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) showed an enhanced inflammatory response and lower 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 inflammation[1,2]. Many mechanisms, including changes in the plasma membrane potential[3,4], Ca^{2+} influx and Ca^{2+}-dependent activation of protein kinase Cα (PKC-α)[5,6], induce the activation of NADPH oxidase. PKC-α phosphorylates the NADPH oxidase Nox2 subunit $p47^{phox}$, resulting in its translocation to the plasma membrane, which is needed for assembly of the oxidase complex[6]. TRPM2 (transient receptor potential-melastatin 2 [7,8] is a nonselective cation channel permeable to Na^+ and Ca^{2+} (the selectivity of TRPM2 for Ca^{2+} rather than Na^+ is 0.5–1.6)[9]. So far, most studies have addressed the influx of Ca^{2+} through the redox-sensitive TRPM2 channel[8,10,11]. We surmised that

TRPM2-induced Ca^{2+} influx should enhance NADPH oxidase activation through activation of Ca^{2+}-dependent PKC isoforms[5,6]; however, here we show 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 an endotoxin (lipopolysaccharide (LPS)). We found, by a patch-clamping approach combined with biochemistry, correlation between lower ROS production and plasma membrane depolarization caused by TRPM2 activation in phagocytic cells. TRPM2 activation resulted in more 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 lowering 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 ulceration[12]. 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 *Trpm2*$^{+/+}$ mice (**Fig. 1d**), which indicated more sequestration of inflammatory PMNs in *Trpm2*$^{-/-}$ mouse lungs. Inflammation induced by LPS 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-κB[13]. 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 lower survival for LPS-challenged *Trpm2*$^{-/-}$ mice compared with LPS-challenged *Trpm2*$^{+/+}$ mice (**Fig. 1e-g**). These results demonstrated a protective role of TRPM2 in LPS-induced lung inflammation.

Oxidative lung injury in TRPM2-deficient mice

As ROS are crucial for the mechanism of lung inflammation and injury[14], we next determined whether TRPM2 participates in regulating ROS production. Given that TRPM2 is a nonselective cation channel permeable to Na^+ and Ca^{2+}, we initially hypothesized that it mediates the production of ROS in phagocytes by conducting Ca^{2+} into cells[6]. However, *Trpm2*$^{-/-}$ mice had more ROS production by PMNs and bone marrow–derived macrophages (BMDMs) stimulated with LPS or the phorbol ester PMA than that in LPS- or PMA-challeged *Trpm2*$^{+/+}$ mice (**Fig. 2a–d**). The greater ROS production required NADPH oxidase, as diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase[15], prevented ROS production in both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ macrophages (**Fig. 2c**). To confirm the inhibitory role for 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 function[16,17]. The production of ROS by *Trpm2*$^{+/+}$ macrophages pretreated for 30 min with 100 μM DPQ was increased to the amount seen in *Trpm2*$^{-/-}$ macrophages, whereas DPQ pretreatment 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 in cells from *Trpm2*$^{-/-}$ mice challenged with PMA or live *Escherichia coli* was substantially greater than that of *Trpm2*$^{+/+}$ cells (**Supplementary Fig. 2a**). *Trpm2*$^{-/-}$ mice also had a lower bacterial burden than that of wild type 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 DNA[18,19], 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 more 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 Ca^{2+} permeable channel, would induce ROS production because of the role of Ca^{2+} in activating NADPH oxidase via Ca^{2+}-dependent PKC-α in phagocytes[6]; 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 $p47^{phox}$ 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 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 linked[3,4,20]. 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 level 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 ± 6 mV to –14 ± 6 mV) but not in the *Trpm2*$^{-/-}$ macrophages (from –82 ± 5 mV to –74 ± 7 mV; **Fig. 4a**,**b**). Although PMA depolarized the plasma membrane of both *Trpm2*$^{+/+}$ macrophages (from –81 ± 2 mV to –10 ± 4 mV) and *Trpm2*$^{-/-}$ macrophages (from –82 ± 2 mV to –50 ± 8), it caused significantly less depolarization of *Trpm2*$^{-/-}$ macrophages (**Fig. 4c**,**d**). We also assessed the difference between *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ cells in membrane potential and its relationship to ROS release (**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 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 membrane[21,22]. 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 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 should 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**). These results together 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, Nudix-type motif 9 homology) in its carboxyl terminus[23–25] and through ROS-mediated generation of ADPR[26]. 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 determined TRPM2 activation by H_2O_2, a known TRPM2-activating ROS[27,28]. Perfusion of cells with H_2O_2 (300 μM) elicited a nonselective cation current in *Trpm2*$^{+/+}$ PMNs, and iso-osmotic replacement of external Na^+ by NMDG abolished the inward current. Although perfusion of cells with H_2O_2 also activated a small outward current in *Trpm2*$^{-/-}$ cells, H_2O_2 failed to induce the inward current in *Trpm2*$^{-/-}$ cells (**Supplementary Fig. 6e**). Blocking the inward Na^+ current with NMDG resulted in left shift of the reversal potential (membrane potential at zero transmembrane current; **Supplementary Fig. 6e**). These findings demonstrated that the cells were hyperpolarized in the absence of Na^+, which therefore favored ROS production.

