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TRPV1 Deletion Enhances Local Inflammation and Accelerates the Onset of Systemic Inflammatory Response Syndrome

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Stephen J. Thompson,‡ Julie E. Keeble,‡ Yanira Riffo-Vasquez,‡ Kenneth D. Bruce,‡ and
Susan D. Brain*

The transient receptor potential vanilloid 1 (TRPV1) is primarily localized to sensory nerve fibers and is associated with the stimulation of pain and inflammation. TRPV1 knockout (TRPV1KO) mice show enhanced LPS-induced sepsis compared with wild type (WT). This implies that TRPV1 may have a key modulatory role in increasing the beneficial and reducing the harmful components in sepsis. We investigated immune and inflammatory mechanisms in a cecal ligation and puncture (CLP) model of sepsis over 24 h. CLP TRPV1KO mice exhibited significant hypothermia, hypotension, and organ dysfunction compared with CLP WT mice. Analysis of the inflammatory responses at the site of initial infection (peritoneal cavity) revealed that CLP TRPV1KO mice exhibited: 1) decreased mononuclear cell integrity associated with apoptosis, 2) decreased macrophage tachykinin NK1-dependent phagocytosis, 3) substantially decreased levels of nitrite (indicative of NO) and reactive oxygen species, 4) increased cytokine levels, and 5) decreased bacteria clearance when compared with CLP WT mice. Therefore, TRPV1 deletion is associated with impaired macrophage-associated defense mechanisms. Thus, TRPV1 acts to protect against the damaging impact of sepsis and may influence the transition from local to a systemic inflammatory state. The Journal of Immunology, 2012, 188: 5741–5751.

Sepsis is an overwhelming and complex response initiated by infectious microorganisms and can be lethal. It is estimated that >0.5 million patients worldwide are annually affected by sepsis (1, 2). The outcome of each episode depends on the severity of the systemic inflammation in response to the initial infection. This systemic inflammatory response syndrome (SIRS) is characterized by activation of the immune and inflammatory systems that, in turn, leads to multiple organ dysfunction and failure (2, 3). Classic signs of sepsis/SIRS include hypothermia/ hyperthermia, tachycardia, tachypnea, and leukocytosis/leukopenia (2). Sepsis can be caused by a variety of microorganisms, and effective treatments have been sought. However, as the mechanisms involved in sepsis are still unclear, few drugs are successful in treating this disease.

Recently, a role for sensory nerves has been described. C and Aδ sensory fibers are distributed throughout the body and, when stimulated, release neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) (4, 5). Transient receptor potential vanilloid 1 (TRPV1) is a nonselective ion channel localized on sensory nerves (6) that acts to integrate sensory and noxious information (7). TRPV1 can be activated by a range of stimuli such as heat, protons, arachidonic acid, lipooxygenase metabolites, and cannabinoids (for review, see Ref. 8). TRPV1 has become known as a “molecular integrator” because its activity can be enhanced by common signaling effectors such as protein kinase C (9).

Evidence shows increased serum levels of CGRP and SP in patients with sepsis (10, 11). SP is suggested to play a proinflammatory role in sepsis (12, 13), whereas a regulatory role for CGRP has been suggested (14). Initial indications that TRPV1 could influence blood pressure and temperature regulation have also been reported in LPS-induced sepsis in rodents (15, 16). Protection against hypotension and hyperthermia has been reported in rats with endotoxemia, where capsazepine, a TRPV1 antagonist, enhances mortality (17). We have previously shown that TRPV1 deletion increases TNF-α, NO, and circulating markers of organ failure during endotoxemia (18). These recent findings indicate a protective role for TRPV1 in sepsis.

To date, little is known of the mechanisms via which TRPV1 is protective and influences SIRS, a characteristic of sepsis. In this study, the role of TRPV1 in sepsis was investigated using the murine cecal ligation and puncture (CLP) model used to serve as a model for human sepsis (2). We provide evidence that TRPV1 protects against the immune and inflammatory responses produced in the peritoneal cavity and also the ensuing SIRS. TRPV1 deletion increases cytokine release, reduces reactive oxygen species...
(ROS) and NO levels, increases apoptosis, and also reduces in vitro phagocytosis in peritoneal macrophages treated with LPS. This impaired macrophage function facilitates bacterial survival, thus promoting a worsening of the systemic inflammatory response. Deletion of TRPV1 leads to enhanced hypotension and hypothermia, indicating TRPV1 has the potential to play an important role in influencing the progression of a local response to infection and the onset of the SIRS.

Materials and Methods

Animals

All procedures were conducted in accordance with the U.K. Animals (Scientific Procedures) Act of 1986. Mice were kept in a climatically controlled environment and given food and water ad libitum. In all studies, female and male age- and sex-matched C57Bl/6J(129SvJ wild type (WT) and TRPV1 knockout (TRPV1KO) mice were used at 8 wk of age. TRPV1KO mice with the exon that encodes part of the fifth and the entire sixth transmembrane domain (including the interconnecting p-loop) of the receptor replaced with a neomycin gene (6) were genotyped as previously described (18, 19). Mice were from established WT and TRPV1KO colonies held at King’s College London and grow normally, although TRPV1KO mice show characteristic loss of thermal responsiveness (18, 19).

