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Transient Receptor Potential Melastatin 2 Is Required for Lipopolysaccharide-Induced Cytokine Production in Human Monocytes

Janine Wehrhahn,* Robert Kraft, † Christian Harteneck, ‡ and Sunna Hauschildt*

Transient receptor potential melastatin 2 (TRPM2) is a Ca2+-permeable nonselective cation channel that is stimulated by oxidative stress and specifically activated by intracellular ADP-ribose. Because TRPM2 is highly expressed in immune cells, a role of this channel in inflammation processes has been proposed. The aim of the current study was to determine the function of TRPM2 in LPS-induced cytokine production of human monocytes. Incubation of human primary monocytes with LPS resulted in an upregulation of TRPM2 mRNA, protein, and of ADP-ribose–induced membrane currents. By using short hairpin RNA to downregulate TRPM2 expression in THP-1 monocytes, we demonstrate that TRPM2 is required for the LPS-induced production of IL-6, IL-8, IL-10, and TNF-α. Application of LPS led to a time-dependent increase in intracellular Ca2+ concentrations in THP-1 cells that was clearly reduced by downregulation of TRPM2. Omission of extracellular Ca2+ strongly decreased TNF-α expression of TRPM2-expressing cells. Thus, TRPM2-mediated Ca2+ entry is a central mechanism for LPS-induced cytokine production in monocytic cells. The identification of TRPM2 as a major player in this LPS-dependent process makes it a promising tool in modulating monocyte functions. The Journal of Immunology, 2010, 184: 2386–2393.

Interaction of human monocytes and macrophages with LPS, the major outer membrane component of Gram-negative bacteria, results in an inflammatory response that includes production of reactive oxygen species (ROS), proinflammatory cytokines, and chemokines. When released in massive amounts, these mediators can cause multiple organ dysfunction syndromes and lethal septic shock (1, 2). Despite much effort either to neutralize the mediators once formed or to inhibit their production, there is no good therapy for these pathological conditions. Because an increase in intracellular calcium concentrations ([Ca2+]i) is a common mechanism used by cells to initiate production of mediators, one strategy to downregulate their production would be to inhibit calcium entry. With the discovery of transient receptor potential (TRP) channels being major players in these pathological conditions, calcium entry into various cell types, much interest has been focused on these channels as pharmacological targets.

Transient receptor potential melastatin 2 (TRPM2) is a Ca2+-permeable ion channel of the melastatin-related TRP channels (3–5), which represent the most diverse group within the TRP superfamily of cation channels (6–8). TRPM2 is unique among known ion channels in that it contains an intracellular enzymatic domain that possesses ADP-ribose (ADPR) hydrolase activity (9). Activation of TRPM2 channels is specifically triggered by ADPR (9, 10) and is modulated by [Ca2+]i (11–13) and temperature (14). Besides ADPR, other intracellular messengers such as NAD, cyclic ADP-ribose (cADPR), NADP, and 2'-oxo-acetylated ADPR have been reported as TRPM2 agonists (10, 15–18). Extracellular signals known to activate TRPM2 channels include stimuli that mediate oxidative stress, such as H2O2 and TNF-α (17, 19). Therefore, a role of TRPM2-mediated Ca2+ influx in H2O2-induced cell death has been proposed, as reviewed by Miller (20). Generation of intracellular ADPR primarily depends on activation of the ubiquitously expressed poly(ADP-ribose) polymerase (PARP-1) (21–23), an enzyme linked to DNA repair upon damage because of chemicals, radiation, and oxidants. It uses NAD+ as a substrate to produce ADPR polymers, which in turn are quickly hydrolyzed into free ADPR by poly(ADPR) glycohydrolase (24). The ectoenzyme CD38, which converts extracellular NAD+ to ADPR (and to a minor extent to cADPR), may also contribute to TRPM2 activation (25).

In addition to a variety of tissues (5, 17), a ubiquitous expression of functional TRPM2 channels has been reported in immune cells, particularly in cells of the monocytic lineage (9, 10, 26, 27). An important function of granulocytes and cells of the monocytic lineage is the generation of ROS, particularly H2O2, to eliminate invading pathogens (28). This process known as the respiratory burst, which is accompanied by an increase in [Ca2+]i, mediated by intracellular store depletion and Ca2+ entry from the extracellular space (29), implies that the cells themselves are also exposed to H2O2. Heiner et al. (25) speculated that the Ca2+-dependent release of H2O2 during respiratory burst is enhanced through Ca2+ entry via TRPM2, thus establishing a positive feedback loop by an autocrine/paracrine pathway. As H2O2 initiates a vast range of cellular processes (30) and induces activation of TRPM2, much interest has focused on the role of TRPM2 in modulating H2O2-mediated signal transduction events. Only recently, Yamamoto et al. (31) showed that application of sublethal doses of H2O2 to human monocytic U937 cells results in chemokine production induced by TRPM2-mediated Ca2+ influx. Activation of TRPM2 by H2O2 has also been observed to occur in activated microglia, the host macrophages of the CNS (27).
Whereas H$_2$O$_2$ has been described as a potent activator of TRPM2 in monocytes, we raised the question whether this channel plays a role in LPS-induced monocyte functions. In this study, we provide evidence that TRPM2-mediated Ca$^{2+}$ entry is essential for LPS-induced cytokine production in human monocytes. Thus, our study describes a new role for TRPM2 channels in the regulation of inflammatory responses.

