (1–5 \(\mu\)M) were sufficient to block Ca\(^{2+}\) influx and chemotaxis in intact primary cells. Importantly, the treatment of mouse fMLF-stimulated neutrophils with 8Br-ADPR did not affect ERK1/2 activation, indicating that this compound is not simply a global suppressor of chemokine/chemoattractant receptor signal transduction. Thus, 8Br-ADPR appears to be a potent and specific antagonist of ADPR-dependent Ca\(^{2+}\) and chemotactic responses.

We previously demonstrated that the chemotactic response of mouse and human neutrophils and monocytes and mouse DCs to multiple chemokines is dependent on Ca\(^{2+}\) influx through a plasma membrane channel. Because cADPR, a catabolite known to mobilize Ca\(^{2+}\) from intracellular stores (7), was also obligatory.
for Ca\(^{2+}\) influx in these cells, we proposed that the plasma membrane Ca\(^{2+}\) channel was likely to be a SOC that was activated by the depletion of cADPR-gated intracellular Ca\(^{2+}\) stores (44). However, our new data suggests that our original hypothesis was incorrect and that the still unidentified plasma membrane channel that controls leukocyte chemotaxis is activated by a combination of cADPR-gated, intracellular free cytosolic Ca\(^{2+}\) and ADPR. Indeed, although large quantities of ADPR are needed to activate cation entry in most cells tested to date (24, 45), far less ADPR is required to activate the channel when free intracellular calcium (24, 60) or cADPR (45) is present. Although it is not yet known how cADPR and ADPR cooperate to facilitate Ca\(^{2+}\) entry in the stimulated leukocytes, we postulate that chemokine/chemoattractant stimulation facilitates cADPR-induced Ca\(^{2+}\) release via RyRs and that this free Ca\(^{2+}\) synergizes with ADPR to induce Ca\(^{2+}\) influx through an ADPR- and Ca\(^{2+}\)-sensitive cation channel. In support of this model, the treatment of mouse neutrophils and DCs with 8Br-cADPR affected both intracellular Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) influx, whereas 8Br-ADPR treatment affected only ADPR-gated Ca\(^{2+}\) influx. This suggests that both cADPR and ADPR work in a concerted and synergistic fashion to activate Ca\(^{2+}\) entry and chemotaxis in chemoattractant/chemokine-stimulated mouse neutrophils and DCs. Although we cannot formally exclude the possibility that the ADPR is generated by other members of the NAD\(^+\)-using PARP family (32), our data clearly negate the role for the most abundant family member, PARP-1, in chemokine receptor-mediated Ca\(^{2+}\) responses and support the concept that CD38 is the key activator of the cation channel in response to chemokine/chemoattractant stimulation.

Although we do not yet know the identity of the Ca\(^{2+}\)/cation channel activated in response to chemokine receptor ligation, we now know that this channel is sensitive to the presence of cADPR antagonists, is dependent on intracellular Ca\(^{2+}\) release, can be blocked with an ADPR analog, and is not a classical SOC like Orai1/CRACM (61–63). Given that TRPM2 is the only known plasma membrane channel activated by ADPR (24–26) and superactivated with cADPR and ADPR (45–47), the data presented here lead us to speculate that chemokine receptor-mediated Ca\(^{2+}\) signaling and chemotaxis might be dependent on TRPM2. In support of this hypothesis, several of the “quasi”-specific inhibitors of TRPM2 (49), including econazole and flufenamic acid, also block Ca\(^{2+}\) signaling and chemotaxis in primary mouse neutrophils and DCs (64), and TRPM2 mRNA (Fig. 1) and protein (64) are found in a number of mouse and human leukocyte populations.

