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*J Immunol* 2012; 189:3689-3699; Prepublished online 29 August 2012; doi: 10.4049/jimmunol.1102969

http://www.jimmunol.org/content/189/7/3689

**Supplementary Material**  
http://www.jimmunol.org/content/suppl/2012/08/29/jimmunol.1102969.DC1

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The TRPM4 Channel Controls Monocyte and Macrophage, but Not Neutrophil, Function for Survival in Sepsis

Nicolas Serafini,*‡*, Albert Dabah,*,††,1 Gaëtan Barbet,*‡‡,1 Marie Demion,*‡‡,1 Tarik Attout,*‡‡,1 Grégoire Gautier,*‡‡,1 Michelle Arcos-Fajardo,*‡‡,1 Hervé Souchet,*‡‡,1 Marie-Hélène Jouvin,§ François Vrtovsnička,*‡‡,‡‡ Jean-Pierre Kinet,§ Marc Benhamou,‡‡,1 Renato C. Monteiro,*,‡‡ and Pierre Launay*,††,‡‡,‡‡ A favorable outcome following acute bacterial infection depends on the ability of phagocytic cells to be recruited and properly activated within injured tissues. Calcium (Ca\textsuperscript{2+}) is a ubiquitous second messenger implicated in the functions of many cells, but the mechanisms involved in the regulation of Ca\textsuperscript{2+} mobilization in hematopoietic cells are largely unknown. The monovalent cation channel transient receptor potential melastatin (TRPM) 4 is involved in the control of Ca\textsuperscript{2+} signaling in some hematopoietic cell types, but the role of this channel in phagocytes and its relevance in the control of inflammation remain unexplored. In this study, we report that the ablation of the Trpm4 gene dramatically increased mouse mortality in a model of sepsis induced by cecal ligation and puncture. The lack of the TRPM4 channel affected macrophage population within bacteria-infected peritoneal cavities and increased the systemic level of Ly6C\textsuperscript{+} monocytes and proinflammatory cytokine production. Impaired Ca\textsuperscript{2+} mobilization in Trpm4 deficient macrophages downregulated the AKT signaling pathway and the subsequent phagocytic activity, resulting in bacterial overgrowth and translocation to the bloodstream. In contrast, no alteration in the distribution, function, or Ca\textsuperscript{2+} mobilization of Trpm4 neutrophils was observed, indicating that the mechanism controlling Ca\textsuperscript{2+} signaling differs among phagocytes. Our results thus show that the tight control of Ca\textsuperscript{2+} influx by the TRPM4 channel is critical for the proper functioning of monocytes/macrophages and the efficiency of the subsequent response to infection.

The Journal of Immunology, 2012, 189: 3689–3699.

Sepsis, which is a major cause of mortality worldwide, is characterized by excessive inflammation in response to infection (1). During septic peritonitis, neutrophil recruitment within the peritoneal cavity is followed by the extravasation of monocytes, which differentiate into inflammatory macrophages (2). Macrophages play a major role in the maintenance of tissue homeostasis as they contribute, together with neutrophils, to the clearance of microorganisms and dying cells (3). In septic patients, macrophage dysregulation has been associated with adverse prognosis (4). However, the mechanisms regulating the functioning of macrophages and their role compared with that of neutrophils in the control of septic peritonitis remain unclear. In the circulation, monocytes show functional heterogeneity and are distinguished based on their expression of specific surface markers. In mice, two main subsets have been characterized, as follows: patrolling (or Ly6C\textsuperscript{−}) monocytes (5, 6) and inflammatory (or Ly6C\textsuperscript{+}) monocytes (7). Ly6C\textsuperscript{+} monocytes contribute to the control of pathogen proliferation during infection (8). In contrast, Ly6C\textsuperscript{−} monocytes are believed to be involved in healing and tissue repair (9). Although macrophages and monocytes are important for the appropriate response to infection, their regulation in the context of bacteria-mediated inflammatory reactions remains to be fully understood.

One potential mechanism involved in the regulation of these myeloid cells is the control of their intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]). Intracellular Ca\textsuperscript{2+} governs a wide range of cellular functions, such as proliferation, cytokine secretion, phagocytosis, and apoptosis (10, 11). Ca\textsuperscript{2+} mobilization through Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels has been observed in macrophages (12, 13) and neutrophils (14), and seems to be a key determinant for the activation of these phagocytes. However, the mechanisms regulating Ca\textsuperscript{2+} homeostasis in these innate immunity cells are largely unknown. Recently, the protein ORAI1 (15-17) and its activator STIM1 (18, 19) have been identified as essential components of the CRAC channel, whose activity is regulated at multiple levels (20–23).