Sublethal sepsis induced by CLP

Experiments were designed with a premortality end point (at 24 h) according to the method described by Baker et al. (20), with minor modifications. Animals received a single i.m. injection containing midazolam (2 mg/kg; Roche, Welwyn, U.K.) and buprenophine (10 µg/kg; Vetsergic, Aloise Animal Health, U.K.), 15 min before the surgery. During the surgical procedure, anesthesia was maintained with isoflurane 2% (Abbott, Kent, U.K.). Surgery was performed using aseptic techniques. In brief, an incision of ∼2 cm was made in the ventral surface of the abdomen and the cecum was exposed through the incision. The cecum was ligated at its base (without causing bowel obstruction) with silk suture 4–0 (Ethicon; Johnson & Johnson) and perforated with a 22-gauge needle resulting in two holes. Sham-operated animals (with incision only) were used as controls. Both sham and CLP mice were sutured with absorbable suture (Vicryl; Ethicon; Johnson & Johnson) and received 1 ml saline 0.9% (s.c.) for postsurgery hydration. Animals were killed 24 h after surgery by cervical dislocation. A laparotomy was performed and the peritoneal cavity was washed with 1.5 ml PBS. The peritoneal exudate lavage fluid (PELF) and tissue samples (lung and liver) were collected for further analysis.

Inflammatory cell accumulation in the peritoneal cavity

Inflammatory cell migration into the peritoneal cavity was evaluated according to Bargon and collaborators (21) with minor modifications. Total cell counts were performed with a Neubauer chamber and microscope (Leitz Diaplan 307-148.001; PL Fluotar ×10 objective; numerical aperture 0.3) after diluting a sample of the collected fluid from the peritoneal cavity with Türk solution (1:20). Cellular smears were prepared with an aliquot (50 µl) of the PELF to determine the differential leukocyte population. For differential cell analysis, we considered only viable cells, that is, cells with normal morphology. The slides were stained with Diff-Quik kit (Gamidor, Didcot, U.K.); then the analysis was carried out under an immersion objective (Leitz Diaplan 307-148.001; ×100 objective; numerical aperture 1.25). Images were acquired with Jenoptik ProResC5 camera and visualized in Capture Pro 2.7.

In vitro phagocytosis

Phagocytosis was analyzed in the peritoneal cells obtained from WT and TRPV1KO mice. For this purpose, animals were injected i.p. with 1 ml PBS containing 1% oyster glycogen. After 18 h, the peritoneal cavity was washed with 10 ml cold PBS, and the peritoneal cells were harvested, centrifuged (10 min, 4˚C), and resuspended (final concentration of 2 × 106 cells/ml) in DMEM containing 10% FCS (v/v), glutamine (2 mM; PAA, Pasching, Austria), penicillin (1×; PAA), and streptomycin (1×; PAA). Cells (6 × 106) were incubated in eight chamber culture slides (BD Falcon) at 37˚C, and after 2 h, nonadhered cells were removed and adherent cells (macrophages) were incubated in the presence and absence of 2 µM fluorescent beads (400; Invitrogen, Paisley, U.K.); for 24 h before analysis. Phagocytosis was evaluated in unstimulated cells and also in cells treated with LPS (100 ng/ml, Escherichia coli 0127:B8). The role SP (10 nM) and CGRP (10 nM) in phagocytosis was evaluated in TRPV1KO macrophages treated with LPS. In addition, TRPV1KO macrophages were incubated in the presence of either the NK1 receptor antagonist SB43998 (5 nM). In a different set of experiments, LPS-stimulated WT macrophages were incubated with either the TRPV1 antagonist SB366791 (20 µM) or vehicle (0.1% DMSO). After the incubation period, the cell culture medium was removed and each well was washed three times with PBS. Wells were fixed in 4% paraformaldehyde for 10 min and washed three times with PBS for the removal of excessive paraformaldehyde. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (H2O2) for 30 min at 4˚C in a dark chamber. Wells were washed twice with PBS (5 min) and then blocked with blocking solution (0.1% rabbit serum, Vectastain ABC kit; Vector Labs; 0.2% BSA and 2% skimmed milk). Two drops of the rabbit polyclonal primary Ab (1:750; diluted in blocking solution) were added to each well. Plates were incubated overnight at 4˚C and then washed three times (10 min) with PBS containing 0.05% Tween 20 and 2 mM levamisole. Two drops of anti-rabbit secondary Ab (Vectastain ABC kit; Vector Labs) were added to each well and incubated for 1.5 h. Wells were then washed three times with PBS containing 0.05% Tween 20 and 2 mM levamisole (10 min). The primary antibodies were counted for each sample, and the average for each sample was considered as an n number. Results are expressed as percentage of cells containing beads and number of phagocytosed beads per 100 cells.

Caspase-3 immunocytochemistry

Caspase-3 expression was evaluated in cultured macrophages obtained from WT and TRPV1KO mice as described earlier. After 24-h incubation with LPS (100 ng/ml), in the presence and absence of either SB366791 (20 µM) or vehicle (0.1% DMSO), the cell culture was removed and the slides were washed three times with PBS. After the incubation period, the cell culture medium was removed and each well was washed three times with PBS. Wells were fixed in 4% paraformaldehyde for 10 min and washed three times with PBS for the removal of excessive paraformaldehyde. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide (H2O2) for 30 min at 4˚C in a dark chamber. Wells were washed twice with PBS (5 min) and then blocked with blocking solution (0.1% rabbit serum, Vectastain ABC kit; Vector Labs; 0.2% BSA and 2% skimmed milk). Two drops of the rabbit polyclonal primary Ab (1:750; diluted in blocking solution) were added to each well. Plates were incubated overnight at 4˚C and then washed three times (10 min) with PBS containing 0.05% Tween 20 and 2 mM levamisole. Two drops of anti-rabbit secondary Ab (Vectastain ABC kit; Vector Labs) were added to each well and incubated for 1.5 h. Wells were then washed three times with PBS containing 0.05% Tween 20 and 2 mM levamisole (10 min). The primary antibodies were counted for each sample, and the average for each sample was considered as an n number. Results are expressed as percentage of caspase-3+ cells.

