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

Janine Wehrhahn, Robert Kraft, Christian Harteneck and Sunna Hauschildt

*J Immunol* 2010; 184:2386-2393; Prepublished online 27 January 2010;
doi: 10.4049/jimmunol.0902474

http://www.jimmunol.org/content/184/5/2386

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/01/27/jimmunol.0902474.DC1

References

This article cites 51 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/184/5/2386.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 immunocytes, 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 down-regulate 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 in 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.
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'-deoxyxymethyloxide 5'-triphosphate, restriction enzymes (Roche, Indianapolis, IN); 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-acetoxymethyl ester, oligonucleotide synthesis, pCMV-mito-myc, Pluronic F-127, and 100-bp DNA ladder (Invitrogen, Karlsruhe, Germany); PGNase F and Quick Ligation Kit (New England Biolabs, Frankfurt/Main, Germany); G-418 sulfate and RPMI 1640 medium (with t-glutamine, 25 mM HEPES, and phenol red) (PAA, Pasching, Austria); penicillin and streptomycin (Seromed Biochrom, 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 (Sigma-Aldrich, Taufkirchen, Germany). Sequencing was performed by GATC Biotech (Konstanz, Germany).

Cell separation and cell culture

Human PBMCs from healthy donors were obtained by centrifugation over a Ficoll-Isoapaque (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) as described previously (32). The purity of the cell preparation was >90% as assessed by morphological screening and immunofluorescence staining with a mAb against CD14 (clone 2G8, Becton Dickinson, Heidelberg, Germany).

Human primary monocytes, untransfected THP-1 human myeloid leukemia cells (gift from Dr. M. Rehli, University of Regensburg, Regensburg, Germany), or stably transfected THP-1 cells were cultured in RPMI 1640 containing 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 was performed in cell culture tubes (2 10$^6$/ml) for 1, 4, and 16 h, respectively, at 37˚C and 5% CO$_2$ in cell culture medium in the presence or absence of LPS at 100 ng/ml, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-R-cysteinyl-(lysyl)-3-hydroxy-4-methyl-2-enoyl-CoA reductase (Pam, CysSK$_2$, EMC Microcollections, Tübingen, Germany) at 100 ng/ml, and muramyl dipeptide (MDP, InvivoGen, Toulouse, France) at 10 ng/ml or human TNF-α (World Health Organization International Standard, code 88786; National Institute for Biological Standards and Control, Hertfordshire, U.K.) at 1 ng/ml.

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

The basic plasmid (pHi-1 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. Niere (University of Bergen, Bergen, Norway). The basic shRNA vector pH1 RNA promoter was modified by adding a GFP expression cassette (multiple cloning site was removed by BglII and AgeI digestion), which was amplified from pEGFP-N1 (BD Clontech, Mountain View, CA) with specific primers, additionally encoding a DraIII restriction site (forward, 5'-AAAAAACACTACCTGTTGATAACCGTGTTCCACCTGAGCG-3', and reverse, 5'-AAAAAACACTACCTGTTGATAACCGTGTTCCACCTGAGCG-3'). The double-stranded oligonucleotides encoding for the TRPM2-specific shRNA (shRNA_TRPM2) and the respective control shRNA (shRNA_scrambled; unspecific sequence derived from shRNA_TRPM2 by scrambling) were designed as follows: shRNA_TRPM2 top strand, 5'-GATCCCCGGG-CCAAGAAGCTTCAACATGAGATCTTGAAGATCTTTGAAGTCTTTGGAAGTTCTTGGCCCGGGG-3'; shRNA_scrambled bottom strand, 5'-GATCCCCCCCTTTTTGGAAA-3'; shRNA_scrambled top strand, 5'-GATCCCCCCCTTTTTGGAAA-3'; and shRNA_scrambled bottom strand, 5'-GATCCCCCCCTTTTTGGAAA-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 Amazexa cell line nucleofector 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 FACSscan 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 iQSYBR 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

![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 Pam, CysSK$_2$, MDP (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 (ΔΔCt method) were standardized to the expression of GAPDH housekeeping gene. Bars represent means ± SEM (n = 3–7). *p ≤ 0.05; **p ≤ 0.01.](http://www.jimmunol.org/)
were confirmed by sequencing to check their identity. Calculations (ΔΔC_{\text{t}} method) were carried out as described previously (36).