Two Ca\textsuperscript{2+}-activated nonselective (CAN) channels, transient receptor potential melastatin (TRPM) 4 and TRPM5, have been characterized in nonexcitable cells (24, 25). These channels are involved in the regulation of Ca\textsuperscript{2+} homeostasis by reducing the driving force of Ca\textsuperscript{2+} influx through the CRAC channel (26). Indeed, under physiological conditions, the opening of TRPM4 by high [Ca\textsuperscript{2+}]\textsubscript{i} allows massive sodium (Na\textsuperscript{+}) entry and membrane

http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1102969

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depolarization, thereby decreasing further Ca\textsuperscript{2+} influx (26). TRPM4 thus acts in concert with the CRAC channel to control [Ca\textsuperscript{2+}]], and subsequent physiological responses in T cells, mast cells, and dendritic cells (DCs) (26–28). The molecular targeting of the Trpm4 gene in the mouse does not result in perinatal death or in an overt spontaneous phenotype. TRPM4-deficient mice are thus valuable tools for the detailed study of the impact of Ca\textsuperscript{2+} influx on cellular functions under pathological conditions.

In this study, we addressed the importance of Ca\textsuperscript{2+} regulation in the control of the acute inflammatory reaction induced by cecal ligation and puncture (CLP). We report that the deregulation of Ca\textsuperscript{2+} homeostasis in the absence of TRPM4 during sepsis had a profound impact on the function of macrophages within the bacteria-infected tissue and on the level of monocyte subsets in the blood. The deficiency of the TRPM4 channel dramatically affected the phagocytosis of bacteria and increased proinflammatory cytokine production, impacting the survival rate of septic mice. However, no alterations of neutrophil function were observed in Trpm4floxed mice, indicating that the mechanisms involved in the regulation of Ca\textsuperscript{2+} signaling differ among phagocytes. These data demonstrate that the resolution of acute infection requires tightly regulated Ca\textsuperscript{2+} mobilization and its regulation by TRPM4 for the appropriate activation of monocytes and macrophages.

Materials and Methods

**Mice**

We have generated the TRPM4-deficient (Trpm4floxed) mouse model (on the mixed 129/B6 background), as previously described (28). In all experiments, mice were 8–12 wk of age and controls were littermates. To selectively reduce TRPM4 expression in myeloid cells, the TRPM4 conditional knockout Trpm4floxed LysMcre (Trpm4floxed LysMcre) mice were generated by serial breeding of the Trpm4floxed (Trpm4floxed) mice with mice expressing Cre recombinase under the control of the LysM promoter (29). Mice were housed at the Bichat Medical School animal facility under specific pathogen-free conditions and were handled according to European directives.

**Cells**

Bone marrow-derived macrophages (BMMs) were obtained from bone marrow cells extracted from femurs and tibias cultured for 5–8 d at 37˚C and 5% CO\textsubscript{2} in presence of M-CSF. Cells were cultured at 1 × 10\textsuperscript{6} cells/ml in RPMI 1640 medium (Invitrogen) containing 10% FBS (v/v; Biowest) and 10% supernatant of L929 cells (v/v) as source of M-CSF. The culture of bone marrow-derived DCs (BMDC) was previously described (28). Peritoneal neutrophils and macrophages were obtained after i.p. injection with 2 ml 3% (v/v) sterile thioglycollate (Sigma-Aldrich). After 3 h and 5 d, respectively, peritoneal cells were harvested by injecting 8 ml PBS (10% FCS) into peritoneal cavity. After injection, cells were dislodged by gentle massage of the abdomen and the cell suspension was collected.

**Cecal ligation and puncture**

Peritonitis was induced by CLP, as described (30). Briefly, mice were anesthetized and the cecum exposed by a 1-cm midline incision on the abdomen. The distal half of the cecum was ligated with a 5-0 silk suture (Ethicon) and punctured with a 21-gauge needle. The cecum was replaced, and 1 ml sterile saline (0.9% NaCl) was injected into the peritoneal cavity. The incision was closed using surgical sutures. Mice were monitored every 8 h for the first 3 d and then every 12 h until death or day 7, when they were euthanized.

**Bacterial quantification**

Peritoneal fluid and blood serum samples were serially diluted in PBS and plated on bacteria-selective agar plates (Chromocult; Merck). Plates were incubated for 18 h at 37˚C, and dark blue to violet colonies were counted.

**Mouse cell phenotyping and flow cytometry**

To analyze the leukocyte distributions in the circulation of inflamed, as well as resting, mice, blood was drawn and RBCs were lysed twice in ACK buffer (10 vol). Peritoneal cells were obtained by injection with 8 ml PBS/0.5% BSA using 25-g needle. After injection, attached cells were dislodged by
room temperature for 30 min. The cells were then washed with Ringer’s solution (in mM: 145 NaCl, 5.4 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, and 0.1% BSA, pH adjusted to 7.5) or in Ca2+-free Ringer’s solution containing 5 mM EGTA, and Ca2+ influx was triggered by adding bacteria directly into the dish.