**Materials and Methods**

**Reagents**

Unless otherwise indicated, materials used in this study were from the following manufacturers: High Fidelity PCR Enzyme Mix, 2’-deoxyriboseol 5’-terminal deoxynucleotidyl transferase enzymes (Promega, Madison, WI), HindIII, DraIII, AgeI, and BglIII, RevertAid H Minus Moloney murine leukemia virus reverse transcriptase, RNase-free DNase I, shrimp alkaline phosphatase, T4 DNA ligase, and T4 DNA polymerase (Fermentas, St. Leon-Rot, Germany); fura-2- acetoxyethyl ester, oligonucleotide synthesis, pCMV-mito-myc, Pluronic F-12, and 100-bp DNA ladder (Invitrogen, Karlsruhe, Germany); FNase F and Quick Ligation Kit (New England Biolabs, Frankfurt/Main, Germany); G-418 sulfate and RPMI 1640 medium (with l-glutamine, 25 mM HEPES, and phenol red) (PAAS, Pasching, Austria); penicillin and streptomycin (Seromed Biocrom, Berlin, Germany); and ADPR, Bradford reagent, FBS, LPS from Escherichia coli 055:B5, mouse anti-human β-actin Ab (clone AC-74), MTT, and peroxidase-conjugated goat anti-mouse Ab (lysyl)3-lysine (Pam3CysSK4; EMC Microcollections, Tübingen, Germany). The basic shRNA vector pH1-RNA promoter was modified by adding a G-418 sulfate at 1 μg/ml to the cell culture medium, stable cell lines were generated. Transfection efficiency was routinely controlled by GFP detection on a FacScan flow cytometer (BD Biosciences, San Jose, CA).

**RNA isolation and reverse transcription**

Total RNA was isolated from monocytes (4 × 10$^6$) and THP-1 cells (3 × 10$^6$) using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. DNase I treatment and reverse transcription were performed as described previously (33).

**Semiquantitative real-time PCR**

The reaction mixture contained 10 μl qSYBR Green supermix (Bio-Rad, Munich, Germany), 125 nM forward and reverse primers, and 1 μl cDNA template in a final volume of 20 μl. Expression of mRNA was analyzed with primers (34, 35) listed in Table I. Samples were run in duplicate in the 7300 real-time PCR cycler system (Applied Biosystems, Darmstadt, Germany). The reactions were performed under the following conditions: initial denaturation at 95˚C for 3 min, followed by 40 cycles of 15 s of denaturation at 95˚C, 30 s of primer annealing at 60˚C, and 30 s of extension/synthesis at 72˚C. Product quantification was optimal at 72˚C. Negative controls were performed with total RNA and water as template. Following PCR, the melting curve for each product was determined, and its correct size was estimated by agarose gel analysis. All cDNA products were designed as follows: shRNA_TRPM2 bottom strand, 5’-AGCTTTTC CAAAAGGCCCAGAAACATTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’; shRNA_scrambled bottom strand, 5’-GATCCCC CCAAATAGCCAGAGATGTCATCTTCACAGGATGCTCTCGG - TCCTATTTTTTTGAAA-3’; and shRNA_scrambled bottom strand, 5’-AGCTTTTC CAAAAGGCCCAGAAACATTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’. The complementary oligonucleotides (5 pmol/μl) were boiled for 5 min at 95˚C and cooled overnight at 4˚C. The double-stranded shRNA oligonucleotides as well as the modified vector pH1-RNA promoter were digested with HindIII and BamHI and then ligated.

THP-1 cells were transfected with the shRNA vectors using Amazka cell line nuclefect kit V (Lonza, Basel, Switzerland) according to the manufacturer’s instruction. By adding G-418 sulfate at 1 μg/ml to the cell culture medium, stable cell lines were generated. Transfection efficiency was routinely controlled by GFP detection on a FacScan flow cytometer (BD Biosciences, San Jose, CA).

