The redox-sensitive cation channel TRPM2 modulates phagocyte ROS production and inflammation

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The NADPH oxidase activity of phagocytes and its generation of reactive oxygen species (ROS) is critical for host defense, but ROS overproduction can also lead to inflammation and tissue injury. Here we report that TRPM2, a nonselective and redox-sensitive cation channel, inhibited ROS production in phagocytic cells and prevented endotoxin-induced lung inflammation in mice. TRPM2-deficient mice challenged with endotoxin (lipopolysaccharide) had an enhanced inflammatory response and diminished survival relative to that of wild-type mice challenged with endotoxin. TRPM2 functioned by dampening NADPH oxidase–mediated ROS production through depolarization of the plasma membrane in phagocytes. As ROS also activate TRPM2, our findings establish a negative feedback mechanism for the inactivation of ROS production through inhibition of the membrane potential–sensitive NADPH oxidase.

The NADPH oxidase-dependent production of reactive oxygen species (ROS) in phagocytic cells in response to infection has a key role in the mechanism of inflammation1–5. Many mechanisms, including changes in the plasma membrane potential3,4, Ca2+ influx and Ca2+-dependent activation of protein kinase C-α (PKC-α)5,6, induce the activation of NADPH oxidase. PKC-α phosphorylates the NADPH oxidase Nox2 subunit p47phox, resulting in its translocation to the plasma membrane, which is needed for assembly of the oxidase complex6. TRPM2 (transient receptor potential-melastatin 2)7,8 is a nonselective cation channel permeable to Na+ and Ca2+ (the selectivity of TRPM2 for Ca2+ over Na+ is 0.5–1.6)9. So far, most studies have addressed the influx of Ca2+ through the redox-sensitive TRPM2 channel9,10,11. We surmised that TRPM2-induced Ca2+ influx should enhance the activation of NADPH oxidase through the activation of Ca2+-dependent PKC isoforms5,6; however, here we found that activation of TRPM2 resulted in less NADPH oxidase–activated production of ROS while at the same time increasing membrane depolarization. We addressed the mechanism of TRPM2 regulation of ROS production in phagocytes and its relationship to changes in membrane potential and the functional importance of TRPM2 in mediating lung inflammatory injury induced by the endotoxin lipopolysaccharide (LPS). We found, by a patch-clamping approach combined with biochemistry, a correlation between less production of ROS and plasma membrane depolarization caused by activation of TRPM2 in phagocytic cells. The activation of TRPM2 resulted in improved survival of endotoxemic mice and less lung oxidative damage, as well as the production of inflammatory cytokines and chemokines. Thus, TRPM2, a nonselective cation channel, protected the lung from inflammatory injury by dampening NADPH oxidase activity in phagocytes and diminishing ROS production.