We took advantage of permeability of TRPM2 to Ca^{2+} to study TRPM2 activation by monitoring Ca^{2+} influx with the calcium indicator Fura-2. The addition of LPS (1 μg/ml) to the extracellular medium induced a transient increase in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in BMDMs from *Trpm2*$^{+/+}$ mice (**Fig. 5a**,**b**). The increase in $[Ca^{2+}]_i$ induced by LPS was significantly lower in cells from *Trpm2*$^{-/-}$ mice. The LPS-induced increase in $[Ca^{2+}]_i$ was also sensitive to the inhibition of NADPH oxidase with DPI (**Fig. 5a**,**b**). These results showed that LPS-induced production of ROS activated TRPM2.

We also used Fura-2 to measure the increase in $[Ca^{2+}]_i$ induced by H_2O_2. This increase was significantly lower in cells from *Trpm2*$^{-/-}$ mice (**Fig. 5c**,**d**). The increase in $[Ca^{2+}]_i$ in *Trpm2*$^{+/+}$ macrophages pretreated with DPQ (100 μM) was diminished to the concentration in *Trpm2*$^{-/-}$ macrophages, whereas DPQ produced no further decrease $[Ca^{2+}]_i$ in *Trpm2*$^{-/-}$ macrophages (**Fig. 5c**,**d**). These results showed a key role for ADPR in mediating ROS-induced activation of TRPM2.

Compensation for Ca^{2+} entry secondary to TRPM2 deletion

We next addressed the possibility that deletion of TRPM2 may lead to enhanced Ca^{2+} signaling induced by stimuli through other redox-sensitive Ca^{2+} channels. We determined changes in intracellular Ca^{2+} induced by platelet-activating factor (PAF), a Ca^{2+}-mobilizing agonist[29] that fails to induce oxidative burst (except at the very high concentration of ~1 μM)[30]. We therefore avoided any confounding effect of the ensuing membrane depolarization in diminishing the

driving force for Ca^{2+} entry in cells as demonstrated above. The addition of PAF (0.1 μM) increased $[Ca^{2+}]_i$ with two peaks—a 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 increase in $[Ca^{2+}]_i$ induced by PAF (**Fig. 5e**,**f**). Therefore TRPM2 deletion did not increase $[Ca^{2+}]_i$ induced by PAF in the absence of ROS production.

