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Targeted endothelial gene deletion of triggering receptor expressed on myeloid cells-1 protects mice during septic shock

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Aims TREM-1 (Triggering Receptor Expressed on Myeloid cells-1) is an immunoreceptor expressed on neutrophils and monocytes/macrophages whose role is to amplify the inflammatory response driven by Toll-Like Receptors engagement. The pharmacological inhibition of TREM-1 confers protection in several pre-clinical models of acute inflammation. In this study, we aimed to decipher the role of TREM-1 on the endothelium.

Methods and results We first showed by qRT-PCR, flow cytometry and confocal microscopy that TREM-1 was expressed in human pulmonary microvascular endothelial cells as well as in mouse vasculature (aorta, mesenteric artery, and pulmonary vessels). TREM-1 expression was upregulated following septic insult. We next observed that TREM-1 engagement impaired mouse vascular reactivity and promoted vascular inflammation. The pharmacological inhibition of TREM-1 (using the synthetic inhibitory peptide LR12) prevented these disorders both in vitro and in vivo. We generated endothelium-conditional Trem-1 ko mice (EndoTREM-1$^{-/-}$) and submitted them to a caecal ligation and puncture-induced septic shock. As compared with wild-type littermates, targeted endothelial *Trem-1* deletion conferred protection during septic shock in modulating inflammatory cells mobilization and activation, in restoring vasoreactivity, and in improving the survival.

Conclusion We reported that TREM-1 is expressed and inducible in endothelial cells and plays a direct role in vascular inflammation and dysfunction. The targeted deletion of endothelial *Trem-1* conferred protection during septic shock in modulating inflammatory cells mobilization and activation, restoring vasoreactivity, and improving survival. The effect of TREM-1 on vascular tone, while impressive, deserves further investigations including the design of endothelium-specific TREM-1 inhibitors.

Keywords TREM-1 • Endothelium • Septic shock

1. Introduction

Septic shock, a complex clinical syndrome resulting from a harmful and damaging host response to infection, is the leading cause of mortality in intensive care units.[1,2] Sepsis develops when the initial appropriate host response to systemic infection becomes deregulated and over-amplified with an intimate crosstalk between inflammation and coagulation.[3]

An important hallmark of sepsis is endothelial activation and dysfunction, which appears to play a pivotal role in the development of this syndrome. The consequences of this endothelial dysfunction include not only alterations in the control of vasomotor and platelet functions, but also an amplification of the inflammatory response associated with programmed cell death, adhesion molecule overexpression and increased leukocyte trafficking.[4–7] Moreover, endothelial dysfunction is associated with an increased risk of cardiovascular events.[8,9] Whereas the protection of the endothelium is of paramount importance in acute (septic shock) or chronic conditions (cardiovascular illnesses), available pharmacological tools are scarce.

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The Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) is an immune-receptor that plays a key role in the amplification of inflammation, crosstalk with other Pattern Recognition Receptors pathways and activation of antigen-presenting cells.[10,11] Membrane TREM-1 presents three distinct domains: an Ig-like structure, a transmembrane part and a cytoplasmic tail which associates with the adaptor molecule DAP12.[12] TREM-1 has been identified with high level of expression in neutrophils, mature monocytes, macrophages and natural killer cells, and with low level of expression on some epithelial cells.[13–15] A recent study from our group also showed that TREM-1 is expressed by platelets where it plays a key role in mediating their activation.[16]

Following the engagement of toll-like receptor (TLR)-2 or -4, TREM-1 is up-regulated in a Myeloid Differentiation factor 88 (MyD88)-dependent way through the involvement of the transcription factors NF-κB, PU.1 and activator protein 1 (AP1).[10–12] On the opposite, TREM-1 silencing down-regulates of several key players of the TLRs pathway, such as MyD88, the cluster of differentiation 14 (CD14), NF-κB Inhibitor alpha (IκBα), as well as several inflammatory mediators such as interleukin 1 beta (IL-1β), monocyte chemotactic protein 1 and IL-10.[17]

Although the pathophysiological role of TREM-1 was first identified during infectious diseases this receptor is involved in many acute and chronic inflammatory disorders.[18] Indeed, TREM-1 pharmacological inhibition or genetic invalidation reduces hyper-responsiveness and death during various experimental septic shock models,[19–21] protects from cardiovascular dysfunction following myocardial infarction,[22,23] and prevents atherosclerosis.24[25] Moreover, pharmacological modulation of TREM-1 impairs platelet activation and prevents from arterial or venous thrombosis.[16]