Phagocytosis assay

BMMs, thioglycolate-elicited peritoneal macrophages, or neutrophils were incubated with Texas Red-coupled E. coli (Molecular Probes) in chamber slides or in suspension (for neutrophils) at 37˚C for 30 min. The phagocytosis was stopped by washing the cells twice in PBS, 0.5% BSA, and 0.1% sodium azide at 4˚C. The cell surface was stained with an anti-CD11b biotinylated Ab (M1/70; BD Biosciences) and streptavidin-Alexa 488 (Molecular Probes) on ice for 1 h before confocal microscopy (Zeiss LSM 510 META).

Monocyte/Macrophage depletion

For the specific depletion of monocytes and macrophages, dichloro-methylene diphosphonic acid (CL2MBP; clodronate)-loaded or PBS-loaded liposomes (Encapsula NanoSciences) were injected i.p. (300 µl per mouse) 18 h before the CLP procedure, as previously described (31). For analysis of cell depletion, blood and peritoneal fluid were harvested from mice, and the presence of neutrophils (CD11b+ Ly-6G+), monocytes (CD11b+ CD115+ Ly6C+/2), and macrophages (CD11b+ F4/80+) was analyzed by flow cytometry.

Immunoblotting

Cells were stimulated with E. coli (50 bacteria/cell), washed, and solubilized with 1% Triton X-100. Proteins were resolved by 8% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Millipore). Protein expression was assessed by immunoblotting with primary Abs to phospho-AKT (P-AKT S473, clone D9E) and AKT (both from Cell Signaling), followed by a goat secondary Ab to mouse (NA931) or rabbit IgG (NA934; both from Amersham) coupled with HRP and visualized by ECL (32).

Statistical analysis

Results are reported as means ± SEM. ANOVA was used to compare results under different conditions. The significance of survival rates on Kaplan–Meier curves was calculated using the log rank test. Data were analyzed using Prism v4 and Origin v7.5.

Results

The TRPM4 channel contributes to survival in septic peritonitis

To evaluate whether TRPM4 is involved in resistance to severe infection, we induced a potent septic inflammation by CLP in Trpm4−/− and littermate control mice. In this model, sepsis originates from a microbial infection within the peritoneal cavity, followed by the translocation of mixed enteric bacteria into the blood compartment (33). Soon after the CLP procedure, we observed a substantial decrease in the survival rate of Trpm4−/− mice as compared with Trpm4+/+ littermate controls (Fig. 1A). Notably, 72 h after CLP, 10% of Trpm4−/− mice survived the inflammation, whereas almost 60% of the Trpm4+/+ mice were still alive. Furthermore, the level of bacteria detected in the peritoneal fluid was markedly higher in Trpm4−/− mice than in littermate controls (Fig. 1B). Six hours after CLP, no bacterial translocation into the blood was detected. However, 24 h after the procedure, a potent bacteremia was observed in both animal groups, although the level of infection was almost 100-fold higher in Trpm4−/− mice than in Trpm4+/+ mice (Fig. 1C). Septic shock
severity is determined by the intensity of the innate immunological overreaction that results in an excess of proinflammatory cytokines (34). We therefore assessed the level of proinflammatory cytokines in the blood, 6 and 24 h after CLP in Trpm4−/− and Trpm4+/+ mice. We noticed a large increase in the levels of MCP-1, IFN-γ, IL-6, and TNF-α in Trpm4−/− mice 24 h after CLP, when compared with littermate controls (Fig. 1D). These results collectively indicate that the TRPM4 channel plays a crucial role in mouse survival during acute bacterial inflammation by controlling microorganism expansion and proinflammatory cytokine release.

Macrophages and monocytes are involved in the decreased survival of Trpm4−/− septic mice

Neutrophils and macrophages play a key role in both the initiation and the development of bacteria-mediated inflammation (35). Because we observed rapid mortality in Trpm4−/− septic mice, we wondered which cell types were involved in the decrease of mouse survival rates after CLP. As early as 6 h after the CLP procedure, a substantial and comparable infiltration of neutrophils was detected in the peritoneal cavity of septic mice. (Fig. 2A). In contrast, there was a major decrease in the number of macrophages in the peritoneum of Trpm4−/− mice compared with Trpm4+/+ mice 24 h after CLP (Fig. 2B). Such a defect was not observed for peritoneal population of eosinophils, B cells, and CD4+ and CD8+ T cells, whether in steady state or after the CLP procedure (Supplemental Fig. 1A, 1B), suggesting that macrophages rather than other immune cells might be involved in the decreased survival rate observed in Trpm4−/− septic mice.