**Western blot analysis**

Western blot analysis was carried out as described previously (37) with some modifications. Briefly, cells (2–4 × 10^7/ml) were suspended in lysis buffer containing 10 mM Tris/HCl, 1 mM EDTA, 4 mM MgCl_2 (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 were run on a 15% SDS-polyacrylamide gel (Protean 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 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.

**Calcium imaging**

Measurements of the LPS-induced changes in [Ca^{2+}]{\text{}} 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 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 = F_{340}/F_{380}, representing [Ca^{2+}], 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 CsOH, 10 mM BAPTA, 8.3 mM CaCl_2, 4 mM

**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), Pam3CysSK_{4} (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.

**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 means ± SEM. **p ≤ 0.01.
Dissolved in DMSO giving stock solutions of 100 and 50 mM, respectively. Anthranilic acid (ACA; obtained from Tocris Cookson, Bristol, U.K.) was dissolved in distilled water, and ADPR was dissolved in Na2ATP, 2 mM MgCl₂, and 10 mM HEPES (pH 7.2 with NaOH). The concentration of free Ca²⁺ in this solution was calculated to be ∼10⁻⁶ M.

To induce activation of TRPM2 channels, the pipette solution using the software WinMAXC (version 2.05; C. Patton, Stanford University, Stanford, CA). To control whether TRPM2 knockdown was successful and whether LPS affects TRPM2 expression in THP-1 cells, shRNA_TRPM2- and shRNA_scrambled-transfected and untransfected cells were incubated in the presence or absence of LPS (100 ng/ml) at 37°C for 16 h. Cells were lysed in permeabilization buffer and sonicated. Proteins (60 μg) were separated by SDS-PAGE and subjected to immunoblot analyses using a polyclonal rabbit-anti-TRPM2 serum and a mouse anti-human β-actin Ab, respectively. Shown is one representative experiment out of three. B, Currents in shRNA_TRPM2- and shRNA_scrambled-transfected THP-1 cells were evoked by obtaining the whole-cell (w.c.) configuration and infusion of a pipette solution containing 1 mM ADPR. Both cell lines were stimulated with 100 ng/ml LPS. The time course of currents at −100 mV and the current-voltage relationships were obtained from responses during voltage ramps from −100 to +100 mV. C, Averaged current responses measured at −100 mV in shRNA_TRPM2- and shRNA_scrambled-transfected THP-1 cells in the presence or absence of LPS represent means ± SEM (n = 5–7 cells each). **p ≤ 0.01; ***p ≤ 0.001.

NAD⁺, ATP, 2 mM MgCl₂, and 10 mM HEPES (pH 7.2 with ∼52 mM CsOH). The concentration of free Ca²⁺ in this solution was calculated to be ∼600 nM using the software WinMAXC (version 2.05; C. Patton, Stanford University, Stanford, CA). To induce activation of TRPM2 channels, the pipette solution contained 1 mM ADPR. For extracellular Na⁺- and Ca²⁺-free conditions, the standard bath solution was exchanged for a solution containing 140 mM N-methyl-D-glucamine-Cl, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4 with HCl). ADPR was dissolved in distilled water, and N-(p-amylocinnamoyl) anthranilic acid (ACA; obtained from Tocris Cookson, Bristol, U.K.) was dissolved in DMSO giving stock solutions of 100 and 50 mM, respectively.

Assays of cell viability

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 (38). After stimulation of THP-1 cells, 0.3 mg/ml MTT was added to the cell culture for 2 h. Cells were lysed overnight at 37°C by adding equal volumes of a buffer containing 20% (v/v) SDS in 50% (v/v) N,N-dimethyl formamide solution. Extinctions were determined in quadruplicates at 570 nm.

Apoptosis or necrosis of monocytes was assessed using the TACS Annexin V FITC apoptosis detection kit (R&D Systems), in which apoptotic cells are labeled with annexin V conjugated to FITC, and necrotic cells are labeled with propidium iodide. The test was performed according to the manufacturer’s instruction on a FACScan flow cytometer (BD Biosciences).