As compensatory expression and activation of ion channels may have been induced in the *Trpm2*$^{-/-}$ mouse model, we next determined whether Ca^{2+} activation of nonselective cation (CAN) channels[31,32] was altered in *Trpm2*$^{-/-}$ cells. We studied CAN channels because they also regulate membrane potential[33]. TRPM4 and TRPM5 have been identified as two channels of the CAN class [31,32,34,35]. The addition of 100 μM Ca^{2+} through the patch pipette elicited an outward-rectified nonselective cation current in both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ 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^{2+} was consistent with reports on TRPM4 and TRPM5 (refs. 32,34,35). Our results showed that the activation of CAN (or TRPM4 and TRPM5) was not affected by the deletion of TRPM2 (**Supplementary Fig. 8**). Thus, the 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*$^{+/+}$ and *Trpm2*$^{-/-}$ 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[36,37]; however, the function of TRPM2 in such cells has remained elusive. The oxidant-dependent mechanism of activation of TRPM2 suggests the involvement of this channel in pathogenic processes such as carcinogenesis, inflammation, ischemia-reperfusion injury, neurodegenerative disorders and diabetes[37]. In such studies, the TRPM2 channel has emerged as an important Ca^{2+}-signaling mechanism that contributes to

cellular functions such as cytokine production, insulin release, cell motility and cell death[37]. In dendritic cells, TRPM2 regulates intracellular Ca^{2+} and is required for optimal maturation and chemotaxis[36]. Dendritic cells express TRPM2 'preferentially' in endolysosomal compartments, which release Ca^{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^{2+} influx[12,37]. Our study has demonstrated a previously unknown function for TRPM2 in which it acts as an essential modulator of plasma membrane potential by conducting the influx of cations, including both Ca^{2+} and Na^{+}. 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 of 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_2^- is generated after assembly of the NADPH oxidase and activation through the transfer of electrons from cytosolic NADPH to extracellular oxygen[38,39]. Electron flux associated with membrane depolarization[40] 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[4,41]. To facilitate ROS production, the transferred extracellular electrons must be neutralized by protons (H^+) transported extracellularly through voltage-gated proton channels[3,42]. Voltage-gated proton channels are open when the plasma membrane is depolarized during NADPH oxidase activation. By moving positive charges (Ca^{2+} and Na^{+}) across the plasma membrane, TRPM2 induces membrane depolarization and thereby inhibits the electrogenic activity of NADPH oxidase and blocks O_2^- 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 has subsequently been shown that NADPH oxidase activity is very voltage dependent and steeply decreases with depolarization in the physiological membrane potential range (–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[41]. Here we have shown that the nonselective cation channel TRPM2 conducted a positive charge (Ca^{2+} and Na^{+}) 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 carrying cations into cells. Our results thus identify the 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[43,44]. The sepsis mediator LPS can either activate or inhibit ion channels[44,45]. 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.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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Figure 1 TRPM2 deletion augmented endotoxin-induced lung inflammation and injury. (**a**–**c**) LPS-induced production of CXCL2 (MIP-2; **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). (**a**) **P* = 0.036, ***P* = 0.0003 and ****P* = 0.0008, compared with *Trpm2*$^{+/+}$ mice (t-test). (**b**) **P* = 0.036 and ***P* = 0.037, compared with *Trpm2*$^{+/+}$ mice (t-test). (**c**) **P* = 0.016 and ***P* = 0.013, 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). A_{460}, 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 challenged for 20 h with LPS (20 mg/kg, administered intraperitoneally). 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**); six experiments (**c**); three (1 h) or five (6 h) experiments (**d**); three experiments (**e**); three experiments (**f**); or four experiments (**g**; mean and s.e.m. in **a**–**d**,**g**). **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 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**) Production of tumor necrosis factor by mouse BMDMs left unstimulated or simulated with LPS with or without DPI. **P* = 0.017, compared with *Trpm2*$^{+/+}$ + LPS; ***P* = 0.0014, compared with *Trpm2*$^{+/+}$ + LPS; ****P* = 0.00006 compared with *Trpm2*$^{-/-}$ + LPS (t-test). Data are representative of three experiments (**a**,**b**), six experiments (**c**,**d**); three experiments (**e**,**f**); ; or four experiments (**g**).