RESULTS

Protective role of TRPM2 in lung inflammation

In the dextran sulfate sodium–induced model of colitis, TRPM2-deficient (Trpm2−/−) mice have less chemokine production, infiltration by polymorphonuclear leukocytes (PMNs) and ulceration12. We therefore determined whether Trpm2−/− mice were similarly protected in a model of endotoxin-induced lung inflammation. Contrary to results obtained for dextran sulfate sodium–induced colitis inflammation, we observed more release of chemokines and proinflammatory cytokines, including tumor necrosis factor, the chemokine CXCL2 (MIP-2) and interleukin 6, in Trpm2−/− mouse lungs than in Trpm2+/+ mouse lungs (Fig. 1a–c). LPS also induced significantly more lung tissue myeloperoxidase activity in Trpm2−/− mice than in Trpm2+/+ mice (Fig. 1d), which indicated more sequestration of inflammatory PMNs in Trpm2−/− mouse lungs. Inflammation induced by LPS in mice is characterized by rapid sequestration of PMNs in response to the release of chemokines and cytokines after activation of the redox-sensitive proinflammatory transcription factor NF-κB13. We also noted higher expression of NF-κB in the lungs of Trpm2−/− mice during LPS-induced inflammation (Supplementary Fig. 1). Furthermore, we observed more lung infiltration by inflammatory cells, more lung edema and diminished survival for LPS-challenged Trpm2−/− mice.
Deletion of TRPM2 augments endotoxin-induced lung inflammation and injury. (a-c) LPS-induced production of CXCL2 (a), tumor necrosis factor (TNF; b) and interleukin 6 (IL-6; c) in lungs after challenge of Trpm2−/− and Trpm2+/+ mice with LPS (10 mg per kg body weight (mg/kg), administered intraperitoneally). *P = 0.036, **P = 0.0003 and ***P = 0.0008 (a); *P = 0.036 and **P = 0.037 (b); and **P = 0.016 and ***P = 0.013 (c), compared with Trpm2+/+ mice (t-test). (d) Sequestration of lung PMNs in mice challenged for 0–6 h with LPS (as in a–c), assessed as tissue myeloperoxidase activity (MPO). A460, absorbance at 460 nm. *P = 0.055 and **P = 0.022, compared with Trpm2+/+ mice (t-test). (e) Hematoxylin-and-eosin staining of sections of lung tissue isolated from Trpm2+/+ and Trpm2−/− mice left unchallenged (left) or challenged for 20 h with LPS (20 mg/kg, administered intraperitoneally; right). Scale bars, 200 µm. (f) Formation of pulmonary edema in Trpm2+/+ and Trpm2−/− lungs after LPS challenge (as in e), assessed as wet weight of lungs. *P = 0.006 (t-test). (g) Survival of Trpm2−/− and Trpm2+/+ mice after intraperitoneal injection of LPS (30 mg/kg). P = 0.0007 (log-rank test). Data are representative of four experiments (a,b,g), six experiments (c), three (1 h) or five (6 h) experiments (d), or three experiments (e,f; mean and s.e.m. in a–d,f,g).

Figure 1

Oxidative lung injury in TRPM2-deficient mice

As ROS are crucial for the mechanism of lung inflammation and injury14, we next determined whether TRPM2 participates in regulating ROS production. Given that TRPM2 is a nonselective cation channel permeable to Na+ and Ca2+, we initially hypothesized that it mediates the production of ROS in phagocytes by conducting Ca2+ into cells6. However, Trpm2−/− PMNs and bone marrow–derived macrophages (BMDMs) stimulated with LPS or the phorbol ester PMA had more ROS production than did LPS- or PMA-challenged Trpm2+/+ cells (Fig. 2a–d). The greater ROS production required NADPH oxidase, as diphenylethiondiamide (DPI), an inhibitor of NADPH oxidase15, prevented ROS production in both Trpm2+/+ and Trpm2−/− macrophages (Fig. 2c). To confirm the inhibitory role of TRPM2 in ROS production, we used DPQ (3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone), a cell-permeable inhibitor of poly(ADP-ribose) polymerase that prevents the generation of adenosine diphosphoribose (ADPR), to block TRPM2 channel function16,17. The production of ROS by Trpm2+/+ macrophages pretreated for 30 min with 100 µM DPQ was increased to the amount in Trpm2−/− macrophages, whereas pretreatment with DPQ did not further increase the release of ROS from Trpm2−/− macrophages (Fig. 2c,d).

To further elucidate the role of TRPM2 in blocking ROS production, we measured intracellular ROS in Trpm2+/+ and Trpm2−/− macrophages. ROS production by cells from Trpm2−/− mice challenged with PMA or live Escherichia coli was much greater than that of Trpm2+/+ cells (Supplementary Fig. 2a). Trpm2−/− mice also had a lower bacterial burden than that of Trpm2+/+ mice (Supplementary Fig. 2b), whereas the extent of phagocytosis was unaltered (Supplementary Fig. 2c). To evaluate the contribution of oxidative damage in mediating the lung inflammatory injury seen in Trpm2−/− mice, we first used immunochemistry to assess 8-hydroxydeoxyguanosine, a sensitive marker of oxidative damage of DNA18,19, in mouse lungs. The expression of 8-hydroxydeoxyguanosine was much higher in Trpm2−/− lungs than in Trpm2+/+ lungs (Fig. 2e,f). The greater production of tumor necrosis factor by macrophages from Trpm2−/− mice was diminished to the same degree as that in macrophages from Trpm2+/+ mice when the former cells were treated with DPI (Fig. 2g). We also observed new injury for endothelial cells interacting with LPS-stimulated macrophages from Trpm2+/+ mice than for those interacting with cells from Trpm2−/− mice (Supplementary Fig. 3), which indicated a greater potential of TRPM2-deficient phagocytes than of wild-type phagocytes to injure the interacting target cells (in this case, endothelial cells). These results showed that inhibition of the production of ROS by phagocytic cells was an important mechanism of TRPM2-mediated protection in LPS-induced inflammation.