**Cell separation and cell culture**

Human PBMCs from healthy donors were obtained by centrifugation over a Ficoll-Isopaque (Pharmacia, Freiburg, Germany) density gradient. After repeated washing in PBS containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-6B elutriation system (Beckman Instruments, Palo Alto, CA) with specific primers, additionally encoding a DraIII restriction site (forward, 5’-CTAGTTTGGCGGGTTTGACATCTTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’; reverse, 5’-GATCCCC CCAAATAGCCAGAGATGTCATCTTCACAGGATGCTCTCGG - TCCTATTTTTTTGAAA-3’; and shRNA_scrambled bottom strand, 5’-AGCTTTTC CAAAAGGCCCAGAAACATTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’).

**Short hairpin RNA vector design and stably transfection of THP-1 cells**

The basic plasmid (pH1-RNA promoter) used to design TRPM2-specific and scrambled short hairpin RNA (shRNA) vectors contains a human H1 RNA promoter and was a gift from Dr. M. Rehli (University of Regensburg, Regensburg, Germany), or stably transfected THP-1 cells were cultured in RPMI 1640 containing 100 U/ml penicillin and 100 μg/ml streptomycin in the presence of 10% FBS (5% CO$_2$, 37˚C). Culture medium for transfected THP-1 cells additionally contained 1 mg/ml G-418 sulfate (Geneticin). Before stimulation of monocytes and vitamin D3-treated THP-1 cells, monocytes were separated in cell culture tubes (2 × 10$^6$) using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. DNase I treatment and reverse transcription were performed as described previously (33).

Total RNA was isolated from monocytes (4 × 10$^6$) and THP-1 cells (3 × 10$^6$) using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. DNase I treatment and reverse transcription were performed as described previously (33).

**Semiquantitative real-time PCR**

The reaction mixture contained 10 μl qSYBR Green supermix (Bio-Rad, Munich, Germany), 125 nM forward and reverse primers, and 1 μl cDNA template in a final volume of 20 μl. Expression of mRNA was analyzed with primers (34, 35) listed in Table I. Samples were run in duplicate in the 7300 real-time PCR cycler system (Applied Biosystems, Darmstadt, Germany). The reactions were performed under the following conditions: initial denaturation at 95˚C for 3 min, followed by 40 cycles of 15 s of denaturation at 95˚C, 30 s of primer annealing at 60˚C, and 30 s of extension/synthesis at 72˚C. Product quantification was optimal at 72˚C. Negative controls were performed with total RNA and water as template. Following PCR, the melting curve for each product was determined, and its correct size was estimated by agarose gel analysis. All cDNA products were designed as follows: shRNA_TRPM2 bottom strand, 5’-AGCTTTTC CAAAAGGCCCAGAAACATTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’; shRNA_scrambled bottom strand, 5’-GATCCCC CCAAATAGCCAGAGATGTCATCTTCACAGGATGCTCTCGG - TCCTATTTTTTTGAAA-3’; and shRNA_scrambled bottom strand, 5’-AGCTTTTC CAAAAGGCCCAGAAACATTCACGTCTTTGAAATCTGTGG - AGTCTTGCCCAGG-3’.

**FIGURE 1. TRPM2 mRNA levels in stimulated human primary monocytes.** Human primary monocytes were incubated in the presence or absence of LPS (100 ng/ml) (A) or in the presence and absence of Pam3CysSK4 (100 ng/ml), MDN (10 μg/ml), and TNF-α (1 ng/ml) (B) for the times indicated (A) and for 16 h (B) at 37˚C. After incubation, total RNA was isolated, and mRNA was reverse transcribed after DNase I digestion. TRPM2 mRNA was quantified by performing a semiquantitative real-time PCR using the IQ SYBR Green Supermix (Bio-Rad). Relative mRNA levels were standardized to the expression of the GAPDH housekeeping gene. Bars represent mean ± SEM (n = 3–7), *p ≤ 0.05; **p ≤ 0.01.
A human primary monocytes were incubated in the presence or absence of LPS (100 ng/ml), Pam3CysSK4 (100 ng/ml), MDP (10 µg/ml), and TNF-α (1 ng/ml) for the times indicated. Cells were lysed in permeabilization buffer and sonicated. Proteins (80 µg) were separated by SDS-PAGE and subjected to immunoblot analyses using a polyclonal rabbit anti-human TRPM2 serum and a mouse anti-human β-actin Ab, respectively. B, Cell lysates were incubated in the presence or absence of PNGase F (60,000 U/ml) for 1 h at 37˚C before performing SDS-PAGE and Western blot analysis. Specificity of TRPM2 detection was ascertained by performing a peptide competition assay (data not shown) and by specific TRPM2 knockdown via shRNA (Fig. 4). Shown is one representative experiment out of three.