To modulate TREM-1 activity, a dodecapeptide (LR12) designed to mimic a highly-conserved sequence across various species (human, monkey, porcine, and murine sequences) and across two genes belonging to the TREM family (*trem-1* and *treml1*) has been used in these former studies. Indeed, LR12 does not bind to TREM-1 but to its (still unknown) ligand(s), acting as a decoy receptor, and as such is not a *direct* TREM-1 inhibitor. However, LR12 seems to specifically inhibit TREM-1 as this peptide shows not effect in the absence of TREM-1.[16,26]

In this study, we demonstrate that TREM-1 is expressed by endothelial cells (ECs) and show that its pharmacological inhibition by LR12 dampens vascular dysfunction induced by endotoxin or bacterial peritonitis. Moreover, we report that targeted endothelial *Trem-1* deletion protects mice during septic shock in modulating inflammatory cells mobilization and activation, in restoring vasoreactivity, and in improving survival.

2. Methods

2.1 Animals

Endothelium-conditional Trem-1 ko mice were generated using a targeting vector designed for conditional deletion of exon 2 that encodes for the extracellular domain of TREM–1.

Breeding of C57Bl/6 Trem-$1^{+/flox}$ chimeric offspring mice with Cadherin 5 Cre deleted mice yielded viable $Trem1^{+/-}$x(Cadh5) $Cre^{+/-}$ offsprings. Interbreeding of $Trem1^{+/-}$ mice gave rise to endothelium-specific Trem-$1^{-/-}$ (EndoTrem$1^{-/-}$) mice at the expected Mendelian frequencies. EndoTrem$1^{-/-}$ mice were equal in size, weight, and fertility to littermate controls. Moreover, the composition and abundance of immune cells in blood, bone marrow, spleen, and lungs did not differ between groups. All animals were housed in plastic cages maintained on a 12-h light/dark cycle at a controlled temperature (24 ± 2°C) and humidity (50 ± 5%), and allowed free access to standard mice chow and water. We used 6- to 8-week-old male EndoTrem-$1^{-/-}$ and WT littermates. Experiments were approved by our institutional Animal Care and Use Committee (number 01079.01), and conducted according to he guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 LR12 peptide

LR12 peptide (LQEEDAGEYGCM) and a scramble inactive peptide (EDGQYECLMEGA) were chemically synthesized (Pepscan Presto BV, Lelystad, The Netherlands) as a COOH terminally amidated peptides. The correct peptides were obtained with >99% yields and were homogeneous after preparative purification, as confirmed by mass spectrometry and analytic reversed-phase high-performance liquid chromatography. These peptides were free of endotoxin. In every experiment involving LR12, the scramble peptide served as control.

2.3 Cell culture and stimulation

Human pulmonary microvascular ECs (HPMECs) were purchased from Promocell (six different batches originating from 6 different donors) (Heidelberg, Germany). The cells were maintained in complete ECs growth medium MV (Promocell) at 37°C in a 5% CO_2 humidified atmosphere incubator. All experiments were performed between Passages 2 and 5.

Cells were stimulated in complete medium supplemented with 10 µg/ml *Escherichia coli* lipopolysaccharide (LPS) (0111: B4; Sigma-Aldrich Saint-Quentin Fallavier, France) in presence or absence of 50 µg/ml LR12 during various times depending of the experiments. Supernatants were collected for cytokines measurements and cells subjected to flow cytometry or lysed for protein phosphorylation and mRNA analyses.

Supernatants from stimulated cells were recovered after 24 h stimulation and the concentrations of IL-6, ICAM-1, and E-selectin were measured using human Quantikine ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's protocol.

2.4 Measurement of intracellular nitric oxide production

The intracellular nitric oxide (NO) production in HPMEC was determined using DAF-FM diacetate (4-amino-5-methylamino- 2′, 7′-difluorofluorescein diacetate; Sigma Aldrich). DAF-FM diacetate is a cell-permeable molecule and non-fluorescent until it reacts with NO. By the interaction with NO, DAF-FM diacetate changes its structure to cell membrane-impermeable and yields fluorescence. After treatment, HPMEC were incubated with 5 µM DAF-2 DA for 1 h at 37°C, washed twice in PBS and collected by trypsinization. DAF-FM fluorescence was compared with unstained controls with 10 000 events recorded, using Accuri C6 flow cytometer. Assays were conducted three times in triplicate.