Modulation of the membrane potential by TRPM2 regulates ROS

We next investigated the mechanism by which TRPM2 inhibited ROS production in phagocytic cells. We initially surmised that TRPM2, as a Ca2+/ permeable channel, would induce ROS production because of the role of Ca2+ in activating NADPH oxidase via Ca2+-dependent PKC-α in phagocytes20; however, the persistence of substantial ROS production in the absence of TRPM2 activation (Fig. 2a–d) indicated another pathway. Indeed PMA-induced activation of PKC-α in BMDMs was unchanged regardless of TRPM2 expression (Fig. 3a). Moreover, the phosphorylation of p47phox induced by PMA was similar in Trpm2−/− and Trpm2+/+ macrophages (Fig. 3b), which suggested that the mechanism of ROS production in Trpm2−/− macrophages was beyond the assembly of the NADPH oxidase complex. Thus, we tested the hypothesis that TRPM2 controls ROS production by regulating the membrane potential of phagocytes because NADPH oxidase activity and membrane potential are linked3,4,20. We measured ROS production at various membrane potentials induced by changes in the extracellular K+ concentration. We found a correlation between lower membrane potential (depolarization) and more extracellular K+ in mouse BMDMs (Supplementary Fig. 4). Likewise, the greater ROS production in Trpm2−/− macrophages was diminished to the amount in Trpm2+/+ macrophages when membrane potential was controlled at the same degree by an increase in extracellular K+ (to 120 mM) in both Trpm2+/+ and Trpm2−/− macrophages (Fig. 3c,d).

We next determined by patch clamping whether TRPM2 activation directly altered the membrane potential. We found that the addition...
of ADPR, the potent activator of TRPM2 (ref. 8), through a patching pipette significantly depolarized the plasma membrane in Trpm2+/+ macrophages (from $-78 \pm 6$ mV to $-14 \pm 6$ mV) but not in the Trpm2−/− macrophages (from $-82 \pm 5$ mV to $-74 \pm 7$ mV; Fig. 4a,b). Although PMA depolarized the plasma membrane of both Trpm2+/+ macrophages (from $-81 \pm 2$ mV to $-10 \pm 4$ mV) and Trpm2−/− macrophages (from $-82 \pm 5$ mV to $-50 \pm 8$), it caused significantly less depolarization of Trpm2−/− macrophages (Fig. 4c,d). We also determined the difference between Trpm2+/+ and Trpm2−/− cells in membrane potential and its relationship to the release of ROS (Fig. 4e).

To confirm the hypothesis that TRPM2 regulates ROS production by altering the membrane potential, we next addressed the role of the entry of Na+ through TRPM2 in mediating the inhibition of ROS production. First we determined whether ROS production can be restored to normal amounts in Trpm2−/− macrophages by providing artificial cationic conductance with gramicidin, a nonselective Na+-permeable channel that depolarizes the plasma membrane21,22. ROS production induced by PMA was diminished to the same amount in both Trpm2+/+ and Trpm2−/− macrophages in the presence of gramicidin (40 µg/ml; Fig. 4f,g). We also determined the contribution of Na+ entry through TRPM2 to the regulation of ROS production by measuring changes in ROS production elicited by PMA in a solution containing NMDG (N-methyl-D-glucamine) instead of Na+. We surmised that Na+ removal would mimic the effects of TRPM2 ablation and Trpm2+/+ cells would therefore produce more ROS in the presence of NMDG. The substitution of NMDG for Na+ augmented ROS production in Trpm2+/+ cells, whereas it had little effect in Trpm2−/− cells (Fig. 4f,g). Together these results demonstrated that TRPM2 profoundly downregulated NADPH oxidase–mediated ROS production through cation influx–dependent depolarization of the plasma membrane potential (Supplementary Fig. 5).