Western blot analysis

Western blot analysis was carried out as described previously (36) with some modifications. Briefly, cells (2–4 × 10⁵/ml) were suspended in lysis buffer containing 10 mM Tris/HCl, 1 mM EDTA, 4 mM MgCl₂ (pH 7.8), and Complete protease inhibitor mixture (Roche, Mannheim, Germany) and sonicated after 20 min on ice. Samples for TRPM2 detection were run on a 7.5%, samples for TNF-α detection on a 15% SDS-polyacrylamide gel (Protein II; Bio-Rad) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Munich, Germany). Following blocking, membranes were probed with a polyclonal rabbit anti-human TRPM2 serum (1/500) or a goat anti-human TNF-α Ab (1/500; R&D Systems, Minneapolis, MN) overnight at 4˚C and with a mouse anti-human β-actin Ab (1/5000) for 1 h at room temperature, respectively. Subsequently, membranes were washed and then incubated with the respective secondary Ab (peroxidase-conjugated goat anti-rabbit Ab, 1/60,000, Dianova, Hamburg, Germany; HRP-conjugated donkey anti-goat IgG, 1/8,000, Santa Cruz Biotechnology, Santa Cruz, CA; peroxidase-conjugated goat anti-mouse Ab, 1/20,000) at room temperature for 1 h. After washing, the proteins were visualized by using Western lightning chemiluminescence (PerkinElmer, Boston, MA). To ascertain specificity of TRPM2 protein, a peptide competition assay was carried out. For this purpose, the polyclonal rabbit anti-human TRPM2 serum (1/500) was preincubated for 30 min with the respective peptide used for rabbit immunization (132 µg/ml Ab solution) before performing Western blot analysis. For PNGase F treatment (deglycosylation), whole-cell lysate (80 µg) of freshly isolated human monocytes was incubated in the presence or absence of PNGase F (60,000 U/ml) according to the manufacturer’s instructions before performing Western blot analysis.

Calcium imaging

Measurements of the LPS-induced changes in [Ca²⁺]i, in stably transfected THP-1 monocytes were carried out using the fluorescent indicator fura-2 in combination with a monochromator-based imaging system (T.I.L.L. Photonics, Gräfelfing, Germany) attached to an inverted microscope (BX51WI; Olympus, Hamburg, Germany). Emitted fluorescence was collected by a charge-coupled device camera. After addition of LPS, THP-1 cells were stored in culture medium at 37˚C. Every 30 min, 50 µl of each cell suspension (shRNA_scrambled and shRNA_TRPM2 transfected) was plated on poly-L-lysine–coated coverslips in 24-well microplates containing standard bath solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH), supplemented with 5 µM fura-2 and 0.01% Pluronic F-127. Cells were stored for 20 min at 20–22˚C and were used for experiments 5–15 min after loading with fura-2-acetoxyethyl ester. For measurements of [Ca²⁺]i, fura-2 fluorescence was excited at 340 and 380 nm, and emission from single cells was acquired in intervals of 2 s for at least 60 s. After correction for the individual background fluorescence, the fluorescence ratio R = F₃₄₀/F₃₈₀, representing [Ca²⁺], values, was calculated.

Electrophysiology

Whole-cell patch clamp experiments were performed in primary monocytes and THP-1 cells placed on coverslips mounted in a recording chamber (chamber volume ∼0.5 ml) connected to a solution drain driven by gravity feed at a rate of 4 ml/min. Membrane currents were recorded using an EPC-8 amplifier (HEKA, Lambrecht, Germany), subsequently low-pass filtered at 1 kHz, digitized with a sampling rate of 5 kHz, and analyzed using pCLAMP software (version 8.0; Axon Instruments, Union City, CA). The pipette resistance varied between 4 and 5 MΩ. Currents were elicited by voltage ramps from −100 mV to +100 mV (400-ms duration) applied every 2 s from a holding potential of 0 mV. Pipettes were filled with a solution composed of 50 mM CsCl, 25 mM CsO, 10 mM BAPTA. 8.3 mM CaCl₂, 4 mM

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**FIGURE 2.** TRPM2 protein levels in stimulated human primary monocytes. A, Human primary monocytes were incubated in the presence or absence of LPS (100 ng/ml), Pam3CysSK4 (100 ng/ml), MDP (10 µg/ml), and TNF-α (1 ng/ml) for the times indicated. Cells were lysed in permeabilization buffer and sonicated. Proteins (80 µg) were separated by SDS-PAGE and subjected to immunoblot analyses using a polyclonal rabbit anti-human TRPM2 serum and a mouse anti-human β-actin Ab, respectively. B, Cell lysates were incubated in the presence or absence of PNGase F (60,000 U/ml) for 1 h at 37˚C before performing SDS-PAGE and Western blot analysis. Specificity of TRPM2 detection was ascertained by performing a peptide competition assay (data not shown) and by specific TRPM2 knockdown via shRNA (Fig. 4). Shown is one representative experiment out of three.