2.5 Adhesion assay

HPMEC plated on Vena8Fluoro+ Biochips were assayed at 80% confluency. Briefly, HPMEC were stimulated in complete medium supplemented with TNFα (10 ng/ml for 2 h). The edium was removed and cell monolayers were washed once with PBS. U937 (expressing TREM-1 at a very low level) or U937 overexpressing Trem-1/Dap12 (U937-TD)[27] were centrifuged at 200 g and resuspended in RPMI at a concentration of 3×10^6 cells/ml. Cell suspension was added to the monolayers coated

channels at a constant shear stress of 0.5 dyne·cm^{-2} for 3 min using the Mirus nanopump (Cellix, Dublin, Ireland). Cell adhesion was recorded on a ZEISS Axiovert 40 CFL fluorescence microscope, using a Q-IMAGING ROLERA-XR digital camera. Cell images of three microscopic fields from each channel were captured, and images were analysed using DucoCell software (Cellix).

2.6 Caecal ligation and puncture polymicrobial sepsis model

Male (6–8 weeks) wild-type (WT) littermates and EndoTrem-1$^{-/-}$ C57Bl/6 mice were anesthetized with isoflurane. The caecum was exposed through a 1.0-cm abdominal midline incision and subjected to a ligation of the distal half followed by puncture with a G21 needle. A small amount of stool was expelled from the punctures to ensure patency. The caecum was replaced into the peritoneal cavity and the abdominal incision closed in two layers. After surgery, all mice were injected s.c. with 0.5 ml 0.9% NaCl solution for fluid resuscitation. Animals were sacrificed by pentobarbital i.p injection at 24 and 72 h after caecal ligation and puncture (CLP) to collect organs (lungs and spleen) and blood.

In another experiment, survival was monitored for 7 days.

2.7 Vascular reactivity procedure

Animals were sacrificed by pentobarbital i.p injection (100 mg/kg) and the thoracic aorta and the mesenteric artery from operated animals were dissected and placed in an ice-cold physiological salt solution with the following composition: 130 mM NaCl, 14, 9 mM $NaHCO_3$, 3.7 mM KCl, 1.2 mM $MgSO_4$, 2, 5 mM $CaCl_2$, 1, 2 mM KH_2PO_4, 5, 5 mM glucose.

Vessels from heathy mice were also sampled and incubated with 10 μg/ml *E. coli* LPS, 20 μg/ml LR12, or 5 μg/ml of the agonistic anti-TREM-1 antibody (αTREM-1) (MAB1278, Biotechne, R&D Systems, USA) during 4 h. Then, a 2 mm-length ring was cut and mounted on a 40 μm stainless steel wire in a small vessel myograph (Danish Myo Technology, Arhus, Denmark). The preparation was supplied with carbogen gas mixture (95%O_2, 5%CO_2). After an equilibration period (at least 20 min) under optimal passive tension, two successive contractions in response to the combination of KCl depolarization (100 mM) and phenylephrine were used to test the maximal contractile capacity of the vessels. Concentration response curves to phenylephrine (1 nM à 100 μM) were performed. After a washout period of 20 min, the vessels were pre-contracted with phenylephrine (1 μM) to 80% of the maximum contraction and the acetylcholine concentration response curve was obtained (1 nM à 100 μM).

2.8 FACS analysis

Cell suspensions are prepared in the following way. The lungs were cut into small pieces and incubated in a cocktail of Collagenase I and DNase I and shaken at 37°C for 1 h. Cells were then triturated and centrifuged at 300 g for 10 min at 4°C. The spleens were crushed in Hank's Balanced Salt Solution (HBSS) at 4°C with the end of a syringe of 2 ml and then filtered on a 70 μm nylon filter. The cell suspension was centrifuged at 300 g for 10 min at 4°C. Pulmonary and spleen cells were then washed with HBSS and re-suspended in HBSS supplemented with 0.2% BSA. Finally, 1 ml of PBS was injected into the mouse peritoneum to recover the peritoneal lavage fluid (PLF). Cells were then obtained as above.

Total viable cell numbers were determined from aliquots using a haemacytometer with Trypan blue staining (BioRad). Cell suspensions were incubated in a cocktail of mAbs (CD45-PerCP, CD11b-PB, Ly6G-PE, Ly6C-FITC, Miltenyi Biotech). The gating strategy has been previously described by our group.[22] Data were acquired on a Gallios FACS analyzer (Beckman Coulter).