**LPS activates TRPM2 through ROS generation**

TRPM2 can be activated by the binding of ADPR to the nudix hydrolase box (NUDT9-H) in its carboxyl terminus23,25 and through ROS-mediated generation of ADPR26. The addition of 200 µM ADPR through the patch pipette elicited a linear, nonselective cation current in both PMNs and BMDMs, and iso-osmotic replacement of external Na+ by the impermeant organic cation NMDG abolished the inward current (Supplementary Fig. 6a–d). Trpm2+/+ PMNs and Trpm2+/− BMDMs failed to respond to ADPR. We also assessed TRPM2 activation by monitoring Ca2+ influx with the calcium indicator

**Figure 2 Enhanced ROS generation and oxidative lung inflammatory injury after deletion of TRPM2.** (a–d) Release of ROS from Trpm2+/+ and Trpm2−/− PMNs left unstimulated (control (Ctrl)) or stimulated with LPS (1 µg/ml; a,b) and from Trpm2+/+ and Trpm2+/− BMDMs left unstimulated or stimulated with PMA (1 µM) with or without pretreatment for 30 min at 37 °C with DPQ (100 µM) or additional treatment with DPI (10 µM; c,d); results are presented as counts per second (c.p.s.) of light emitted. *P = 0.00007 (b) or 0.007 (d; t-test). (e,f) Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHdG; brown) in lung sections from Trpm2+/+ and Trpm2−/− mice left unchallenged or challenged for 20 h with LPS (20 mg/kg); nuclei (N) are stained with hematoxylin (blue). Scale bars, 100 µm (e) or 20 µm (f). (g) Release of tumor necrosis factor from mouse BMDMs left unstimulated or stimulated with LPS with or without DPI. *P = 0.017 and **P = 0.0014, compared with Trpm2+/+ + LPS; ***P = 0.00006, compared with Trpm2+/− + LPS (t-test). Data are representative of three experiments (a,b,e,f), six experiments (c,d) or four experiments (g; error bars (b,d,g), s.e.m.).
Figure 3 TRPM2 has no effect on the phosphorylation of PKC-α or p47phox, and a high concentration of extracellular K+ inhibits ROS production in Trpm2+/− and Trpm2+/+ macrophages. (a) Phosphorylation (p-) of PKC-α in Trpm2+/− and Trpm2+/+ BMDMs stimulated for 2 min with PMA (1 μM); total PKC-α (below) serves as a loading control. (b) Phosphorylation of p47phox in Trpm2+/− and Trpm2+/+ BMDMs pretreated for 2 min with PMA (1 μM), evaluated by immunoprecipitation of p47phox and immunoblot analysis with antibody to phosphorylated serine (p-p47) and to p47phox (p47). (c) Release of ROS in BMDMs over time from untreated or PMA-treated Trpm2+/− and Trpm2+/+ BMDMs, with or without DPI, in 5 mM or 120 mM extracellular K+ (key). (d) Summary of data obtained in c. *P = 0.002, compared with Trpm2+/+ cells (t-test). Data are representative of three experiments (a) or four experiments (b–d; mean and s.e.m. in d).

Fura-2. The addition of LPS (1 µg/ml) to the extracellular medium induced a transient increase in the intracellular concentration of Ca2+ ([Ca2+]i) in BMDMs from Trpm2−/− mice (Fig. 5a,b). The LPS-induced increase in [Ca2+]i was significantly lower in cells from Trpm2−/− mice and was also sensitive to the inhibition of NADPH oxidase with DPI (Fig. 5a,b). These results showed that the LPS-induced production of ROS activated TRPM2.

We also used Fura-2 to measure the increase in [Ca2+]i, induced by H2O2. This increase was significantly lower in cells from Trpm2−/− mice (Fig. 5c,d). The increase in [Ca2+]i in Trpm2+/+ macrophages pretreated with DPQ (100 µM) was diminished to that in Trpm2+/+ macrophages, whereas DPQ produced no further decrease in [Ca2+]i in Trpm2−/− macrophages (Fig. 5c,d). These results demonstrated a key role for ADPR in mediating ROS-induced activation of TRPM2.