Because an increase in cytokine production in the blood (as observed in Trpm4−/− septic mice) is usually associated with leukocytosis during the acute phase of infection (35), we next assessed the number of monocytes and neutrophils in the blood of Trpm4−/− mice before and after CLP. We observed an increase in monocyte numbers in the blood of Trpm4−/− mice when compared with littermate controls 24 h after CLP (Fig. 3A). In contrast, no difference in the number of blood neutrophils was detected between Trpm4−/− and Trpm4+/+ mice (Fig. 3B). Inflammatory (Ly-6C+) and patrolling (Ly-6C−) monocytes are equivalently represented in the mouse blood in steady state condition (9).

The distribution of these two monocyte subsets in Trpm4+/+ control mice was comparable before and after CLP (Fig. 3C, upper middle panel). By contrast, their distribution in the blood of Trpm4−/− mice changed dramatically under septic conditions (Fig. 3C, upper lower panel). Twenty-four hours postinfection, when bacteria had reached the blood compartment, almost 80% of Trpm4−/− monocytes were inflammatory monocytes due to a robust (3-fold in Fig. 3A) increase in monocyte numbers and a moderate (33%) decrease in Ly6C− monocytes (Fig. 3C, right panel). Furthermore, the production of the inflammatory cytokine TNF-α after cell stimulation with E. coli was 10–30 times higher in Ly-6C+ monocytes than in Ly-6C− monocytes (Fig. 3D), indicating that the expansion of the Ly-6C+ monocytes in the blood of Trpm4−/− septic mice most likely affected the mice inflammatory status, hence impacting Trpm4−/− mice survival. Together, our data suggest that the decrease in the survival rate observed in Trpm4−/− mice after CLP is due to a defect in monocytes and macrophages, but not in neutrophils.

Monocytes and macrophages confer a survival advantage on mice during sepsis

To further assess the role of the TRPM4 channel in myeloid cells during sepsis, we generated a mouse model in which the Trpm4 gene is ablated only in macrophages and neutrophils (Trpm4f/f LysMCre mice), by crossing Trpm4 floxed mice with LysMCre mice that express Cre recombinase under the control of the LysM promoter (29) (Supplemental Fig. 2A). After the CLP procedure, Trpm4f/f LysMCre mice presented a decrease in survival compared with control mice (Trpm4f/f), with increased bacterial titers in the blood and peritoneal fluid (Fig. 4A, 4B), suggesting that it is mainly through its expression in macrophages and/or neutrophils that TRPM4 contributes to the survival of mice postinfection. We therefore evaluated the specific contribution of monocytes and macrophages to the increased mortality observed in TRPM4-deficient mice during sepsis, as follows. After clodronate liposome treatment of mice (36), nearly all monocytes and macrophages (Fig. 4C, 4D), but not neutrophils (Supplemental Fig. 2B, 2C), were depleted from the peritoneal cavity of both Trpm4−/− and Trpm4+/+ mice. Hence, 18 h after liposome treatments, mice were...
subjected to CLP. Macrophage depletion resulted in a striking decrease in the survival rate of Trpm4+/+ control mice to the point that survival rates of both Trpm4+/− and Trpm4−/− mice were now similar (Fig. 4E). Moreover, bacterial titers in peritoneal lavages and sera were also identical in Trpm4+/+ and Trpm4+/− monocyte/macrophage-depleted mice (Fig. 4F). These results thus underscore the critical role of the TRPM4 channel in monocytes/macrophages for efficient bacterial clearance and mouse survival after CLP.

Intracellular Ca2+ mobilization is regulated by TRPM4 in monocytes/macrophages, but not in neutrophils

Intracellular Ca2+ mobilization is critical for all hematopoietic cells, and it is assumed that there is a redundant mechanism responsible for its regulation. However, we observed that monocytes/macrophages, but not neutrophils, were affected in septic mice by the deletion of Trpm4. Therefore, we assessed the presence and the function of this channel in both cell types. Monocytes and neutrophils were directly isolated from the blood, and both macrophages and DCs were derived in vitro from the bone marrow of wild-type and Trpm4−/− mice. As reported for several immune cells including DCs (28), Trpm4 transcripts were readily detected in monocytes and macrophages by RT-PCR. In contrast, the level of TRPM4 mRNA was barely detectable in neutrophils (Fig. 5A). Notably, the TRPM5 channel, which is closely related to TRPM4 in terms of electrophysiological properties, was detected only in DCs, but not in monocytes/macrophages or neutrophils (Fig. 5A).