The expression of TREM-1 on HPMEC was detected by FACS after staining with 5 μl allophycocyanin-conjugated mouse monoclonal anti-human TREM-1 antibody (Miltenyi Biotech) for 30 min at 4°C in dark. After washing with PBS twice, the cells were re-suspended and fixed with 4% paraformaldehyde. Accuri C6 flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for analysis.

2.9 ELISA assay

Soluble TREM-1 levels were assessed in plasma, lung and PLF using enzyme-linked immunosorbent assay (mouse/rat TREM-1 Quantikine ELISA Kits; R&D Systems) according to the manufacturer's protocol. sVCAM-1 and IL-6 concentrations were also measured in plasma and lung in enzyme-linked immunosorbent assay with murine cytokine assay (mouse IL-6 or sVCAM-1/CD106 Quantikine ELISA Kits; R&D Systems).

2.10 Real-time PCR

Total RNAs were extracted from HPMEC or tissues (aorta and mesenteric artery) using RNeasy Plus Mini Kit (Qiagen, Venlo, The Netherlands) and quantified with NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) before being reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and quantified by quantitative polymerase chain reaction (PCR) using Qiagen probes (Quantitect Primers) for human TREM-1 and ActB, or murine TREM-1, IL-6, IL-10, TNF-α, and ActB. ActB serves as housekeeping gene.

2.11 Immunoblotting

HPMEC and cells from aortas or mesenteric arteries were lysed in PhosphoSafe Extraction Reagent (Novagen, Merck Biosciences, Nottingham, UK) and centrifuged for 5 min at 16 000 g at 4°C to collect the supernatant. Protein concentration was determined (BCA Protein Assay Kit, Pierce; ThermoScientific), and thirty micrograms of each sample were electrophoresed on a Criterion XT Bis-Tris Gel 4–12% (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Millipore, Saint-Quentin en Yvelines, France). The membrane was blocked with 5% w/v skim milk powder in TBST (0.1 M Tris-HCl pH 8, 1.5 M NaCl and 1% Tween-20) for 2 h at room temperature, and subsequently incubated with anti-(p)ERK1/2, anti-(p)Akt, anti-(p)P38 (p38 mitogen activated protein kinase), anti-(p)P65 (Nuclear Factor-κB p65), anti-inducible nitric oxide synthase (iNOS), anti-COX-1, and anti-COX-2 (Cell Signaling, USA) antibodies overnight at 4°C. After vigorous washing in TBST, the membrane was incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Immunocomplexes were detected with the SuperSignal West Femto Substrate (Pierce; ThermoScientific). Non-phosphorylated forms, tubulin or actin (Cell Signaling) were used for normalization. Acquisition and quantitative signal density analyses were performed by a LAS-4000 imager (FSVT) and Multi-Gauge software (LifeScience Fujifilm, Tokyo, Japan).

2.12 Confocal microscopy

The mouse lungs or aortas were frozen in Tissue-Tek OCT (VWR, PA, USA) and cut by cryostat into 10 μm sections and mounted on slides. After fixation, the frozen section slides were incubated with the indicated primary antibodies (TREM-1, Cadherin and TO-PRO3) (BIOSS, MA, USA) overnight at 4°C. Then, the slides were washed, mounted, and visualized through sequentially scanning on TCS SP5 X confocal

microscope (Leica, Wetzlar, Germany). Images were processed using LAS AF Lite blue software (Leica).

HPMEC were seeded and stimulated on Nunc LabTek chambers (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. After stimulation, cells were then washed and fixed with paraformaldehyde (4%) for 20 min, permeabilized with Triton 0.1% for 30 min, and blocked in 1% bovine-serum albumin for 1 h prior to incubation with indicated primary antibodies at 4°C overnight (TREM-1, vWF) (BIOSS, MA, USA). Nuclei were stained with 1 µg/ml TO-PRO3 (Invitrogen, USA) for 1 h at 37°C. After washing with PBS coverslips were mounted on Vectashield (Vector Laboratories, CA, USA) and examined with the same confocal microscope and analysing software.

2.13 Statistical analysis

All data, unless indicated, were normally distributed and then are presented as mean ± SD. Statistical significance between groups was analysed using Student *t*-test, One-way ANOVA with Tukey's *post hoc* testing for repeated measures, regular two-way ANOVA test. Kaplan–Meier survival curves were analysed using the log-rank test. Statistical analysis was performed using Prism Version 6 software (GraphPad) and a P value < 0.05 was deemed significant.