Compensation for Ca2+ entry secondary to TRPM2 deletion

We next addressed the possibility that deletion of TRPM2 may lead to enhanced Ca2+ signaling induced by stimuli through other redox-sensitive Ca2+ channels. We assessed changes in intracellular Ca2+ induced by platelet-activating factor (PAF), a Ca2+-mobilizing agonist that fails to induce an oxidative burst (except at the very high concentration of ~1 μM). We therefore avoided any confounding effect of the ensuing membrane depolarization in diminishing the driving force for Ca2+ entry in cells as demonstrated above. The addition of PAF (0.1 μM) increased [Ca2+]i with two peaks—an initial rapid response of high amplitude, followed by a slow response of lower amplitude—in cells from Trpm2+/+ and Trpm2−/− mice (Fig. 5e,f). Notably, PAF did not induce ROS production in BMDMs from Trpm2+/+ or Trpm2−/− mice (Supplementary Fig. 7). There was also no difference between Trpm2+/+ and Trpm2−/− BMDMs in the

Figure 4 TRPM2 inhibits ROS production in macrophages through plasma membrane depolarization. (a) Change in plasma membrane potential over time in Trpm2+/+ and Trpm2−/− BMDMs treated with ADPR. (b) Summary of the data in a. *P = 0.0005, compared with Trpm2+/− + ADPR (t-test). (c) Change in plasma membrane potential over time in Trpm2+/+ and Trpm2−/− BMDMs treated with PMA (1 μM) in the presence of 5 mM or 120 mM K+. (d) Summary of the data in c. *P = 0.000004, compared with Trpm2−/− cells (t-test). (e) Correlation of membrane potential with ROS production in BMDMs stimulated with PMA (1 μM) in the presence of 5 mM K+ in the extracellular solution (based in part on data in Figs. 3d and 4d). (f) Release of ROS over time from Trpm2+/+ and Trpm2−/− BMDMs left unstimulated or stimulated with PMA (1 μM) alone (+ PMA) or with gramicidin (40 µg/ml; + PMA + GC) or with replacement of Na+ with NMDG (+ PMA + NMDG). (g) Summary of the data in f. *P = 0.0003 and **P = 0.0006, compared with Trpm2+/+ + PMA (t-test). Data are representative of six experiments (a,b), fifteen experiments (c,d), five experiments (e), or six or four experiments (f,g; mean and s.e.m. in b,d,g).
increase in [Ca\textsuperscript{2+}], induced by PAF (Fig. 5e,f). Therefore, deletion of TRPM2 did not increase the [Ca\textsuperscript{2+}], induced by PAF in the absence of ROS production.

As compensatory expression and activation of ion channels may have been induced in the Trpm2\textsuperscript{−/−} mouse model, we next determined whether Ca\textsuperscript{2+} activation of nonselective cation channels\textsuperscript{31,32} was altered in Trpm2\textsuperscript{−/−} cells. We studied these channels because they also regulate membrane potential\textsuperscript{33}. TRPM4 and TRPM5 have been identified as two channels of this class\textsuperscript{31,32,34,35}. The addition of 100 µM Ca\textsuperscript{2+} through the patch pipette elicited an outward-rectified nonselective cation current in both Trpm2\textsuperscript{+/+} and Trpm2\textsuperscript{−/−} macrophages (Supplementary Fig. 8). The property of this outward-rectified current (the amplitude of the current at positive potential was greater than the currents at negative potential) induced by Ca\textsuperscript{2+} was consistent with published reports of TRPM4 and TRPM5 (refs. 32,34,35). Our results showed that the activation of nonselective cation channels (or TRPM4 and TRPM5) was not affected by deletion of TRPM2 (Supplementary Fig. 8). Thus, deletion of TRPM2 was not compensated for by changes in the activation of other related family members (TRPM4 and TRPM5), and compensation for these channels probably does not explain the substantial differences between Trpm2\textsuperscript{+/+} and Trpm2\textsuperscript{−/−} mice in lung inflammatory injury shown above.