Assessment of NO levels by measurement of NO2⁻/NO3⁻

The NO2⁻/NO3⁻ content was measured by the Griess assay as an indicator of NO production in the PELF obtained from sham- and CLP-operated animals. NO2⁻ was reduced to nitrite (NO2⁻) by incubating 80 µl sample with 20 µl of 1 U/ml nitrate reductase and 10 µl of 1 mM NADPH at 30 min at 37˚C in a 96-well plate. Next, 100 µl Griess reagent (5% v/v H3PO4 containing 1% w/v sulfamic acid and 0.1% w/v N-1-naphthylethylenediamine) was added and incubated for 15 min at 37˚C. Absorbance at 550 nm was measured immediately using a spectrophotometer (Spectra Max 190; Molecular Devices, Palo Alto, CA). After subtraction of background readings, the absorbance in each sample was compared with that obtained from a sodium nitrite (0–100 µM) standard curve and expressed as NO3⁻ levels (µM).

Superoxide release from the peritoneal inflammatory cells

Superoxide (O2⁻) release from the peritoneal inflammatory cells obtained from sham- and CLP-operated animals was measured by chemiluminescence using lucigenin (bis-N-methylacridinium nitrate; Sigma Chemical) as a probe (22). For this, 100 µl modified Krebs buffer (pH 7.4, composition: NaCl 131 mM, KCI 5.6 mM, NaHCO3 25 mM, NaH2PO4·H2O 1 mM, glucose 5 mM, HEPES 5 mM, t-arginine 100 µM, CaCl2 2.5 mM, MgCl2 1 mM, and NADPH 100 µM) was pipetted into each well of a 96-well white plate (Nunc). Then, 50,000 cells obtained from the PELF were added and incubated with or without O2⁻ dismutase (50 U/ml; Sigma Aldrich, U.K.) in 100 µl modified Krebs buffer. Immediately, the plate was read in a chemiluminescence reader (Plate Chameleon V Microplate Reader; Nokki Technologies PVT, Hidex Oy, Finland) at 37˚C, 1 s/read, each 90 s, for 1 h. Results are expressed as the difference in the cpm in the presence and absence of O2⁻ dismutase.

H2O2 production by peritoneal inflammatory cells

H2O2 production by peritoneal inflammatory cells obtained from sham- and CLP-operated animals was measured by using a H2O2/peroxidase assay (Amplex Red; Molecular Probes; Invitrogen, Paisley, U.K.). The assay was performed according to the manufacturer’s instructions with minor modifications. In brief, 25,000 cells from the PELF were incubated with 100 µl Krebs buffer (pH 7.4, com-
position: NaCl 131 mM, KCl 5.6 mM, NaHCO₃ 25 mM, NaH₂PO₄·H₂O 1 mM, glucose 5 mM, HEPES 5 mM, CaCl₂ 2.5 mM, MgCl₂ 1 mM, and NADPH 100 µM and 100 µl of a solution containing NaPO₄ 0.05 M (pH 7.4), HRP 0.2 U/ml, and Amplex Red Reagent (10-acetyl-3,7-dihydroxyphenoxazine) 25.7 µg/ml, for 2 h, at 37°C. Samples incubated with Krebs buffer only were used as controls. After incubation, 100 µl of each sample was pipetted into 96-well plates, and reaction was read at 560 nm. Absorbance readings, obtained for samples incubated in the absence or presence of Amplex Red Reagent, were compared with a H₂O₂ standard curve (0–40 µM). Results are expressed as the difference between absorbance readings obtained for samples incubated in the presence of Amplex Red Reagent or Krebs (in µM).

Cytokine and chemokine measurement

PELF IL-6, IL-10, TNF-α, IL-1β, and keratinocyte-derived chemokine (KC) levels were evaluated using a proinflammatory multiplex cytokine kit (Mouse Proinflammatory multiplex kit; Meso-Scale Discovery, Gaithersburg, MD) according to the manufacturer’s instructions. Sample readings for each cytokine were compared with a standard curve (0–10,000 pg/ml). MCP-1 and MIP-1β were evaluated by using a multiplex assay (Searchlight; Aushon Biosystems, Billerica, MA). Results are expressed as protein levels in pg/ml. All detection Abs exhibited <1% cross-reactivity with other analytes.

Blood pressure evaluation

The effects of sepsis on blood pressure were assessed 24 h after surgery. Blood pressure was assessed noninvasively in conscious, restrained mice by the tail cuff technique using the CODA 6 System (Kent Scientific, Torrington, CT), which assesses tail blood pressure by means of volume pressure recording (18). Baseline measurements were taken before surgery.

Body temperature

Body temperature was monitored using a rectal probe (Harvard Apparatus, Holliston, MA) just before and 24 h after surgery (18).

Assessment of organ damage

Dysfunction of heart/liver, kidneys, and pancreas was assessed by measuring aspartate aminotransferase (AST), creatinine, and lipase levels, respectively, present in plasma obtained from WT and TRPV1 KO mice 24 h after surgery. In a separate group, CLP WT mice were treated s.c. for 6 d (twice daily) with the TRPV1 antagonists, capsaizinpe (30 µg/animal) or SB366791 (0.5 mg/kg), or with vehicle (120 µl; 10% DMSO in saline). Measurements were made by Nationwide Laboratories (Lancashire, U.K.). Results are expressed as UI/l (lipase and AST) and µmol/l (creatinine).