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**FIGURE 3.** TRPM2 currents activated by ADPR in LPS-stimulated human primary monocytes. A, Time-dependent changes of inward currents (at −100 mV) elicited by obtaining the whole-cell (w.c.) configuration and infusion of a pipette solution containing 1 mM ADPR in a cultured unstimulated monocyte. The current-voltage relationships were obtained from responses during voltage ramps from −100 to +100 mV at the time points indicated. NMDG⁺-containing bath solution suppressed inward currents and ACA (20 µM) blocked inward and outward currents. B, Statistical analysis of ADPR-induced current responses measured at −100 mV in different monocyte preparations and under conditions as shown in A. In some cases, ADPR was not added to the pipette solution. Bars represent mean ± SEM. **p < 0.01.

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**FIGURE 4.** Expression of TRPM2 and ADPR-activated currents in human primary monocytes. A, TRPM2 protein levels were ascertained by Western blotting. B, ADPR-activated currents were measured in human primary monocytes with stable knockdown of TRPM2 via lentiviral shRNA.
Dissolved in DMSO giving stock solutions of 100 and 50 mM, respectively. Anthranilic acid (ACA; obtained from Tocris Cookson, Bristol, U.K.) was contained 1 mM ADPR. For extracellular Na+- and Ca2+-free conditions, the standard bath solution was exchanged for a solution containing 140 mM methyl-D-glucamine-Cl, 1 mM MgCl2, and 10 mM HEPES (pH 7.4 with ∼600 mM CsOH). The concentration of free Ca2+ in this solution was calculated to be ∼52 mM.

Viability of THP-1 cells was assessed by using the colorimetric MTT assay, based on the fact that viable cells are able to reduce MTT to purple formazan, which is measured spectrophotometrically. All data concerning levels of gene expression or secretion are expressed as means ± SEM of at least five cells from at least three independent experiments. Statistical significance was evaluated with unpaired Student’s t test.

Statistics

All data concerning levels of gene expression or secretion are expressed as means ± SEM from at least three independent experiments. Data from patch-clamp and calcium-imaging experiments are expressed as means ± SEM of at least five cells from at least three independent experiments. Statistical significance was evaluated with unpaired Student’s t test.

Results

TRPM2 expression in human primary monocytes is induced by diverse stimuli

Incubating human primary monocytes for 16 h in the presence of LPS led to a strong upregulation of TRPM2 mRNA expression (Fig. 1A, Table I). To test whether other ligands than LPS known to activate monocytes also share this effect, cells were incubated with Pam3CysSK4, MDP and TNF-α. Pam3CysSK4 is a synthetic analog derived from the N terminus of bacterial lipoprotein (39) and MDP has been identified as the minimal active structure of peptidoglycan (40). Signal transduction initiated by the four stimuli differs in so far as LPS and Pam3CysSK4 bind to TLR4 and TLR2, respectively. MDP in-
Stimulation with Pam3CysSK4, MDP, and TNF-α resulted in an increase in TRPM2 mRNA expression (Fig. 1B), indicating that upregulation of TRPM2 mRNA seems to be a common event associated with monocyte activation.

To determine whether regulation at the mRNA level was reflected by TRPM2 protein expression, Western blot analysis was carried out. Treatment of human primary monocytes with LPS markedly enhanced TRPM2 protein expression. This up-regulation also occurred after incubation of the cells with Pam3CysSK4, MDP, and TNF-α (Fig. 2A). The Ab used to detect TRPM2 recognized two proteins with molecular weights of ~160 and 175 kDa. Assuming that the difference in m.w. is due to glycosylation of the protein (TRPM2 is predicted to have five glycosylation sites), we incubated cell lysates with PNGase F, an enzyme that cleaves the Asn-linked glycoproteins (41). As seen in Fig. 2B, the higher m.w. protein disappeared after PNGase F treatment and the amount of the lower m.w. protein increased, indicating the presence of asparagine-linked glycosyl moieties on TRPM2.