**DISCUSSION**

TRPM2 has been found in many cell types of the immune system, including dendritic cells, PMNs, monocytes, macrophages and lymphocytes\textsuperscript{36,37}; however, the function of TRPM2 in such cells has remained elusive. The oxidant-dependent mechanism of the activation of TRPM2 suggests the involvement of this channel in pathogenic processes such as carcinogenesis, inflammation, ischemia-reperfusion injury, neurodegenerative disorders and diabetes\textsuperscript{38}. In such studies, the TRPM2 channel has emerged as an important Ca\textsuperscript{2+}-signaling mechanism that contributes to cellular functions such as cytokine production, insulin release, cell motility and cell death\textsuperscript{39}. In dendritic cells, TRPM2 regulates intracellular Ca\textsuperscript{2+} and is required for optimal maturation and chemotaxis\textsuperscript{30}. Dendritic cells express TRPM2 ‘preferentially’ in endolysosomal compartments, which release Ca\textsuperscript{2+} after exposure to intracellular ADPR or stimulation by chemokines. In monocytes, the impairment of chemokine production in cells lacking TRPM2 has been linked to a defect in TRPM2-mediated Ca\textsuperscript{2+} influx\textsuperscript{31,37}. Our study has demonstrated a previously unknown function for TRPM2 in which it acts as an essential modulator of plasma membrane potential by conducing the influx of cations, including both Ca\textsuperscript{2+} and Na\textsuperscript{+}. Our results obtained with phagocytic cells showed that activation of the redox-sensitive TRPM2 inhibited ROS production, which suggests that TRPM2 serves as a crucial negative feedback mechanism for ROS production.

The question arises of how TRPM2, a member of the TRP family of nonselective cation channels, downregulates ROS production. The superoxide anion radical O\textsubscript{2}\textsuperscript{−} is generated after assembly of the NADPH oxidase and activation through the transfer of electrons from cytosolic NADPH to extracellular oxygen\textsuperscript{38,39}. Electron flux associated with membrane depolarization\textsuperscript{60} would therefore inhibit any further transfer of electrons across the membrane by NADPH oxidase if a compensating charge is not provided. This could explain the voltage dependence of NADPH oxidase activity\textsuperscript{4,41}. To facilitate ROS production, the transferred extracellular electrons must be neutralized by protons (H\textsuperscript{+}) transported extracellularly through voltage-gated proton channels\textsuperscript{3,42}. Voltage-gated proton channels are open when the plasma membrane is depolarized during NADPH oxidase activation. By moving positive charges (Ca\textsuperscript{2+} and Na\textsuperscript{+}) across the plasma membrane, TRPM2 induces membrane depolarization and thereby inhibits the electrogenic activity of NADPH oxidase and blocks O\textsubscript{2}\textsuperscript{−} production. Although there is no doubt about the voltage-dependent nature of NADPH oxidase activity, the range of voltage dependence has varied in different studies. It has been reported that NADPH oxidase is voltage independent from −100 mV to >0 mV but is steeply inhibited by further depolarization and is abolished at about +190 mV (ref. 3). It was subsequently shown that NADPH oxidase activity is very voltage dependent and steeply decreases with depolarization in the physiological range of membrane potential (−60 mV to 60 mV). The apparent voltage independence has been attributed to limitation of the substrate NADPH and not to any intrinsic property of NADPH oxidase\textsuperscript{41}. Here we have shown that the nonselective cation channel TRPM2 conducted a positive charge (Ca\textsuperscript{2+} and Na\textsuperscript{+}) into phagocytes and thus suppressed NADPH oxidase activity when the plasma membrane was negative. Under those conditions, TRPM2 activation is expected to diminish electron transfer through NADPH oxidase by...
the transport of cations by activated TRPM2 into cells. Our results have thus identified a key role of TRPM2-dependent inhibition of NADPH oxidase activity in inactivating ROS production.