Gene expression analysis in peritoneal inflammatory cells

Quantitative mRNA expression in peritoneal inflammatory cells was determined by real-time PCR. In brief, peritoneal cells were collected from WT and TRPV1 KO mice 24 h after CLP and stored in RNA later until RNA extraction was performed. DNA-free total RNA was extracted from the samples using the RNaseasy Microarray kit (Qiagen). A total of 0.5 µg total RNA was reverse transcribed to cDNA using a Corbett Rotorgene (hold: 10 min at 95°C; cycling: 45 cycles: 10 s at 95°C, 15 s at 57°C, and 5 s at 72°C; melt: 68–90°C), using the SensiMixTM SYBR No-ROX Kit (Bioline). The following primers were obtained from Sigma: caspase-3 [forward: 5’-GGAGGTGACTTCTCTGATGCTT-3’; reverse: 5’-AAACAGCGACCCTCTTT-3’], p53 [forward: 5’-ATGCCCCGAGCAGAACCAAGCGGGG-3’; reverse: 5’-GGGCTCTCTGCCTCTTCTC-3’], PI3Ka [forward: 5’-GACAAGAACAGGCGGACATGT-3’; reverse: 5’-CAGTACCCAGGCGGAGC-3’], PI3KB [forward: 5’-GACAAGAACAGGCGGACATGT-3’; reverse: 5’-CAGTACCCAGGCGGAGC-3’], TACPA [forward: 5’-CTCGAGAATGGGCTCCACC-3’; reverse: 5’-CTGGAGAATGGGCTCCACC-3’], PAS [forward: 5’-TTCAGATCTTGGTGATGTTGTA-3’; reverse: 5’-CTCTGGCAAGATGGCCAGAC-3’], NOX4 [forward: 5’-GAAACATTGGTATCTCTGATTCA-3’; reverse: 5’-AAGGACACAAAGGCTAGAACAGA-3’], TAC-1 [forward: 5’-AACGCTCATGAGGTTCTTTG-3’; reverse: 5’-TTCGGTGATTGCTCTGAAAAG-3’], oCRP [forward: 5’-AGGAGAGAAGAGAGAGAG-3’; reverse: 5’-CAGATTTCCCAACGGCTTAGA-3’], TLR4 [forward: 5’-GGATCTGGATGGCAGACATTC-3’; reverse: 5’-CTGTGATGTGGTGATGGTGAG-3’], CD14 [forward: 5’-GGACTCTGTGCACTGGTGAG-3’; reverse: 5’-CTGTGATGTGGTGATGGTGAG-3’], triggering receptor expressed on myeloid cell 1 (TREM1) [forward: 5’-TCTGGGATTGTCTGGTTGTGA-3’; reverse: 5’-GGAGTGAAACATCACTGGAAGCC-3’], TREM2 [forward: 5’-TGGGACTCTCCACAGGTGTT-3’; reverse: 5’-GTGGTGTGAGGGCTTGG-3’], PLA2G12A [forward: 5’-TGGAATAACCATCTCCACACA-3’; reverse: 5’-GGGAGAGGATACCTATGTTCAGA-3’], and B2M [forward: 5’-CTCAGAAGGTTAAGCTGAGG-3’; reverse: 5’-GATGCTTGTACATGTTCCTG-3’]. Results are expressed as copy number per microgram of pure cDNA normalized by comparison with B2M and PLA2G12A using GeoNorm version 3.1 (23). All experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real Time PCR Experiments guidelines.

Detection of bacteria in liver and lung

The presence of bacteria in liver and lung samples obtained from CLP WT and TRPV1 KO mice after 24-h sepsis was assessed. For this purpose, the levels of 16S rRNA, transcribed from a gene expressed by all bacteria (23) were determined. In brief, samples were collected and stored in RNase-free tubes. The TRPV1 KO mouse group were treated s.c. for 2 h, at 37°C. Samples incubated with the presence of Amplex Red Reagent, were compared with a H₂O₂ standard curve (0–40 µM). Results are expressed as copy number per microgram by using a chromogenic endotoxin detection system (Genscript), which mainly measures endotoxin that is dissociated from bacteria (free endotoxin) (24). All samples were collected and kept in endotoxin-free tubes at −80°C until the endotoxin test. Just before the assay, tissue samples were homogenized in endotoxin-free PBS. All samples were diluted 1:10 and heated at 75°C for 5 min to minimize protein interference with the assay. Assay was performed according to manufacturer’s instructions. Results obtained from plasma samples were expressed as endotoxin units per milliliter. Results obtained from tissue samples were normalized by protein content and expressed as endotoxin units per milligram of protein. Protein levels were quantified by Bradford protein assay (Bio-Rad, Hemel Hempstead, U.K.).

Tissue and plasma endotoxin levels

Endotoxin levels were analyzed in plasma and liver and lung samples obtained from CLP WT and TRPV1 KO mice by using a ToxinSensor chromogenic endotoxin detection system (Genscript), which mainly measures endotoxin that is dissociated from bacteria (free endotoxin) (24). All samples were collected and kept in endotoxin-free tubes at −30°C until the endotoxin test. Just before the assay, tissue samples were homogenized in endotoxin-free PBS. All samples were diluted 1:10 and heated at 75°C for 5 min to minimize protein interference with the assay. Assay was performed according to manufacturer’s instructions. Results obtained from plasma samples were expressed as endotoxin units per milliliter. Results obtained from tissue samples were normalized by protein content and expressed as endotoxin units per milligram of protein. Protein levels were quantified by Bradford protein assay (Bio-Rad, Hemel Hempstead, U.K.).

Data analysis

The results are presented as mean ± SEM. The percentages of inhibition are reported as mean ± SEM of inhibitions obtained in each individual experiment compared with control samples. Statistical comparisons of the data were performed by ANOVA followed by Newman–Keuls and unpaired or paired t test when appropriate. The p values <0.05 were considered significant.