Because TRPM4 is involved in the regulation of Ca2+ homeostasis, we next analyzed Ca2+ mobilization in blood monocytes and neutrophils from wild-type and Trpm4−/− mice after stimulation of the cells with bacteria, known to induce a potent influx of Ca2+ (28). We observed a sustained increase in intracellular Ca2+ in Trpm4−/− monocytes when compared with Trpm4+/− monocytes after E. coli stimulation (Fig. 5B). In contrast, the ablation of the TRPM4 channel did not affect Ca2+ mobilization in neutrophils (Fig. 5C), indicating that this channel plays a minor role, at best, in Ca2+ regulation in neutrophils during infection.

To confirm functionally the presence of the TRPM4 channel in monocytes/macrophages, we used the patch-clamp technique to
measure its channel activity in BMMs. By subtracting the mean residual current-voltage trace of Trpm4−/− BMMs (unfilled circles) from those of Trpm4+/+ BMMs (filled circles), we unmasked a TRPM4-like current in these wild-type macrophages (gray circles) (Fig. 5D). Because TRPM4 is directly activated by intracellular Ca2+ and is a nonselective monovalent cation channel, we further analyzed the specificity of the TRPM4-like current detected in macrophages. Ca2+ rise experiments confirmed the Ca2+-dependent activation of this current in BMMs (0.1, 1, and 10 μM [Ca2+]i) (Fig. 5E). The inward current was completely abolished when extracellular Na+ was replaced by the large cation NMDG. The substitution of extracellular Cs+ for Na+ did not modify the inward current (Fig. 5F), showing the nonselectivity of the TRPM4 channel for monovalent cations, as previously reported (24, 37, 38). These data collectively demonstrate that TRPM4 is expressed in and controls the Ca2+ homeostasis of monocytes/macrophages.

**TRPM4 channel regulates peritoneal macrophage phagocytosis**

The number of peritoneal macrophages was not affected by Trpm4−/− mouse in steady state or in sterile inflammation such as the one induced by thioglycollate, suggesting that there was no impairment of peritoneal macrophage differentiation in Trpm4−/− mice (Supplemental Fig. 1). Thus, we wondered whether the decrease in the number of macrophages observed in the peritoneal cavity of Trpm4−/− mice after CLP was due to a decreased macrophage viability. Therefore, the viability of macrophages, withdrawn from infected peritoneal cavity 10 h after CLP, was assessed by annexin V-FITC/PI staining. We found that macrophage cell death induced during acute peritonitis was significantly higher in Trpm4−/− mice than in control mice (Fig. 6A).

We next explored whether the regulation of Ca2+ homeostasis by the TRPM4 channel would affect macrophage function. Because we detected a dramatic bacterial expansion in the peritoneal cavity of Trpm4−/− septic mice, we assessed the phagocytic activity of peritoneal macrophages from Trpm4−/− and Trpm4+/+ mice. Thioglycollate-elicited macrophages were incubated with different doses of Texas Red-labeled E. coli for 30 min, and the extent of phagocytosis was determined by confocal microscopy. Macrophages from Trpm4−/− mice displayed significantly less phagocytosis of E. coli than those from control mice (Fig. 6B). However, no difference in phagocytosis was observed between Trpm4−/− and Trpm4+/+ thioglycollate-elicited neutrophils (Fig. 6C). These results demonstrate that the absence of TRPM4 is responsible for bacterial overgrowth most likely by impairing macrophage phagocytic activity, and thus contributes to the increased mortality observed in Trpm4−/− mice.

**Ca2+ mobilization regulated by TRPM4 controls peritoneal macrophage functions through AKT signaling**

We next sought to understand the molecular mechanism controlled by TRPM4 in the clearance of bacteria by peritoneal macrophages. Phagocytosis of bacteria is dependent on both Ca2+ mobilization and the activation of the PI3K/AKT pathway (33, 39, 40). Keeping in mind the decrease in phagocytosis observed in Trpm4−/− peritoneal macrophages, we next assessed both the level of Ca2+ mobilization and AKT phosphorylation in thioglycollate-elicited Trpm4−/− and Trpm4+/+ macrophages after their stimulation with E. coli. Intracellular Ca2+ mobilization was significantly lower in Trpm4−/− than in Trpm4+/+ peritoneal macrophages (Fig. 7A), as also observed in Trpm4−/− peritoneal macrophages after CLP (Supplemental Fig. 3A). In line with this result, a reduced AKT phosphorylation was observed in these Trpm4−/− macrophages (Fig. 7B). Although the reduced calcium mobilization and reduced AKT phosphorylation were in agreement with the decreased phagocytic activity of Trpm4−/− peritoneal macrophages,
the reduced calcium signal observed in these cells was surprising because we and others have shown that the TRPM4 channel negatively regulates Ca²⁺ entry. Because Trpm4²/² blood monocytes have an increase of Ca²⁺ influx when stimulated with E. coli (Fig. 5B), we hypothesized that Ca²⁺ overload during blood monocyte extravasation within peritoneal cavity (41) could drive the newly generated Trpm4²/² peritoneal macrophages into an unresponsive state. To test this hypothesis, we generated BMMs in presence of M-CSF, which does not induce Ca²⁺ mobilization, thus avoiding a Ca²⁺ overload during maturation (Supplemental Fig. 3B). These BMMs were then tested for Ca²⁺ mobilization, AKT phosphorylation, and phagocytosis. After bacterial stimulation, we noticed an increase in Ca²⁺ mobilization in Trpm4²/² BMMs when compared with Trpm4⁺/+ BMMs (Fig. 7C), which correlated with increased AKT phosphorylation (Fig. 7D). Furthermore, phagocytosis of bacteria was increased in Trpm4²/² BMMs compared with Trpm4⁺/+ BMMs (Fig. 7E). Thus, in the inflamed peritoneal cavity of Trpm4⁻/⁻ mice, the Ca²⁺ overload in recruited monocytes impairs subsequent macrophage phagocytosis. We conclude that TRPM4, through the regulation of Ca²⁺ signaling, ensures proper macrophage function that permits the control of infection and survival during acute peritonitis.