Ion channels have a critical role in the array of pathological responses to sepsis. The sepsis mediator LPS can either activate or inhibit ion channels. Our study has described a protective anti-inflammatory role for TRPM2 in LPS-induced lung injury and has demonstrated a previously unknown function for TRPM2 in dampening NADPH oxidase–mediated production of ROS by phagocytic cells. TRPM2 mediated the transmembrane flux of cations down the electrochemical gradient, raising the concentration of intracellular cations and depolarizing the plasma membrane. The depolarization inhibited ROS production in phagocytes and prevented lung inflammatory injury induced by LPS. Our findings have identified an intrinsic negative feedback mechanism that functions at the level of the assembled NADPH oxidase complex. TRPM2 activation in phagocytic cells during sepsis may thus have a unique ability to prevent NADPH oxidase–derived ROS production and thereby protect the host against inflammation and tissue injury.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

A.D. and A.B.M. designed the study; A.D., X.-P.G., F.Q., T.K., J.H., C.H. and S.M.V. did experiments and data analysis; and A.D., R.D.Y. and A.B.M. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice, cell cultures and reagents. C57BL/6 mice were from Charles River Laboratory. Trpm2+/− mice (obtained from B.A. Miller) were generated and originally provided by GlaxoSmithKline46. Another group has independently generated Trpm2−/− mice47; those were not used here. Trpm2−/− mice were backcrossed with C57BL/6 mice for five generations to eliminate any background effects on the observed phenotypes. Trpm2−/− and wild-type (Trpm2+/+) littermates were used. All mice were housed in the University of Illinois Animal Care Facility in accordance with institutional guidelines and guidelines of the US National Institutes of Health. Veterinary care of these animals and related animal experiments was approved by the University of Illinois Animal Resources Center. Mouse BMDMs were isolated and cultured as described37. Mouse PMNs were isolated from mouse bone marrow with Percoll density gradients as described48. Mouse lung vascular endothelial cells were isolated from Trpm2+/+ and Trpm2−/− mice as described49. PMA (phorbol-12-myristate-13-acetate), LPS (from E. coli strain 0111:B4) and H2O2 were from Sigma. Polyclonal antibody to PKC-α (sc-208) was from Santa Cruz Biotechnology; polyclonal antibodies to p47phox (06-822) were from Millipore; and antibody to p47phox phosphorylated at Ser473, after immunoprecipitation with antibody to p47phox, was from Invitrogen.

Whole-cell recordings. Electrophysiological recordings were obtained by the voltage-clamp technique. All experiments were done at 22–24 °C with an EPC-10 patch-clamp amplifier and the Pulse V 8.8 acquisition program (HEKA Electronik). TRPM2 whole-cell currents were measured by a method similar to that described before50. Currents were elicited by a series of test pulses ranging from −110 mV to +110 mV (200 ms in duration and delivered at intervals of 2 s). The holding potential was 0 mV. The pipette solution contained 135 mM CsSO4, 8 mM NaCl, 2 mM MgCl2, 0.5 mM CaCl2, 1 mM EGTA and 10 mM HEPES, pH 7.2. ADPR (200 µM) or PMA (1 µM) was included in the pipette solution, or H2O2 (300 µM) was perfused into the cells to induce TRPM2 current. For the induction of Ca2+-activated cation currents, 100 µM free intracellular Ca2+ was included in the pipette solution. The bath solution contained 145 mM NaCl, 2 mM CaCl2, 1 mM MgCl2 and 10 mM HEPES, pH 7.4. In some experiments, NaCl in the bath solution was replaced by NMDG (also 145 mM). For perforated patch clamps, ~500 mg solubilized amphotericin B was added to the pipette solution. Whole-cell currents were analyzed with IGOR software (WaveMetrics).

Plasma membrane potential recordings. Plasma membrane potentials were recorded by current clamp. The pipette solution contained 135 mM KCl, 8 mM NaCl, 2 mM MgCl2, 0.5 mM CaCl2, 1 mM EGTA and 10 mM HEPES, pH 7.2. The bath solution contained 5 mM KCl, 140 mM NaCl, 2 mM CaCl2, 1 mM MgCl2 and 10 mM HEPES, pH 7.4. For study of the effect of the extracellular K+ concentration on membrane potential in resting cells, Na+ in the bath solution was replaced by an equal amount of K+. In perforated patch clamp, ~500 mg solubilized amphotericin B was added to the pipette solution. Changes in membrane potential were analyzed with IGOR software.