Results

TRPV1 protects against organ damage and hypothermia in sepsis

Systemic sepsis was evaluated 24 h after CLP by measuring plasma levels of AST, creatinine, and lipase as indicators of heart/liver, kidney, and pancreas failure, respectively. Sepsis induction by CLP led to a significant increase in AST, but not creatinine and lipase levels, when compared with sham-operated animals, indicating organ dysfunction in WT mice (Fig. 1A–C). TRPV1 KO exhibited further increases of AST, creatinine, and lipase production, compared with CLP-operated WT mice (Fig. 1A–C), showing that mice lacking TRPV1 suffer accelerated organ damage. No difference was observed between the sham-operated WT and TRPV1 KO mice. Similarly, 6-d treatment with the
TRPV1 antagonists capsazepine or SB366791 led to increased levels of AST (Fig. 1D) and lipase (Fig. 1E) in CLP WT compared with vehicle-treated mice. Significant hypothermia was seen in CLP-operated TRPV1KO when compared with baseline (Fig. 1F) and also when compared with CLP WT animals. No changes were observed in sham-

FIGURE 1. Effect of TRPV1 deletion or blockade on the onset of early multiple organ failure, hypothermia, and hypotension in sepsis. Plasma levels of (A) AST, (B) creatinine, and (C) lipase in WT and TRPV1KO mice; plasma levels of (D) AST and (E) lipase in WT mice treated s.c. for 6 d (twice daily) with the TRPV1 antagonists, capsazepine (50 μg/animal) or SB366791 (0.5 mg/kg), or vehicle (10% DMSO in saline; 120 μl/animal). AST, creatinine, and lipase were used as indicative of heart/liver, kidney, and pancreas failure, respectively, 24 h after CLP induction. (F) Body temperature and (G) systolic blood pressure were measured before and 24 h after CLP induction. Bars represent the mean ± SEM of all mice per group (n = 6, sham; n = 8, CLP group), obtained from three independent experiments. *p < 0.05, **p < 0.01 compared with sham-operated (A–C) or baseline (D, E), #p < 0.05 compared with CLP-operated WT mice.
operated mice. A decreased systolic blood pressure was observed in both CLP WT and TRPV1KO when compared with baseline (Fig. 1G), which was significant in CLP TRPV1KO mice. These results suggest TRPV1 has a role in maintaining the body homeostasis during the transition of local to systemic inflammatory disease.

**TRPV1 is expressed on peritoneal inflammatory cells and is critical for peritoneal mononuclear cell viability**

TRPV1 is expressed in peritoneal inflammatory cells obtained from both sham and CLP WT mice (Fig. 2A). Thus, we hypothesized that TRPV1 played a role in peritoneal cell functioning. We first evaluated the naive peritoneal cell population. TRPV1KO presented lower cell counts when compared with WT mice (Fig. 2B), suggesting a possible intrinsic mechanism for lack of peritoneal protection in these animals.

We analyzed the possible participation of TRPV1 in influencing cell migration into the peritoneal cavity following CLP. Total cell counts (Fig. 2C) were increased in both CLP WT and TRPV1KO mice when compared with shams. For differential analysis, only viable cells were considered (Fig. 2F). CLP WT mice exhibited increased numbers of both polymorphonuclear and mononuclear cells (Fig. 2D, 2E). CLP TRPV1KO and WT mice presented similar polymorphonuclear cell increase when compared with shams (Fig. 2D, 2E). In contrast, the number of intact mononuclear cells was reduced (59 ± 11%) in CLP-operated TRPV1KO mice compared with CLP WT mice (Fig. 2E).

**Increased apoptosis and changes in cytokine and chemokine release in the peritoneal cavity of TRPV1KO mice**

Cytokines and chemokines play a fundamental role in modulating sepsis (25, 26). Increased levels of TNF-α, IL-6, IL-10, and IL-1β were observed in CLP-operated WT mice when compared with shams (Fig. 3A–D). However, TNF-α, IL-6, and IL-10 levels in CLP TRPV1KO were significantly enhanced in comparison with CLP WT mice (Fig. 3A–D). MCP-1 and MIP-1β, but not KC levels, were reduced in CLP TRPV1KO when compared with WT mice (Fig. 3E–G). Analysis of the expression of FAS, a protein associated with apoptosis, revealed that peritoneal cells obtained from CLP TRPV1KO mice present higher FAS mRNA copy.

![FIGURE 2. TRPV1 role in peritoneal cells.](http://www.jimmunol.org/)
numbers when compared with CLP WT samples (Fig. 4A). Similarly, the mRNA for the apoptosis effectors p53 and caspase-3 was higher in CLP TRPV1KO than in CLP WT mice (Fig. 4B, 4C). In addition, we evaluated caspase-3 protein expression in adherent macrophages cultured from CLP WT peritoneal samples treated or not with the TRPV1 antagonist SB-366791 (20 μM).

Results show that the percentage of caspase-3+ cells was greater in SB-366791–treated samples (Fig. 4D, 4E).

Reduced ROS and NO levels in the peritoneal cavity in the TRPV1KO mice

Sepsis induced by CLP in WT mice led to an increase of NO, O$_2^-$, and H$_2$O$_2$ release into the peritoneal cavity (Fig. 5A–C). Negligible amounts of these mediators were detected in sham-operated animals. Surprisingly, the levels of NO, H$_2$O$_2$, and O$_2^-$ were substantially less in TRPV1KO by 85 ± 7, 77 ± 12, and 56 ± 10%, respectively, compared with levels in WT mice at 24 h after CLP. In addition, both NOX2 and NOX4 mRNA levels were reduced in CLP TRPV1KO in comparison with CLP WT peritoneal cells (Table I).