It has been reported that TRPM2 regulates cation entry in some oxidant-stimulated cell types and that this response is dependent on PARP-1 (56–58). Our data indicates that the channel activated in mouse neutrophils in response to fMLF is clearly distinct from the channel activated in response to oxidant exposure. First, 8Br-ADPR treatment blocks Ca\(^{2+}\) influx in chemoattractant-stimulated mouse neutrophils but not in H\(_2\)O\(_2\)-stimulated neutrophils. Second, CD38 is required for Ca\(^{2+}\) influx in chemoattractant-stimulated neutrophils but not in oxidant-stimulated neutrophils. Finally, PARP-1 is not obligate for chemoattractant-stimulated Ca\(^{2+}\) influx in mouse neutrophils but is required for Ca\(^{2+}\) influx in H\(_2\)O\(_2\)-treated neutrophils. Importantly, these differences were not limited to mouse neutrophils, as we obtained very similar results using mouse DCs (data not shown). Thus, there are clearly differences in the regulation of these two Ca\(^{2+}\) responses despite the fact that both responses are postulated to be dependent on TRPM2. It is possible that TRPM2 is gated by the ADPR generated by the PARP/PARG pathway in response to certain stimuli and is gated by the ADPR- and cADPR-dependent intracellular Ca\(^{2+}\) generated via the CD38 pathway in response to other stimuli. Alternatively, as reported by Wehage et al. (65), it is possible that oxidative stress-induced activation of TRPM2 may not be dependent on ADPR. In either case, the resolution of these questions will have to wait until TRPM2-deficient primary leukocytes are available for study.

Regardless of whether ADPR and cADPR activate Ca\(^{2+}\) entry through TRPM2 or another unknown cation channel, it is still difficult to understand how an ectoenzyme, like CD38, regulates Ca\(^{2+}\) responses that are activated by metabolites, such as cADPR and ADPR, that appear to function inside cells. Indeed, the proposed mechanism for ADPR-induced cation entry requires the binding of ADPR to the cytoplasmic NUDT9-H domain of TRPM2 (57, 66). Interestingly, there are reports indicating that CD38, in at least some cell types, is localized in intracellular nuclear membranes (67, 68), perhaps allowing for access to intracellular NAD\(^+\) and for the formation of ADPR and cADPR inside the cell. In addition, it has been reported that the extracellular second messengers made by CD38 can be transferred by nucleoside transporters from the outside to the inside of the cell (69), thereby allowing the second messengers to access their cytosolic binding sites. Unfortunately, measurements of intracellular ADPR content currently require very large numbers of cells (22), precluding an analysis in primary leukocytes at this time. However, once the sensitivity of the technology improves, it will be important to measure intracellular ADPR in leukocytes that have been activated by chemoattractants like fMLF to determine whether ADPR is either made or transported specifically in response to ligation of the chemoattractant receptors.

Our data show that CD38, the metabolites made by CD38, and their downstream targets, namely the RyR (cADPR target) and TRPM2 or another ADPR-activated channel (ADPR targets), play critical roles in regulating leukocyte migration. However, it is important to point out that this CD38/RyR/ADPR channel-regulated signaling pathway does not control signaling through all of the known chemokine/chemoattractant receptors. Indeed as shown here, the activation of Ca\(^{2+}\) influx by ADPR is not required for either Ca\(^{2+}\) or chemotactic responses of mouse neutrophils responding to the CXCR1/CXCR2 agonists IL-8 and MPI2. Likewise, although we have found that the chemotaxis of peripheral blood human neutrophils activated with hFPRL1 ligands is dependent on cADPR (30) and ADPR (data not shown), there is a report demonstrating that human DCs migrate normally in the presence of the cADPR antagonist 8Br-cADPR (70). Therefore, our results cannot necessarily be extrapolated across species to all of the different chemokine receptors or to all cell types, and additional analyses will be needed to understand why chemotaxis can proceed independently of the CD38/RyR/ADPR-gated channel pathway in one setting and not in another context.

Taken altogether, our data show that compounds that block the activity of either cADPR or ADPR inhibit leukocyte trafficking, at least in vitro, and strongly suggest that inhibitors of CD38, RyRs, or the ADPR-gated cation channel could also be used to inhibit DC and neutrophil migration in vivo. In addition, the data predict that NAD\(^+\) analogues, such as 8Br-NAD\(^+\), could be used to specifically target leukocyte migration without necessarily affecting the activities of other intracellular NAD\(^+\)-metabolizing enzymes such as PARP-1. In conclusion, we show a previously unappreciated important role for ADPR in regulating chemokine receptor-mediated signal transduction and demonstrate that nucleotide analogues that target ADPR-sensitive cation channels block cell migration and may be useful for the treatment of inflammatory disease.
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Disclosures

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