Discussion

The control of inflammatory responses is a crucial challenge for the immune system, and innate immune cells, such as neutrophils and macrophages, play a major role in controlling inflammation in time and space (35). Neutrophils possess a large number of antimicrobial functions, including phagocytosis and the release of antimicrobial peptides and reactive oxygen species. Macrophages, involved in the clearance of dead cells as well as of bacteria, are also implicated in Ag presentation to lymphocytes and therefore

![FIGURE 5.](http://www.jimmunol.org/Downloadedfrom)
involved in the control of the intensity of the inflammatory response. Most of the biological functions of these phagocytes are dependent on an increase in \([\text{Ca}^{2+}]_\text{i}\). ORAI1 is believed to be the main channel responsible for \(\text{Ca}^{2+}\) entry in these cells following the activation of membrane-bound receptors, and its expression is both ubiquitous and critical in most hematopoietic cells (22). Yet, the regulation of \(\text{Ca}^{2+}\) mobilization in myeloid cells remains poorly understood. As well, the impact of \(\text{Ca}^{2+}\) influx on the onset and resolution of the inflammatory reaction has not been studied in detail mainly due to the absence of adequate animal models (23). In our study, we show that TRPM4 is crucial for proper function of monocytes and macrophages. The deregulation of \(\text{Ca}^{2+}\) homeostasis through the deletion of \(\text{Trpm4}\) gene led to severe defects responsible for a drastic drop in the survival of mice during peritonitis. Bacteria are powerful inducers of \(\text{Ca}^{2+}\) influx. This massive \(\text{Ca}^{2+}\) entry is likely the result of direct interaction of bacteria with chemokine receptors such as CCR1 and CCR5 that are highly expressed on macrophages (28, 42). Our data are in agreement with the notion that intracellular \(\text{Ca}^{2+}\) entry after membrane receptor triggering needs to be tightly regulated to allow the cell to return in resting state and be able to answer to another round of activation. Indeed, we have previously shown the absence of TRPM4 channel in DCs leads to uncontrolled \(\text{Ca}^{2+}\) influx impairing subsequent wave of activation (28). We observed that a first calcium overload in \(\text{Trpm4}^{-/-}\) immature DCs had a dramatic effect on their subsequent migration to inflamed tissue induced by CCL21 due to a lower calcium response to this cytokine. Likewise, in \(\text{Trpm4}^{-/-}\) monocytes, the in vitro generation of macrophages in presence of M-CSF, which does not trigger massive \(\text{Ca}^{2+}\) influx, leads to resting macrophages that can subsequently develop robust \(\text{Ca}^{2+}\) mobilization and phagocytic activity upon \(E.\ coli\) stimulation. By contrast, \(\text{Trpm4}^{-/-}\) monocytes that have experienced \(\text{Ca}^{2+}\) overload during extravasation to infected peritoneum give rise to unresponsive macrophages with reduced level of \(\text{Ca}^{2+}\) mobilization in the presence of \(E.\ coli\) (Supplemental Fig. 4). Thus, control of the amplitude of \(\text{Ca}^{2+}\) response by TRPM4 during maturation of immune cells appears important for subsequent proper cell function. Whether this can be extended to other immune cells is an attractive hypothesis that remains to be evaluated.