Figure 3 TRPM2 has no effect on phosphorylation of PKC-α or p47^{phox} and high extracellular potassium 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 serves as a loading control. (**b**) Phosphorylation of p47^{phox} in *Trpm2*$^{-/-}$ and *Trpm2*$^{+/+}$ BMDMs pretreated for 2 min with PMA (1 μM), evaluated by immunoprecipitation of p47^{phox} and immunoblot analysis with antibody to phosphorylated serine (p-p47) and to p47^{phox} (p47). (**c**) Release of ROS in BMDMs over time from PMA-treated *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDMs in 5 mM or 120 mM extracellular K^+ (key). (**d**) Summary of data obtained in **c**. **P* = 0.002 compared with *Trpm2*$^{+/+}$ group. Data are representative of three experiments (**a**) or four experiments (b–**d**; mean and s.e.m. in **d**).

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* = 0.00004, 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**).

Figure 5 LPS-induced entry of Ca^{2+} into macrophages depends on TRPM2 expression. (**a**) $[Ca^{2+}]_i$ over time in *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDMs treated with LPS (1 μg/ml) alone or with DPI (1 μM), presented as the ratio of basal $[Ca^{2+}]_i$ to steady-state $[Ca^{2+}]_i$. (**b**) Average of results in **a**. **P* = 0.016, compared with *Trpm2*$^{-/-}$ mice (t-test). (**c**) $[Ca^{2+}]_i$ over time in *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDMs given no pretreatment or pretreated with DPQ, and then treated with H_2O_2. (**d**) Average of results in **c**. **P* = 0.008, compared with *Trpm2*$^{-/-}$ cells (t-test). (**e**) $[Ca^{2+}]_i$ over time in *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDMs treated with PAF (0.1 μM), presented as the summary of the amplitudes of the two peaks (fast increase and slow increase), calculated by measurement of the changes in ratio from basal $[Ca^{2+}]_i$ to steady-state $[Ca^{2+}]_i$. (**f**) Average of results in **e**. Data are representative of six experiments (**a**,**b**), seven experiments (**c**,**d**) or four experiments (**e**,**f**; mean and s.e.m. in **b**,**d**,**f**).

ONLINE METHODS

Mice, cell cultures and reagents. C57BL/6 mice were from Charles River Laboratory. *Trpm2*$^{-/-}$ C57BL/6 mice were a gift from B.A Miller). *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 described[46]. Mouse PMNs were isolated from mouse bone marrow with Percoll density gradients as described[47]. Mouse lung vascular endothelial cells were isolated from *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice as described[48]. PMA (phorbol-12-myristate-13-acetate), LPS (from *E. coli* strain0111:B4) and H_2O_2 were from Sigma. Polyclonal antibody to PKC-α was from Santa Cruz Biotechnology (catalog number sc-208,), polyclonal antibody to p47 (catalog number 07-500) and polyclonal antibody to phosphorylated PKC-α (catalog number 06-822) were from Millipore and antibody to p47^{phox} phosphorylated at Ser473 were from Invitrogen (catalog number 61-8100).