TRPM2 currents are upregulated in LPS-treated primary monocytes

Because expression of both TRPM2 mRNA and protein was increased in LPS-stimulated monocytes compared with unstimulated cells, we investigated whether this upregulation is also reflected by ion channel activity. We therefore recorded whole-cell currents in human primary monocytes by using an intracellular pipette solution appropriate to full ADPR-induced activation of TRPM2 channels. TRPM2-mediated inward currents were evoked by infusion of the specific channel agonist ADPR (1 mM) (Fig. 3A). Indeed, omission of ADPR from the pipette solution prevented the activation of currents (Fig. 3B). To quantify the contribution of unspecific leak conductance to the ADPR-induced inward currents, we used an extracellular solution containing the TRPM2-impermeable cation NMDG⁺ instead of Na⁺ and Ca²⁺, or we applied the TRPM2 inhibitor ACA (42). Thus, ADPR-evoked inward currents reflect activity of TRPM2 cation channels. TRPM2 inward currents normalized to the cell capacitance were ~54 ± 5 pA/pF (n = 10) in freshly isolated monocytes. Cells stored for 16 h in culture medium showed inward current densities of −29 ± 5 pA/pF (n = 10) and −62 ± 5 pA/pF (n = 10) in the absence and presence of LPS, respectively (Fig. 3B). This increase in current density suggests a functional upregulation of TRPM2 channels in LPS-treated human primary monocytes, which is in agreement with the effect of LPS on TRPM2 mRNA and protein expression.

TRPM2 expression and activity is reduced in shRNA-treated cells

To address the question whether TRPM2 has a role in the inflammatory response, the human monocytic cell line THP-1 was used for additional experiments. Its stage of differentiation, that is closest to human monocytes, and its molecular manipulability make THP-1 cells an appropriate cell model for our purpose. Comparable with the effect of LPS on TRPM2 expression in primary monocytes, stimulation of THP-1 cells with LPS resulted in an upregulation of TRPM2 mRNA (Supplemental Fig. 1). THP-1 cells were stably transfected with shRNA to downregulate TRPM2 expression. As shown in Fig. 4A, knockdown of TRPM2 led to a drastic reduction of detectable protein in untransfected and LPS-treated cells. By contrast, transfection of THP-1 cells with the respective scrambled shRNA did not result in any significant modification of TRPM2 expression. The cell viability of the shRNA_TRPM2-transfected cells, incubated for 4 and 16 h in the presence or absence of LPS, did not differ from shRNA_scrambled-transfected or untransfected cells as determined by an MTT test (data not shown).

Next, we investigated whether the decrease in TRPM2 protein expression was mirrored by TRPM2 activity. ADPR-evoked whole-cell currents in shRNA_scrambled-transfected THP-1 cells showed densities of −71 ± 13 pA/pF (n = 5) and −131 ± 27 pA/pF (n = 7) in the absence and presence of LPS, respectively (Fig. 4B). However, transfection with shRNA_TRPM2 resulted in inward current densities of −11 ± 2 pA/pF in the absence of LPS (n = 5) and of −8 ± 2 pA/pF after LPS stimulation (n = 7), indicating a functional downregulation of TRPM2 under both conditions (Fig. 4C).

Ca²⁺ influx via TRPM2 plays a key role in LPS-induced cytokine production

Given the importance of Ca²⁺ signaling for immunocyte functions, we asked whether TRPM2-mediated Ca²⁺ influx contributes to
LPS-induced cytokine production in monocytic cells. We exposed shRNA_TRPM2- and shRNA_scrambled-transfected as well as untransfected THP-1 cells to LPS and measured TNF-α, IL-6, IL-8, and IL-10 expression at the mRNA (Fig. 5A–D, Table I) and protein level (Fig. 5E–H). As a result, TNF-α, IL-6, IL-8, and IL-10 mRNA expression was significantly reduced in shRNA_TRPM2-transfected cells compared with shRNA_scrambled-transfected cells (Fig. 5A–D). The decrease in mRNA expression was mirrored by a diminished cytokine production (Fig. 5E–H).

To analyze the TRPM2-induced decrease in TNF-α secretion in shRNA_TRPM2-transfected cells in more detail, we next studied the expression of the precursor form of TNF-α (pro–TNF-α), a 26-kDa transmembrane type II polypeptide, and the soluble 17-kDa polypeptide, which is derived from the precursor form by proteolytic cleavage (43, 44). After incubating the cells for 4 h in the absence or presence of LPS, cell lysates were analyzed by Western blotting using a polyclonal goat anti-human TNF-α Ab and a mouse anti-human β-actin Ab, respectively. Shown is one representative experiment out of three.