**TRPV1 deletion affects phagocytosis via the NK₁ receptor for SP and by affecting PI3K expression**

Unstimulated WT and TRPV1KO-derived macrophages presented a similar ability to phagocytose beads (Fig. 6A, 6B). After LPS stimulation, the number of beads phagocytosed (Fig. 6A) by WT macrophages, as well the number of cells able to phagocytose (Fig. 6B), remained unaltered when compared with unstimulated cells. Surprisingly, TRPV1KO macrophages were less able to phagocytose beads after LPS with a 67 ± 6% reduction when compared with KO unstimulated cells (Fig. 6A). Similarly, the number of TRPV1KO macrophages containing beads was also reduced by 36 ± 9% (Fig. 6B). SP and CGRP are released from sensory nerves when TRPV1 is activated, but they are also present in resident macrophages and circulating leukocytes (27–29). Indeed, SP treatment has been shown to stimulate macrophage-
mediated phagocytosis in vitro (30). Cotreatment of LPS-stimulated TRPV1KO macrophages with SP restored their phagocytic ability (Fig. 6C, 6D). Preincubation of cells treated with LPS and SP with the selective SP NK1 receptor antagonist SR140333, but not the selective NK2 receptor antagonist SR48968, blocked this stimulatory effect of SP on TRPV1KO macrophages (Fig. 6C, 6D). In contrast, CGRP coincubation had no significant effect in the TRPV1KO macrophage response treated with LPS (Fig. 6C). LPS-stimulated WT macrophages incubated with the TRPV1 antagonist SB366791 (20 μM) exhibited less phagocytosis when compared with vehicle-treated cells (Fig. 6E, 6F).

PI3Kα and β are involved in phagolysosome formation and also ROS production by macrophages (31, 32). CLP TRPV1KO peritoneal cells express less PI3Kα and β when compared with CLP WT cells (Fig. 6G, 6H, respectively).

**FIGURE 4.** TRPV1KO mice exhibit enhanced apoptosis markers. (A) FAS, (B) p53, and (C) caspase-3 mRNA levels in peritoneal cells obtained from WT and TRPV1KO mice 24 h after sepsis induction. (D and E) Caspase-3 protein expression by immunocytochemistry in LPS-challenged WT cells treated with the TRPV1 antagonist SB366791 (20 μM) or vehicle (0.1% DMSO). Immunocytochemistry staining was visualized under ×40 magnification. Bars represent the mean ± SEM of all mice per group obtained from four independent experiments performed in duplicate. n is indicated on the graphs. *p < 0.05 compared with vehicle-treated cells.

**FIGURE 5.** TRPV1 deletion decreases NO and ROS production in the peritoneal cavity. (A) NO2/NO3 levels are shown indicating NO formation (NOx); (B) H2O2 and (C) O2•- release from the peritoneal lavage cells 24 h after CLP induction. Bars represent the mean ± SEM of all mice per group obtained from at least three independent experiments performed in duplicate. n is indicated on the graphs. *p < 0.05, **p < 0.01 compared with sham-operated; ##p < 0.01 compared with CLP-operated WT mice.
Table I. Analysis of gene expression of various genes by quantitative real-time PCR in peritoneal cell samples obtained from CLP WT and TRPV1KO mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>CLP WT (×10^3 Copies/μl)</th>
<th>CLP TRPV1KO (×10^3 Copies/μl)</th>
</tr>
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<tbody>
<tr>
<td>NOX2</td>
<td>2114.9 ± 880.3</td>
<td>852.5 ± 532.7</td>
</tr>
<tr>
<td>NOX4</td>
<td>0.003 ± 0.002</td>
<td>0.0006 ± 0.0004</td>
</tr>
<tr>
<td>TAC-1</td>
<td>0.23 ± 0.13</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>αCGRP</td>
<td>423 ± 175.7</td>
<td>415.6 ± 151.4</td>
</tr>
<tr>
<td>TLR4</td>
<td>4757.3 ± 2500.3</td>
<td>4955 ± 1880.3</td>
</tr>
<tr>
<td>CD14</td>
<td>10,900 ± 3704.7</td>
<td>7900.2 ± 3123.6</td>
</tr>
<tr>
<td>TREM1</td>
<td>355.7 ± 177.9</td>
<td>283.7 ± 110.7</td>
</tr>
<tr>
<td>TREM2</td>
<td>206.6 ± 116.2</td>
<td>256.5 ± 91.9</td>
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</tbody>
</table>

Samples were collected 24 h after surgery, and results are expressed as number of copies/μl (×10^3). Results express the mean ± SEM of all mice per group obtained from at least two independent experiments performed in duplicate; n = 5 each group.

Table I shows the mRNA copy numbers for TAC-1 (a precursor of SP) were reduced in CLP TRPV1KO when compared with CLP WT samples, whereas no difference was observed for CGRPαx gene.

**TRPV1 does not affect pathogen recognition by TLR4**

Considering that CLP is a model that involves mainly Gram-negative bacteria (2) and that we found that TRPV1KO peritoneal cells behave differently to WT peritoneal cells in the presence of LPS, we quantified the mRNA levels of TLR4. No difference in TLR4 expression between peritoneal cell samples of CLP WT and TRPV1KO mice was observed (Table I). Also, CLP WT and TRPV1KO samples showed similar levels of mRNA for molecules involved in TLR4 signaling such as CD14, TREM1, and TREM2 (Table I).

**TRPV1 deletion facilitates local bacterial survival**

Our results show increased 16S rRNA levels in liver but not lung samples obtained from CLP TRPV1KO when compared with WT mice (Table II). Endotoxin is mainly released from bacteria after being shed or after bacterial lysis (33). CLP WT and TRPV1KO mice lung and liver samples exhibited similar levels of endotoxin per milligram of tissue (Table II). In contrast, CLP TRPV1KO plasma samples presented reduced endotoxin units when compared with CLP WT samples (Table II).