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 current were measured by a method similar to that described[8]. Currents were elicited by a series of test pulses range from –110 mV to +110 mV (test pulses were 200 ms in duration and delivered at intervals of 2 s). The holding potential was 0 mV. The pipette solution contained 135 mM $CsSO_3CH_3$, 8 mM NaCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM EGTA and 10 mM HEPES, pH 7.2. ADPR (200 μM) or PMA (1 μM) was included in the pipette solution, or H_2O_2 (300 μM) was perfused into the cells to induce TRPM2 current. For the induction of Ca^{2+}-activated cation currents, 100 μM free intracellular Ca^{2+} was included in the pipette solution. The bath solution contained 145 mM NaCl, 2 mM $CaCl_2$, 1 mM MgC_2 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 MgC_2, 0.5 mM CaC_2, 1 mM EGTA and 10 mM HEPES, pH 7.2. The bath solution contained 5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM MgC_2 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 replace 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 (5×10^6) from *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice were treated with PMA or LPS. ROS generation in cultured medium was measured by luminol-enhanced chemiluminescence as described[49]. Chemiluminescence was measured in 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 $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was recorded in single cells as described[50]. For this, an Axiovert 200 inverted microscope (Zeiss) was illuminated with a DeltaRam dual-wavelength monochrometer-based illumination system (Photon Technology International) with a xenon lamp and an R928 photon-counting photomultiplier (Hammamatsu) and optical coupling to the microscope. Cells were loaded for 30 min at 37 °C with 2 μM Fura-2 (acetoxymethyl 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 $p47^{phox}$ was assessed with antibody to p47 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 described[48]. 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) H_2O_2). 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.

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Supplemental information

Redox-Sensitive Cation Channel TRPM2 Modulates Phagocytic Cell ROS Production and Inflammation

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Supplemental Figure Legends.

Supplemental Figure 1. TRPM2 ablation augments LPS-induced NF-κB activation in mouse lung. *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice were challenged with LPS (10 mg/kg, i.p.) for the indicated times. Nuclear extracts from lung tissue were used for EMSA analysis.

Supplemental Figure 2. TRPM2 ablation enhances intracellular ROS production and bacteria killing without affecting phagocytosis. (**a**). TRPM2 ablation enhances intracellular ROS generation in bone marrow derived macrophage (BMDM) stimulated with *E. coli* (multiplicity of infection is 10:1) and PMA (1 μM). Intracellular ROS was detected by loading cells with 25 μM H2DCFDA which is not fluorescent until oxidized by ROS. Oxidized fluorescence of H2DCF (DCF) was detected with a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences). (**b**). TRPM2 ablation enhances bacterial killing *in vivo*. Both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice were injected intravenously with *E. coli* (strain 25922, ATCC) 2 x 10^6/body weight (g). After 4 or 24 h, blood and lung tissue were collected, the homogenized suspensions were diluted and plated on Soy-broth plates, and colony formation units (CFU) were counted. Bars indicate mean ± SEM. **p* =0.02 (n = 5), ***p* = 0.002 (n = 5) in *Trpm2*$^{-/-}$ mice compared with *Trpm2*$^{+/+}$ mice post-*E. coli*. (**c**). Phagocytosis occurs independently of TRPM2 expression. Representative raw data from flow cytometry of BMDM from *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice. Macrophages were incubated with AlexaFluor488-conjugated *E.coli* (Molecular Probes, E-13231) for 30 min at 37 °C. Data were collected using a MoFlo flow cytometer (DakoCytomation). The strength of fluorescence was used as the index of phagocytosis. Note there is no shift of fluorescence strength between

Trpm2$^{+/+}$ and *Trpm2*$^{-/-}$ macrophages. The figure shows representative curves from one of four independent experiments that produced similar results.

Supplemental Figure 3. *Trpm2*$^{-/-}$ BMDM-mediated endothelial cytotoxicity. Endothelial cytotoxicity was assayed by a fluorimetric microassay as in [2]. Mouse lung vascular endothelial cells (MLVECs) were labeled with calcein-AM at a final concentration of 10 mM for 1 h. LPS-activated macrophages (2 x 10^6) from *Trpm2*$^{+/+}$ or *Trpm2*$^{-/-}$ mice were co-cultured with labeled wild type of MLVECs at an effector-to-target ratio of 50:1 for 3 h. Cytotoxicity was measured by fluorimetry as dye release in supernatants after addition of macrophages. * p =0.02 (n=3) compared to *Trpm2*$^{+/+}$ *group*.