Because store-operated \(\text{Ca}^{2+}\) influx is critical for the functioning of all hematopoietic cells, one would assume that the mechanisms underlying its regulation would be redundant. Unexpectedly, we found that the disruption of one regulatory channel, TRPM4, induced a profound deregulation of \(\text{Ca}^{2+}\) homeostasis in macrophages with no effect on neutrophils, indicating that \(\text{Ca}^{2+}\) homeostasis is regulated differently among phagocytic cells. Indeed, neutrophils are to date the only immune cells in which the \(\text{Trpm4}\) gene led to severe defects responsible for a drastic drop in the survival of mice during peritonitis. Bacteria are powerful inducers of \(\text{Ca}^{2+}\) influx. This massive \(\text{Ca}^{2+}\) entry is likely the result of direct interaction of bacteria with chemokine receptors such as CCR1 and CCR5 that
each type of immune cells. Studies have shown that CAN, to which the TRPM4 channel belongs, are involved in the regulation of Ca^{2+} homeostasis in immune cells (24, 25). The absence of a detectable effect of a TRPM4-channel deletion on neutrophils both under steady state and inflammatory conditions, and the absence of the closely related channel TRPM5 in neutrophils suggest that channels other than CAN channels may be implicated in the regulation of Ca^{2+} entry after membrane receptor engagement in these cells. The fact that enhanced Ca^{2+} entry mediated either by the nonstore-operated TRPM2 channel or indirectly by the VSOP/Hv1 proton channel induces an increase in neutrophil migration (43, 44) supports the view that these cells might have a special mechanism of regulation of Ca^{2+} homeostasis. Furthermore, Weber et al. (45) reported that the TRPM4 channel is expressed differently in Th1 and Th2 lymphocytes, with a differential role in Ca^{2+} mobilization and NFATc1 localization. Therefore, it appears from our analysis and the studies above that the expression of Ca^{2+}-related channels in a given cell type is related to its specific functions, and that whereas Ca^{2+} is one of the most common second messengers involved in cell activation, there is no ubiquitous mechanism responsible for its regulation in hematopoietic cells (46, 47).

In peritonitis, neutrophils are the first phagocytes to be recruited within inflammatory sites. However, our study underlines the critical role of macrophages during bacterial infection. Indeed, 6 h after the CLP procedure, when the number of neutrophils that had reached the peritoneal cavity was maximal in both Trpm4^{−/−} and Trpm4^{+/+} mice, the concentration of bacteria was 50 times higher in Trpm4^{−/−} mice than in littermate controls. Furthermore, the peritoneal depletion of macrophages, but not neutrophils, using clodronate liposomes led to a dramatic drop in the survival rate of Trpm4^{+/+} control mice to levels similar to that of Trpm4^{−/−} mice.
A recent work has revealed that neutrophils are capable of producing large amounts of the anti-inflammatory cytokine IL-10 under infection with Gram-negative bacteria, in contrast to monocytes, which under the same infectious conditions produce proinflammatory mediators (32). More recently, another study has suggested that during peritonitis, the IL-10 produced by extravasated neutrophils suppresses inflammatory monocyte/macrophage functions within the peritoneal cavity, and that neutrophils might be dispensable for the survival of septic mice (48). Therefore, even though the number and kinetics of migrating neutrophils are higher than those of inflammatory monocytes in the peritoneal cavity, the latter play a crucial role in mounting an appropriate inflammatory response during peritonitis and in the control of mortality.

Currently, the treatment of sepsis is mainly based on anti-biotherapy. Extensive efforts to use neutralizing Abs to inflammatory cytokines, such as anti-TNF-α Ab, have not achieved all the therapeutic benefits expected for patients. The inflammatory reaction needs to be controlled to avoid chronic collateral damage or acute shock. Most of the time, this is efficiently performed by physiological mechanisms. Yet, sometimes, inadequate control mechanism leads to pathological conditions. By specifically modulating particular inflammatory cells, one could foresee the control of inappropriate or overly aggressive inflammation. Thus, the regulation of Ca2+ homeostasis by TRPM4, which specifically affects the functions of monocytes and macrophages, suggests that this channel could be proposed as a new therapeutic target in acute inflammatory reactions, such as sepsis.

In summary, our study demonstrates that the timely regulation of Ca2+ homeostasis by TRPM4, which specifically affects the functions of monocytes and macrophages, suggests that this channel could be proposed as a new therapeutic target in acute inflammatory reactions, such as sepsis.

Acknowledgments

We thank M. Aloulou (INSERM U699) for training in both CLP and phagocytosis experiments. We thank A. Lehuen, L. Baudouin, and J. Diana (INSERM U561) for advice and help in cell sorting, E. Ferrary (INSERM U773) for help with the patch clamp setup, C. Cordier and J. Mègez (IFR94, IRNEM) for cell sorting, B. Ryffel (CNRS UMR6218) for providing the LysMcRfp mice, S. Perruche (INSERM U645) for help with monocytes/macrophage depletions experiments, S. Benadda for assistance in confocal microscopy, and J. Bex, A. Bouhalfa, and E. Couchi for help with animal care.

Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTAL FIGURE 1

A  Steady state

![Bar graph showing cell numbers (10^6) for various cell types under steady state conditions.](image)

B  CLP

![Bar graph showing cell numbers (10^6) for various cell types under CLP conditions.](image)

C  Thioglycollate

![Bar graph showing cell numbers (10^6) for various cell types under thioglycollate conditions.](image)
A  PCR analysis

B  Blood

C  Peritoneal cavity
SUPPLEMENTAL FIGURE 3

A

2 mM Ca\(^{2+}\)

E. coli

Iono

(B/F0) - 1

Time (s)

[Graph showing time (s) and fluorescence (B/F0) - 1 for Trpm4\(^{+/+}\) and Trpm4\(^{-/-}\) in 2 mM Ca\(^{2+}\) with E. coli and Iono.

B

2 mM Ca\(^{2+}\)

M-CSF

Iono

(B/F0) - 1

Time (s)

[Graph showing time (s) and fluorescence (B/F0) - 1 for Trpm4\(^{+/+}\) and Trpm4\(^{-/-}\) in 2 mM Ca\(^{2+}\) with M-CSF and Iono.


SUPPLEMENTAL FIGURE 4

Controlled inflammatory reaction

- **Regulated Ca^{2+} influx**
  - ORAI1
  - TRPM4
  - Ca^{2+}
  - Na^{+}
  - STIM1
  - Cytoplasm

- **Ly6C^+ monocyte**
- **Ly6C^- monocyte**
- **Macrophage**
- **Peritoneal cavity**
- **Blood**
- **Bacteria**

**Uncontrolled inflammatory reaction**

- **Deregulated Ca^{2+} influx**
  - ORAI1
  - STIM1
  - Ca^{2+}

- **Neutrophil**
- **Uncontrolled inflammatory reaction**
SUPPLEMENTAL FIGURE 1. Peritoneal leukocyte populations in Trpm4+/+ and Trpm4−/− mice in steady-state and inflammatory conditions. Right panels: Numbers of peritoneal macrophages (MΦ), neutrophils (PMN), eosinophils (Eos), B and T lymphocytes measured in (A) steady-state, (B) CLP and (C) Thioglycollate conditions. Left panels: CD80 and CD86 labeling on peritoneal macrophages in each experimental conditions.

SUPPLEMENTAL FIGURE 2. Deletion of the Trpm4 gene in Trpm4f/f LysMCre macrophages and preservation of neutrophils viability in presence of clodronate liposomes. (A) RT-PCR analysis of TRPM4 expression in bone marrow derived macrophages from Trpm4f/f LysMCre and Trpm4f/f mice (737 pb PCR product). Rps15, small ribosomal protein (as positive control) and H2O: no cDNA (as negative control). (B) Monocytes and neutrophils analysis in the blood of Trpm4+/+ and Trpm4−/− mice 18 h after depletion with clodronate-loaded liposomes (CL2MBP-lip) or PBS-loaded liposomes (PBS-lip). Representative scatter plots of blood monocytes (CD115+CD11b+ cells) and neutrophils (CD11b Ly6G+ cells) from individual Trpm4+/+ and Trpm4−/− liposomes-treated mice. (C) Macrophages and neutrophils analysis in the peritoneum of Trpm4+/+ and Trpm4−/− mice 18 h after depletion with CL2MBP-lip or PBS-lip. Flow cytometric analysis of peritoneal macrophages (CD11b F4/80hi cells) and neutrophils (CD11b Ly6G+ cells) from individual Trpm4+/+ and Trpm4−/− liposomes-treated mice.

SUPPLEMENTAL FIGURE 3. Calcium mobilization is reduced inflammatory peritoneal macrophages and not detected following M-CSF triggering in resting monocytes. (A) Measurement of Ca2+ mobilization, recorded by flow cytometry, and triggered with bacteria in peritoneal macrophages after over-night CLP. Histograms show the mean ±SEM of the area under the curve (AUCs) in arbitrary units. Iono: ionomycin. *: p value < 0.05. (B) Measurement of Ca2+ mobilization, by flow cytometry, after M-CSF incubation in Trpm4+/+ and Trpm4−/− Ly-6C+ monocytes.

SUPPLEMENTAL FIGURE 4. TRPM4 regulates macrophage-mediated inflammation. Left: In the mouse model of sepsis, monocytes extravasate from the bloodstream into the peritoneal cavity and differentiate into macrophages. Cytokine release and phagocytic activity by macrophages regulate the inflammatory reaction. Right: The deregulation of Ca2+ homeostasis due to the absence of the TRPM4 channel impair macrophage viability and phagocytosis within the infected peritoneal cavity, and increase the level of inflammatory Ly6C+ monocytes, both processes that result in lethal inflammation.