FIGURE 6. Expression of pro–TNF-α in lysates of shRNA-treated THP-1 cells. Transfected (shRNA_TRPM2 and shRNA_scrambled) and untransfected (shRNA_scrambled-transfected) THP-1 cells were incubated in the presence or absence of LPS (100 ng/ml) at 37˚C. After 4 h, cells were lysed in permeabilization buffer and sonicated. Proteins (120 µg) were separated by SDS-PAGE and subjected to immunoblot analyses using a polyclonal goat anti-human TNF-α Ab and a mouse anti-human β-actin Ab, respectively. Shown is one representative experiment out of three.

FIGURE 7. TRPM2-mediated Ca2+ influx is required for LPS-induced TNF-α-secretion. A, Transfected (shRNA_TRPM2, shRNA_scrambled) and untransfected THP-1 cells were incubated in the presence or absence of LPS (100 ng/ml) and EGTA (0–5 nM) for 4 h at 37˚C. Culture supernatants were collected from stimulated THP-1 cells. The concentrations of TNF-α in the culture supernatants were determined by ELISA. Bars represent means ± SEM from (n = 3). *p ≤ 0.05; **p ≤ 0.01. Statistical difference within each cell population was tested between EGTA-treated and -untreated cells. B, LPS-induced changes of basal [Ca2+], indicated by the ratio of fluorescence intensities (F340/F380) in fura 2-loaded, transfected (shRNA_TRPM2, shRNA_scrambled) THP-1 cells. Data points are means ± SEM (n = 31–61 cells from three independent experiments). **p ≤ 0.01, shRNA_TRPM2 versus shRNA_scrambled cells.
data clearly indicate that Ca\textsuperscript{2+} influx through TRPM2 channels contributes to the LPS-induced increase in [Ca\textsuperscript{2+}], which appears to be necessary for cytokine production.

**Discussion**

In this study, we show that the Ca\textsuperscript{2+}-permeable channel TRPM2 plays a key role in LPS-induced cytokine production in human monocytes. When analyzing the TRPM2 expression at the mRNA and protein level, we found strong signals in freshly isolated monocytes. The signals further increased when the cells were exposed to LPS and by far exceeded those detected in unstimulated controls. In addition to the enhanced protein synthesis, another means to regulate protein activity involves posttranslational protein modification such as glycosylation. We could show that monocytes express TRPM2 in a glycosylated and nonglycosylated version, whereas a regulation of TRPM2 protein by stimuli such as LPS could only be shown for the nonglycosylated form of TRPM2. Dietrich et al. (45) suggested a role of N-linked protein glycosylation as a major determinant for basal activity of TRPC3 and TRPC6 channels, which raises the possibility that the N-glycosylation state may be associated with differences in functional activities.

Besides LPS, other potent monocyte activators, including Pam3CysSK\textsubscript{4}, MDP, and TNF-\alpha, affect TRPM2 expression. Although the stimuli do not act by the same signal transduction mechanism, they all seem to initiate biochemical reactions, resulting in enhanced levels of TRPM2 protein synthesis. Thus, expression of sufficient functionally active TRPM2 channels seems to be one mean by which activated monocytes are enabled to fulfill some of their multiple functions.

We could indeed show that downregulation of TRPM2 results in an impaired TNF-\alpha, IL-6, IL-8, and IL-10 secretion. These data together with the finding that THP-1 cells produce little TNF-\alpha in the absence of extracellular Ca\textsuperscript{2+} clearly demonstrate that the uptake of extracellular Ca\textsuperscript{2+} via TRPM2 plays an important role in LPS-induced cytokine production. However, as TRPM2 knockdown and omission of extracellular Ca\textsuperscript{2+} did not completely block LPS-induced TNF-\alpha production, the involvement of additional Ca\textsuperscript{2+}-mobilizing pathways may account for the residual TNF-\alpha response. Treatment of monocytes with LPS has been reported to cause an increase in [Ca\textsuperscript{2+}], which is related to the production of TNF-\alpha (46, 47). These Ca\textsuperscript{2+} signals appeared within minutes after addition of micromolar LPS (46, 47). When extending the time course of Ca\textsuperscript{2+} measurements, we found the most dramatic changes in basal Ca\textsuperscript{2+} to take place in the first 2 h after exposure of 100 ng/ml LPS. A similar LPS-dependent kinetics of [Ca\textsuperscript{2+}], has been observed in mouse microglial cells (48). Having shown that the elevation in [Ca\textsuperscript{2+}], after LPS exposure was strongly reduced in shRNA_TRPM2-transfected cells, we suggest that TRPM2 represents one of the so far unknown channels that mediate Ca\textsuperscript{2+} uptake in LPS-stimulated monocyte cells.