**Discussion**

Recent evidence shows that TRPV1 blockade or deletion in LPS-induced endotoxemia is associated with poorer survival in rats (17) and enhanced symptoms, with raised sepsis markers in mice (18). Although this protective role for TRPV1 has been demonstrated in two species, the in vivo mechanisms are unknown. In this article, we demonstrate that TRPV1 protects against CLP-induced sepsis, considered to model human disease. This protection is linked to inflammatory cell functioning, specifically the macrophage and its ability to kill and phagocytose bacteria, pivotal to the efficiency of local defense mechanisms at the site of infection and the transition from a local to a systemic inflammatory response as observed in SIRS.

We show that TRPV1KO mice exhibited decreased body temperature and systolic blood pressure after CLP when compared with baseline measurements at 24 h, indicative of the onset of systemic sepsis, whereas no significant changes were observed in the CLP WT group. These results complement our findings that TRPV1 deletion is associated with decreases in both temperature and mean blood pressure at 4 h after LPS treatment (18). In this study, we used the decreased temperature as a marker of impending morbidity (34) in keeping with welfare considerations. AST, creatinine, and lipase levels were measured as markers of heart/liver, kidney, and pancreas dysfunction, indicative of early signs of organ failure. We show that TRPV1 protects against liver, kidney, and pancreatic failure as the release of AST, creatinine, and lipase are increased in CLP TRPV1KO mice. Similarly, increased organ failure was also observed in CLP WT animals treated with the TRPV1 antagonists capsazepine and SB366791. These data confirm that an SIRS has been initiated by 24 h after CLP, with enhanced hypotension, hypothermia, and markers of organ damage indicating an accelerated sepsis onset after either TRPV1 deletion or pharmacological blockade.

Both human dendritic cells and T lymphocytes express functional TRPV1 (35, 36). We show that WT peritoneal inflammatory cells express TRPV1 gene in both sham and septic conditions. This would enable TRPV1-mediated protection to occur at an early stage of local gut infection after CLP. The regulation of cell migration and inflammatory mediator release involves a fine cross talk between several systems in the body and is critical for sepsis outcome (1, 23, 37), but the mechanisms involved in the modulatory effect of TRPV1 are unclear. Our results show that TRPV1 can modulate sepsis at several steps of the CLP-induced inflammatory response. SIRS and organ damage caused by 24-h CLP in mice with disrupted TRPV1 signaling was correlated with the demonstration of a distinct enhanced inflammation in the peritoneal cavity compared with that in WT mice. The CLP-induced peritoneal inflammation in TRPV1KO mice was associated with a reduced number of viable mononuclear cells, increased expression of apoptosis markers, and increased production of key cytokines, but reduced ROS, NO, and chemokine generation in the peritoneal cavity, facilitating an increased bacterial survival in the peritoneal cavity. Moreover, the TRPV1KO peritoneal macrophages exhibited impaired phagocytosis to LPS, caused by a tachykinin NK1-receptor–dependent mechanism.

Mortality in sepsis is preceded by a simultaneous release of both proinflammatory and anti-inflammatory cytokines (38). Our results show that TNF-α, IL-6, and IL-10 levels are increased in the peritoneal cavity of CLP TRPV1KO compared with CLP WT mice. This suggests that TRPV1 can influence cytokine generation during septicemia and reinforces the concept that the inflammatory response observed in TRPV1KO mice is being accelerated. Indeed, there is evidence of enhanced TNF-α levels in LPS-treated TRPV1KO, compared with WT mice (18). In addition, administration of a low dose of the TRPV1 agonist capsaicin has been shown to reduce sepsis in septic rats, by decreasing plasma levels of TNF-α and IL-6, but not IL-10, which was increased in that study (39). These evidences suggest that TRPV1 activation represents an important step in the inflammatory response associated to sepsis.

We also found that TRPV1 influences ROS and NO release from peritoneal cells. Indeed, links between O2− production and TRPV1 upregulation, involving NOX production and subsequent O2− release, exist (40, 41). Moreover, there is evidence that TRPV1 can be activated in the presence of H2O2 (42). We postulate that sepsis triggers O2− and H2O2 release from cells located in the peritoneum that, in turn, amplify TRPV1 expression to enhance ROS generation. We show that high levels of O2−, H2O2, and NO are released from the peritoneal cells of CLP WT mice. However, one of the most striking findings from this study is that substantially less ROS and NO release was detected in CLP TRPV1KO mice. This surprised us, because NO release was enhanced after 4 h in TRPV1KO in the acute response to intraperitoneal LPS (18). The opposing effects of TRPV1 on ROS and NO release may be model dependent. Previous studies show that capsaicin treatment reduces
plasma NO release in CLP-operated rats (39) and also reduces NO release from RAW264.7 macrophages stimulated with LPS in vitro (43), supporting the idea that TRPV1 activation can regulate NO. We found that the reduced generation of ROS and NO from CLP TRPV1KO peritoneal cells correlated with reduced NOX2 and NOX4 mRNA expression in these cells. Indeed, it has

![FIGURE 6. TRPV1 deletion impairs phagocytosis in peritoneal macrophages.](http://www.jimmunol.org/)

Table II. Detection of bacterial 16S rRNA by quantitative real-time PCR in tissue samples and endotoxin levels quantification in plasma and tissue samples obtained from CLP WT and TRPV1 KO mice

<table>
<thead>
<tr>
<th>Tissue Endotoxin Levels (×10^−5 Endotoxin Units/Mg Protein)</th>
<th>Plasma Endotoxin Levels (Endotoxin Units/Ml)</th>
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<tbody>
<tr>
<td>16S rRNA (Copies/μl)</td>
<td>Lang</td>
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been demonstrated that NOX4 can interact with TLR4, thus regulating H$_2$O$_2$ production (44), whereas NOX2 activation in the phagosomal membrane of macrophages leads to O$_2^-$ generation (45). One interpretation of our data is that TRPV1 is involved in mediating the ability of phagocytes to release ROS and NO$^*$, and kill microorganisms. This defensive mechanism is impaired in TRPV1KO mice, resulting in increased bacterial survival in the peritoneal cavity such as in the liver, and this may lead to the acceleration of the systemic phase of the disease.