Supplemental Figure 4. Regulating membrane potential by extracellular K^+ concentration ($[K^+]_{out}$). (**a**) A typical recording of plasma membrane potential changes in wild type BMDM under different concentrations of extracellular potassium as indicated in the figure. Membrane potential was recorded with the current patch clamp technique. The stepwise increases in membrane potential (depolarization) with increased extracellular K^+ (the intracellular K^+ concentration $[K^+]_{in}$ was kept same at 135 mM). (**b**) Correlation of membrane potential with $[K^+]_{out}$. Measured membrane potential (pink circle) as described in (**a**) was compared with estimated K^+ equilibrium potential (E_K, black diamond). E_K was calculated by equation $E_K = 58 \log_{10}([K]_{out}/[K]_{in})$. The tight correlation of measured membrane potential with estimated E_K demonstrates that K^+ is the major ion controlling resting membrane potential in macrophages.

Supplemental Figure 5. Illustration of the protective role of TRPM2 in endotoxin-induced lung inflammatory injury. Endotoxin-induced ROS generation in a negative feedback manner dampens NADPH oxidase-derived ROS production in phagocytes, and therefore protects lungs from endotoxin-induced inflammary injury. TRPM2 activation functions by depolarizing the plasma membrane (indicated by "+++") and thus inhibits the membrane potential sensitive NADPH oxidase ROS production. Because TRPM2 can itself be activated by ROS (hydroperoxide, H_2O_2, other ROS), the products of NADPH oxidase, TRPM2 serves as a crucial negative feedback regulator of ROS production. As shown in the right part of the figure, deletion of TRPM2 reduces positive charge (Ca^{2+} and Na^{+}) flux into the cell, and the reduced depolarization of the plasma membrane favors transfer of electrons carried by NADPH oxidase; therefore, ROS production is markedly enhanced as seen in *Trpm2*$^{-/-}$ cells (indicated by thicker pink).

Supplemental Figure 6. Whole cell current induced by ADPR, H_2O_2 and PMA. TRPM2 activation was evaluated by whole cell patch clamp method. Currents were elicited by 200 ms voltage steps from -110 to +110 mV in 10 mV increments from a holding potential of 0 mV. (**a-b**) Whole-cell currents evoked in response to introduction of ADPR (200 μM) into voltage-clamped bone marrow derived macrophage (BMDM) from *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice. Raw whole cell currents is shown in **a** and averaged current-voltage (membrane potential) plot (*I-V* plot) is shown in **b**. *Trpm2*$^{-/-}$ BMDM lack detectable currents. (**c-d**). Whole-cell currents elicited in response to introduction of ADPR (200 μM) into voltage-clamped PMN isolated from bone marrow from both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice. Raw whole cell currents is shown in **c** and

averaged current-voltage (membrane potential) plot (*I-V* plot) is shown in **d**. Note that isosmotic replacement of external Na^+ by the impermeant organic cation N-methyl-D-glucamine (NMDG) abolished the ADPR-induced inward current. Also *Trpm2*$^{-/-}$ PMN lack detectable currents. (**e**). Whole-cell currents induced in response to H_2O_2 (300 μM) in *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ PMN. H_2O_2 failed to induce inward current but a small outward current in *Trpm2*$^{-/-}$ PMN (pink color), compared with the current induced in *Trpm2*$^{+/+}$ PMN (black color). Left-shift membrane potential due to NMDG replacement in *Trpm2*$^{+/+}$ PMN (blue color) was shown with a larger scale in the inset.

Supplemental Figure 7. Platelet-activating factor (PAF) fails to induce ROS production in BMDM. ROS generation was determined using luminol-ECL assay; *cps* = counts per second of light emitted. PAF (0.1 μM) failed to induced detectable ROS production in both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ BMDM. PMA was used here as a positive control. **p* = 0.006 (n= 3).