Detection of cytokines in culture supernatants

Culture supernatants were collected from THP-1 cells for measurement of TNF-α, IL-6, IL-8 and IL-10 concentrations. The concentrations of TNF-α were determined by ELISA. Primary anti-TNF-α Abs as well as the secondary purified rabbit polyclonal TNF-α Abs were provided by W. Buttmann, Maastricht University, Maastricht, The Netherlands. The peroxidase-labeled goat anti-rabbit Ab was from Dianova. TNF-α standard was purchased from NIBSC (World Health Organization International Standard, human, natural, code: 88/786; National Institute for Biological Standards and Control). The peroxidase substrate tetramethylbenzidine was from Calbiochem (Merck, Darmstadt, Germany).

Secreted IL-6, IL-8, and IL-10 were detected by the BD Cytometric Bead Array (Human IL-6, IL-8, and IL-10 Flex Set and Human Soluble Protein Master Buffer Kit; BD Biosciences) on a BD FACSAarray bioanalyzer, according to the manufacturer’s instructions.

Statistical analyses

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 Pam₃CSK₄, MDP and TNF-α. Pam₃CSK₄ and MDP have been identified as the minimal active structure of peptidoglycan (40). Signal transduction initiated by the four stimuli differs in so far as LPS and Pam₃CSK₄ bind to TLR4 and TLR2, respectively. MDP interacts with the intracellular nucleotide-binding oligomerization domain-like receptors (40) and TNF-α binds to TNF-α receptors.

Table I. List of primers used in real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH real time forward (34)</td>
<td>CAGTTCCAGCCATGACTGAC</td>
</tr>
<tr>
<td>GAPDH real time reverse (34)</td>
<td>CTGAACTGGCGTCCGAG</td>
</tr>
<tr>
<td>TRPM2 real time forward</td>
<td>TCAAGCTGCTTCTCTCTCTG</td>
</tr>
<tr>
<td>TRPM2 real time reverse</td>
<td>GGTTGCTGAGGTTGTTAT</td>
</tr>
<tr>
<td>TNF-α real time forward</td>
<td>AAAACGGACGAGGAGATG</td>
</tr>
<tr>
<td>TNF-α real time reverse</td>
<td>CAGTACAGGCGGTGATCAT</td>
</tr>
<tr>
<td>IL-6 real time forward (35)</td>
<td>GGTACGAGGCAGGAGCATG</td>
</tr>
<tr>
<td>IL-6 real time reverse (35)</td>
<td>CAGTTCAGGCTCTCTCTCTT</td>
</tr>
<tr>
<td>IL-8 real time forward (35)</td>
<td>GGTACGAGGCAGGAGCATG</td>
</tr>
<tr>
<td>IL-8 real time reverse (35)</td>
<td>CAGTTCAGGCTCTCTCTCTT</td>
</tr>
<tr>
<td>IL-10 real time forward</td>
<td>AAGGGTATCTCACCCTGCTC</td>
</tr>
<tr>
<td>IL-10 real time reverse</td>
<td>AAGGGTATCTCACCCTGCTC</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jimmunol.org/ by guest on April 30, 2017
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, we 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 asparagine-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 pipeline solution appropriate for 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. Comparably 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 unstimulated 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...
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.

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 assess the role of extracellular Ca²⁺ influx in TNF-α production, THP-1 cells were incubated for 4 h in culture medium containing the chelating agent EGTA to remove extracellular Ca²⁺. EGTA led to a concentration-dependent decrease in LPS-mediated TNF-α production of untransfected as well as shRNA_scrambled-transfected cells and to a minor extent also of shRNA_TRPM2-transfected cells (Fig. 7A). Treatment with EGTA did not affect the viability as assessed by the use of an annexin V apoptosis test (data not shown).