The decrease in ROS and NO levels in the absence of TRPV1 twinned with evidence that peritoneal cells do express TRPV1 led us to investigate cell migration. We have shown that peritoneal lavage from CLP TRPV1KO mice contained lower levels of chemokines involved in macrophage recruitment (MCP-1 and MIP-1b) (46, 47) than that from CLP WT mice, whereas levels of KC, a neutrophil-related chemokine (48), was similar in both WT and TRPV1KO mice. Also, in vivo analysis of cell populations showed that naive TRPV1KO possess fewer peritoneal resident inflammatory cells than WT mice. However, after CLP, both WT and TRPV1KO mice exhibited similar numbers of viable PMN cells in the peritoneal cavity, whereas the number of viable mononuclear cells was significantly lower in TRPV1KO compared with WT mice.

The reduction of viable macrophages and increased TNF-α levels observed in CLP TRPV1KO led us to investigate the expression of apoptosis markers such as FAS (a death receptor member of the TNF superfamily expressed on inflammatory cells) (49), and the apoptosis effector molecules caspase-3 and p53. TNF-α itself is known to mediate apoptosis via its TNFR1 (50). In addition, FAS, p53, and caspase-3 mRNA levels were all increased in peritoneal inflammatory cell samples from CLP TRPV1KO when compared with CLP WT mice. Similarly, WT macrophages treated with the TRPV1 antagonist SB366791 expressed more caspase-3 than WT cells after LPS treatment. These findings lead us to hypothesize that the increased release of TNF-α and increased expression of FAS and apoptosis effectors in the peritoneal cavity favors mononuclear cell apoptosis in the TRPV1KO septic mouse. This would explain, at least in part, the reduction of the infection in the liver, whereas the number of viable mononuclear cells was significantly lower in TRPV1KO compared with WT mice.

In conclusion, we provide substantial new evidence that TRPV1 activation is associated with sepsis outcome. We show that TRPV1 activation at the site of infection is directly linked to a series of events in the immune and inflammatory response, central to macrophage functioning such as apoptosis, ROS, and NO production, and also the release of key controlling cytokines and chemokines from inflammatory cells. The sum effect of TRPV1 activation is associated with sepsis outcome. TRPV1 regulates bacterial killing instead.

TRPV1 is best known for its ability to release SP and CGRP (5), neuropeptides considered as predictors of lethal sepsis outcome in patients (11). Previously, we have shown that the enhanced LPS-induced endotoxemia observed in TRPV1KO mice is unlikely to be due to SP (18). In this study, we show reduced levels of TAC-1 mRNA in CLP TRPV1KO when compared with CLP WT macrophages treated with the TRPV1 antagonist SB366791 expressed more caspase-3 than WT cells after LPS treatment. These findings lead us to hypothesize that the increased release of TNF-α and increased expression of FAS and apoptosis effectors in the peritoneal cavity favors mononuclear cell apoptosis in the TRPV1KO septic mouse. This would explain, at least in part, the reduction of the infection in the liver, whereas the number of viable mononuclear cells was significantly lower in TRPV1KO compared with WT mice.

We also evaluated the impact of TRPV1 disruption on pathogen recognition. TRPV1 has been found colocalized to the LTR4 and CD14 receptor complex in sensory nerves (52). In addition, TLR4 could sensitize TRPV1 via protein kinase C activation (53). Although these pieces of evidence suggest a sensing mechanism for bacterial infection in sensory nerves, we could not find differences in the mRNA expression of TLR4 or in molecules involved in the pathogen recognition mediated by TLR4 and TLR4 signaling such as CD14, TREM1, and TREM2 (52–56). This suggests that TRPV1 does not interfere with TLR4 functioning, but rather reinforces the idea that TRPV1 regulates bacterial killing instead.

TRPV1 is best known for its ability to release SP and CGRP (5), neuropeptides considered as predictors of lethal sepsis outcome in patients (11). Previously, we have shown that the enhanced LPS-induced endotoxemia observed in TRPV1KO mice is unlikely to be due to SP (18). In this study, we show reduced levels of TAC-1 mRNA in CLP TRPV1KO when compared with CLP WT peritoneal inflammatory cells. SP is suggested to modulate immune cell function and inflammation via a range of mechanisms (28, 29, 55, 57). This article shows for the first time, to our knowledge, that SP mediates TRPV1 protection by positively regulating macrophage phagocytosis via NK1 receptor activation. By comparison, we found no evidence for a role of CGRP in this response.

In conclusion, we provide substantial new evidence that TRPV1 activation is associated with sepsis outcome. We show that TRPV1 activation at the site of infection is directly linked to a series of events in the immune and inflammatory response, central to macrophage functioning such as apoptosis, ROS, and NO production, and also the release of key controlling cytokines and chemokines from inflammatory cells. The sum effect of TRPV1 activation is an impairment of phagocytosis and bacterial clearance during sepsis. This directly influences the enhancement of hypotension and hypothermia, and evidence of organ damage. This study demonstrates the importance of TRPV1 to the initial site of infection and highlights the need to learn more about fundamental mechanisms in humans and also potential therapeutic strategies at this early stage (e.g., when patients are undergoing operations) to attempt to combat local infections before they disseminate into SIRS.

Disclosures
The authors have no financial conflicts of interest.

References