Supplemental Figure 8. Whole cell current induced by increased intracellular Ca^{2+} ($[Ca^{2+}]_i$). Whole-cell currents evoked in response to increased $[Ca^{2+}]_i$ (100 μM) in PMN isolated from mouse bone marrow. Raw whole cell currents is shown in **a** and averaged *I-V* plot is shown in **b**. Increased $[Ca^{2+}]_i$ from 100 nM to 100 μM induced a highly outward rectified current with a reversal potential at 0 mV (**b**) in both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ PMN.

Supplemental Methods

Nuclear protein isolation and electrophoretic mobility shift assay (EMSA). Nuclear protein was extracted from both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ lungs using salt buffers. Lung tissue was homogenized in 500 µl of ice-cold buffer A (10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% Igepal CA-630 detergent, and 0.5 mM PMSF) and incubated on ice for 30 min. The samples were then centrifuged at high speed for 10 min at 4°C. The supernatant was collected into separate containers and labeled as cytosolic extract. The nuclear pellet was crushed and resuspended in 200 µl of buffer B (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM KCl, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 10 µg/ml leupeptin). After being incubated on ice for 30 min, the resuspended samples were again centrifuged at high speed for 10 min at 4°C. The resulting supernatant (nuclear extract) was collected and stored at -80°C. EMSA was performed as in[1]. Briefly, nuclear extract (10 µg) was incubated with 1 µg of poly (deoxyinosinic-deoxycytidylic acid) in a binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, and 10% glycerol) for 15 min at room temperature. Next, end-labeled double-stranded oligonucleotides containing the NF-κB site (15,000 cpm each) were added to the above reaction mixture and incubated at room temperature for 15 min. The sequence of the oligonucleotide was as follows: NF-κB, 5'-AGTTGAGGGGACTTTCCCAGGC-3'. It contained the consensus NF-κB binding site in the Ig κL chain enhancer. The labeled nuclear proteins were resolved by native PAGE using 0.25 x TBE buffer (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA; pH 8.4). The gel was then transferred to blotting paper and exposed to film at -80°C for 8 h.

***Measurement of intracellular ROS generation*.** Macrophages from both *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice were loaded with 25 µM H2DCFDA which is not fluorescent until oxidized by reactive

oxygen species. After loading with H2DCFDA for 20 min, cells were exposed to various stimulation at 37^0C, data were collected from dye labeled cells 10-30 min later with a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences).

E. coli infection and bacterial load determinations. *E. coli* strain 25922 (ATCC) was cultured at 37 °C for overnight. After washed twice with PBS, E-coli were adjusted to 2 X 10^8/ml in PBS. *Trpm2*$^{+/+}$ and *Trpm2*$^{-/-}$ mice were intravenously injected with 2 X 10^6/g E-coli. After 4 or 24 h, blood and lung were collected. Lung tissue were weighted and homogenized in 1 ml cold PBS. Then blood and tissue homogenized suspension were diluted and plated on Soy-broth plate. The colony formation units (CFU) were counted.

Macrophage-mediated endothelial cytotoxicity. Macrophage-mediated endothelial cytotoxicity was assayed by a fluorimetric microassay as described previously [2]. Mouse lung vascular endothelial cells (MLVECs) were plated in 96-well tissue culture plates at a cell density of 4 x 10^4 cells/well with or without TNF-α (20 ng/mL) for 18 hr. The adherent MLVECs were washed with PBS and labeled with calcein-AM at a final concentration of 10 mM for 1 h. Excess dye was removed by washing with HBSS. LPS-activated macrophages (2 X 10^6) from *Trpm2*$^{+/+}$ or *Trpm2*$^{-/-}$ mice were co-cultured with labeled MLVECs at an effecter-to-target ratio of 50:1 for 3 h. The plate was centrifuged at 2000 rpm for 5 min. The supernatant was transferred to a 96-well plate and the fluorescence read at 485 nm excitation and 535 nm emission. Cytotoxicity was measured as dye release in the supernatants by fluorimetry and expressed as increased times in intensity after addition of macrophages.

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