Measurement of ROS production in phagocytic cells. BMDMs or PMNs (5 × 106) from Trpm2+/+ and Trpm2−/− mice were treated with PMA or LPS. ROS generation in cultured medium was measured by luminol-enhanced chemiluminescence as described50 with a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences). For this, luminol was added to the coculture medium to a final concentration of 50 µM, and horseradish peroxidase was added to a final concentration of 40 units per ml. The release of ROS into the culture medium was assessed after cells were stimulated with the appropriate stimulus (or stimuli) for the desired time.

Fluorometric measurement of [Ca2+]i. [Ca2+]i was recorded in single cells as described51. For this, an Axiocam 200 inverted microscope (Zeiss) was illuminated with a DeltaRay dual-wavelength monochromer-based illumination system (Photon Technology International) with a xenon lamp and an R928 photon-counting photomultiplier (Hamamatsu) and optical coupling to the microscope. Cells were loaded for 30 min at 37 °C with 2 µM Fura-2 (acetoxyethyl ester) in Hank’s balanced-salt solution. The fluorescence of Fura-2 at 340 nm and 380 nm was acquired and analyzed offline with Felix 32 software (Photon Technology International).

Mortality studies. Trpm2+/+ and Trpm2−/− mice (40 per group) were injected intraperitoneally with LPS (30 mg/kg) and survival was documented every 2 h.

Immunoprecipitation and immunoblot analysis. Activation of PKC-α was assessed with an antibody to phosphorylated PKC-α and activation of p47phox was assessed with antibody to p47phox phosphorylated at Ser473, after immunoprecipitation of p47 with antibody to p47. Antibody to nonphosphorylated PKC-α or p47 was used to assess protein loading.

Measurement of cytokine and chemokine concentrations by enzyme-linked immunosorbent assay. Trpm2+/+ and Trpm2−/− mice were killed by intraperitoneal injection of anesthesia at the appropriate time after LPS injection. Whole lungs were then obtained for measurement of cytokine and chemokine concentrations. Lungs were homogenized and centrifuged. Supernatants were collected for measurement of chemokine concentrations by enzyme-linked immunosorbent assay with a Bio-Plex Multiplex Cytokine Assay (Bio-Rad).

Lung tissue myeloperoxidase activity. Myeloperoxidase activity in lung tissues was measured as described52. Lungs were homogenized and centrifuged and supernatants were then collected and mixed with assay buffer (o-dianisidine hydrochloride (0.2 mg/ml) and 0.0005% (vol/vol) H2O2). The change in absorbance was measured at 460 nm for 3 min, and MPO activity was calculated as the change in absorbance over time. PMN sequestration was quantified as MPO activity normalized by the final dry-lung weight.

Statistical analysis. Differences in survival rates were assessed by the log-rank test. Other statistical comparisons were made with the two-tailed Student’s t-test. The significance of differences between groups was determined with a two-tailed t-test.

Additional methods. Information on the isolation of nuclear proteins and electrophoretic mobility-shift assays, measurement of the generation of intracellular ROS, E. coli infection and determination of bacterial loads, and macrophage-mediated endothelial cytotoxicity is available in the Supplementary Methods.

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Corrigendum: The redox-sensitive cation channel TRPM2 modulates phagocyte ROS production and inflammation

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In the version of this article initially published, the description of the Trpm2−/− mice in the first paragraph of the Online Methods was incomplete. That section should read as follows: "Trpm2−/− mice (obtained from B.A. Miller) were generated and originally provided by GlaxoSmithKline. Another group has independently generated Trpm2−/− mice; those were not used here." The new reference (46) is as follows: Knowles, H. et al. Transient receptor potential melastatin 2 (TRPM2) ion channel is required for innate immunity against Listeria monocytogenes. Proc. Natl. Acad. Sci. USA 108, 11578–11583 (2011). The error has been corrected in the HTML and PDF versions of the article.