The extended transient Ca\textsuperscript{2+} increase after LPS exposure observed in our study occurs in a time frame that would allow for regulating long-term effects such as cytokine production. Long-term effects of other immune cells, such as activation and differentiation of B and T cells, that require transcriptional programming also depend on sustained Ca\textsuperscript{2+}/calcineurin signaling, whereas brief activation of Ca\textsuperscript{2+} suffices for acute secretory processes such as mast cell degranulation (49). In contrast, a rapid transient rise in [Ca\textsuperscript{2+}], induced in monocytes by the application of H\textsubscript{2}O\textsubscript{2} has been reported to trigger chemokine production in monocytes (31). Thus, the physiological responses induced by Ca\textsuperscript{2+} strongly depend on the type of immune cells studied, the stimulus used, and the timescale of Ca\textsuperscript{2+} signaling.

At present it is unknown which second messengers link LPS to TRPM2 activation and Ca\textsuperscript{2+} entry. Possible candidates are ADPR, NAD\textsuperscript{+}, cADPR, and nicotinic acid–adenine dinucleotide phosphate. In neutrophils stimulated with iMLP, ADPR has been identified as the activating molecule, which in cooperation with Ca\textsuperscript{2+} ions released from intracellular stores, enables Ca\textsuperscript{2+} influx through TRPM2 (50). Data showing that the iMLP-induced Ca\textsuperscript{2+} response and in vitro migration are suppressed in TRPM2-deficient neutrophils point to an important role of TRPM2 in the activation process of these cells (31). Another well-described activator of TRPM2 is H\textsubscript{2}O\textsubscript{2} (17, 19, 22, 51), an experimental paradigm of oxidative stress. Because granulocytes and monocytes at sites of inflammation not only generate reactive oxygen intermediates, including H\textsubscript{2}O\textsubscript{2}, but are also exposed to H\textsubscript{2}O\textsubscript{2}, much interest has focused on the biological significance of H\textsubscript{2}O\textsubscript{2}-induced TRPM2 activation. Whereas in most experiments the role of TRPM2 in H\textsubscript{2}O\textsubscript{2}-mediated cell death has been studied [review by Miller (20)], Yamamoto et al. (31) could demonstrate that the application of moderate amounts of H\textsubscript{2}O\textsubscript{2} induced TRPM2-dependent IL-8 production in monocytes. Although using a different stimulus, the data of Yamamoto et al. (31) are in line with the results presented in this study. Furthermore, the authors found that TRPM2-knockout mice were largely protected from dextran sulfate sodium-induced experimental colitis, an ROS-associated inflammatory model, suggesting that TRPM2 has major roles in the progressive severity of inflammation.

In summary, we have identified TRPM2 at the mRNA, protein, and functional levels in human peripheral blood monocytes. By silencing protein expression in THP-1 cells using TRPM2-specific shRNA, we demonstrated that TRPM2 channels regulate LPS-induced cytokine production by allowing Ca\textsuperscript{2+} entry across the plasma membrane. Considering its involvement in inflammatory processes, TRPM2 could serve as an important target for therapeutic intervention in diseases such as sepsis or rheumatoid arthritis.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Supplementary information

TRPM2 is required for LPS-induced cytokine production in human monocytes

Supplementary Figure 1. TRPM2-mRNA levels in stimulated THP-1 cells.
THP-1 cells were incubated in the presence or absence of LPS (100 ng/ml) for 16 h (C) at 37 °C. After incubation, total RNA was isolated and mRNA was reverse transcribed after DNAseI digestion. TRPM2-mRNA was quantified by performing a semiquantitative real time PCR using the IQ™ SYBR® Green Supermix (BioRad). Relative mRNA levels (ΔΔCt-method) were standardized to the expression of the GAPDH housekeeping gene. Bars represent means ± SEM (n = 5). ** p ≤ 0.01.
Supplementary Figure 2. Kinetics of LPS-induced TNF-α production in THP-1 cells.

Transfected (shRNA_TRPM2, shRNA_scrambled) and untransfected THP-1 cells were incubated at 37 °C in the presence or absence of LPS (100 ng/ml) for the times indicated. (A) After incubation, total RNA was isolated and mRNA was reverse transcribed after DNAseI digestion. TNF-α mRNA was quantified by performing a semiquantitative real time PCR using the IQ™ SYBR® Green Supermix (BioRad). Relative mRNA levels (ΔΔCt-method) were standardized to the expression of the GAPDH housekeeping gene and mRNA concentrations of shRNA_scrambled cells at 0 h were set as the 100% reference. (B) Culture supernatants were collected from stimulated THP-1 cells. The concentrations of TNF-α in the culture supernatants were determined by ELISA. Bars represent means ± SEM from (n = 3).