Having shown the crucial role of extracellular Ca²⁺ for TNF-α production, we next tested the effect of TRPM2 channel activation on [Ca²⁺], in LPS-treated cells. Therefore, [Ca²⁺], was measured in shRNA_TRPM2 and shRNA_scrambled THP-1 cells at different time points up to 5 h after the addition of LPS. In control cells (shRNA_scrambled), LPS caused a gradual increase in [Ca²⁺], which peaked after 2 h and then returned to values slightly exceeding normal levels (Fig. 7B). According to the kinetics, elevated Ca²⁺ concentrations can already be observed at time points (30 min) that precede maximum RNA (Supplemental Fig. 2A) and protein expression (Supplemental Fig. 2B) of the cytokines studied in this article. Although LPS also induced a continuous yet small increase in [Ca²⁺], in shRNA_TRPM2-transfected cells, Ca²⁺ values leveled off after 3 h without having reached a peak.
data clearly indicate that Ca\(^{2+}\) influx through TRPM2 channels contributes to the LPS-induced increase in [Ca\(^{2+}\)]\(_i\), which appears to be necessary for cytokine production.

**Discussion**

In this study, we show that the Ca\(^{2+}\)-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\(_4\), 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\(^{2+}\) clearly demonstrate that the uptake of extracellular Ca\(^{2+}\) via TRPM2 plays an important role in LPS-induced cytokine production. However, as TRPM2 knockdown and omission of extracellular Ca\(^{2+}\) did not completely block LPS-induced TNF-\(\alpha\) production, the involvement of additional Ca\(^{2+}\)-mobilizing pathways may account for the residual TNF-\(\alpha\) response. Treatment of monocytes with LPS has been reported to cause an increase in [Ca\(^{2+}\)]\(_i\), which is related to the production of TNF-\(\alpha\) (46, 47). These Ca\(^{2+}\) signals appeared within minutes after addition of micromolar LPS (46, 47). When extending the time course of Ca\(^{2+}\) measurements, we found the most dramatic changes in basal Ca\(^{2+}\) to take place in the first 2 h after exposure of 100 ng/ml LPS. A similar LPS-dependent kinetics of [Ca\(^{2+}\)]\(_i\) has been observed in mouse microglial cells (48). Having shown that the elevation in [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\) uptake in LPS-stimulated monocytes.

The extended transient Ca\(^{2+}\) 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\(^{2+}\)/calcineurin signaling, whereas brief activation of Ca\(^{2+}\) suffices for acute secretory processes such as mast cell degranulation (49). In contrast, a rapid transient rise in [Ca\(^{2+}\)]\(_i\) induced in monocytes by the application of H\(_2\)O\(_2\) has been reported to trigger chemokine production in monocytes (31). Thus, the physiological responses induced by Ca\(^{2+}\) strongly depend on the type of immune cells studied, the stimulus used, and the timescale of Ca\(^{2+}\) signaling.

At present it is unknown which second messengers link LPS to TRPM2 activation and Ca\(^{2+}\) entry. Possible candidates are ADPR, NAD\(^+\), cADPR, and nicotinic acid–adenine dinucleotide phosphate. In neutrophils stimulated with IL-1\(\beta\), ADPR has been identified as the activating molecule, which in cooperation with Ca\(^{2+}\) ions released from intracellular stores, enables Ca\(^{2+}\) influx through TRPM2 (50). Data showing that the IL-1\(\beta\)-induced Ca\(^{2+}\) 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\(_2\)O\(_2\) (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\(_2\)O\(_2\), but are also exposed to H\(_2\)O\(_2\), much interest has focused on the biological significance of H\(_2\)O\(_2\)-induced TRPM2 activation. Whereas in most experiments the role of TRPM2 in H\(_2\)O\(_2\)-mediated cell death has been studied [review by Miller (20)], Yamamoto et al. (31) could demonstrate that the application of moderate amounts of H\(_2\)O\(_2\) 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\(^{2+}\) 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.

**Acknowledgments**

We thank Dr. Marc Niere from the University of Bergen for supporting us with the basic shRNA vector pH1-RNA promoter. IL-6, IL-8, and IL-10 cytokometric bead arrays were performed by Dr. Manja Kamprad (Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany). Vitamin D\(_3\) was a gift from F. Hoffmann-La Roche. We also thank Prof. Stefan Feske (New York University Langone Medical Center, New York, NY) for critically reading the manuscript.

**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).