3. Results

3.1 TREM-1 is expressed by HPMECs

The expression of *Trem-1* in HPMEC was analysed by qRT-PCR. Although *Trem-1* was barely detectable under basal condition, LPS stimulation induced a significant increase in expression with a peak reached at 6 h (*Figure 1A*). Addition of LR12, a synthetic dodecapeptide that inhibits TREM-1, dampened this LPS-induced TREM-1 upregulation. To confirm these results, TREM-1 expression was monitored by flow cytometry (*Figure 1B* and *C*). LPS stimulation induced a time-dependent increase of the percentage of TREM-1^{+} cells as well as their level of expression (mean fluorescence intensity), which was again modulated by LR12.

To get insights on the localization of TREM-1 in HPMEC, we stained these cells for TREM-1, Von Willebrand factor and TO-PRO3 (*Figure 1D*). TREM-1 was not observed in resting cells (upper panel). LPS stimulation induced TREM-1 expression that seemed to co-localize with the Von Willebrand factor in HPMEC (lower panel).

Using three different techniques we thus demonstrated that TREM-1 is expressed in human microvascular ECs upon LPS stimulation.

3.2 TREM-1 is expressed by vascular endothelium

To confirm the expression of TREM-1 by ECs, we studied its expression by qRT-PCR in isolated mouse aortas and mesenteric arteries stimulated with LPS for 6 hours. *Trem-1* expression was very low in resting condition and sharply increased upon LPS stimulation both in aortas (*Figure 2A*) and mesenteric arteries (*Figure 2B*). Interestingly, *Trem-1* became undetectable after endothelium removal (*Figure 2A*). Confocal imaging of aortas confirmed this LPS-induced TREM-1 upregulation (*Figure 2E*). The same findings were observed during septic shock induced by CLP: Trem-1 was over-expressed in aortas and mesenteric arteries harvested 18 h after the onset of sepsis (*Figure 2C* and *D*). LR12 injection 2 h after the surgery prevented from this up-regulation.

Confocal microscopy of the lung vasculature reveals that the expression of TREM-1 was low in healthy animals while strongly increased during septic shock (*Figure 2F*).

TREM-1 is therefore expressed by the vascular endothelium following acute septic insult.

3.3 TREM-1 modulation inhibits LPS-induced vascular dysfunction

We next examined the function of endothelial TREM-1. Aortas from healthy WT mice were harvested and vascular reactivity assessed. As expected, incubation of aortas' rings with LPS severely impaired both contractility and endothelium-dependent relaxation (*Figure 3A*). Co-incubation with LR12 almost completely restored vasoreactivity. By contrast, TREM-1 activation induced by an anti-TREM-1 agonistic monoclonal antibody (αTREM-1) decreased contractility and relaxation to the same extent of that obtained with LPS. Similar results were observed on mesenteric arteries (not shown). As shown in *Figure 2*, although TREM-1 is very low in resting vessels, its expression is not null and therefore sufficient for its engagement by anti-TREM-1 antibody. Of note, we used a control isotypic antibody that showed no effect on vasoreactivity.

These data suggest that TREM-1 engagement (either induced by LPS or by agonistic mAb) impairs vascular reactivity.

To evaluate the effects of LR12 on LPS-stimulated HPMEC, the production of NO, s-ICAM-1, s-E-selectin and IL-6 was examined. LPS induced an increased production of NO, s-ICAM-1, s-E-selectin and IL-6 that was partially reduced by LR12 (*Figure 3B–E*). Using PCR-array (84 innate immune response genes) we observed that LR12 impacted the expression of 28 genes with a fold change superior > 4 as compared with LPS alone (see Supplementary material online, *Figure S1*).

A kinetic analysis of protein expression in HPMEC by western blot revealed an increase phosphorylation of ERK1/2, p38 and p65 and an increased expression of COX-2 upon LPS stimulation that was again reduced by LR12 (*Figure 3F*).

Beyond being responsible for maintaining vascular reactivity, endothelium is involved in leukocyte trafficking. We thus performed cell–cell adhesion assays under flux to address the role of TREM-1 on leukocyte adhesion to the endothelium. We used two different monocytic cell lines: U937 that express TREM-1 at a very low level and U937-TD that over-express TREM-1.[25] Although few U937 adhered to unstimulated ECs, TNFα increased the number of adherent monocytes (*Figure 3G*). Monocytes adhesion was exaggerated when using U937-TD cells. This approach revealed that TREM-1 over-expression on U937 favours adhesion to ECs.

Altogether, these findings point to a role of TREM-1 in endothelium activation and vascular reactivity.

3.4 TREM-1 inhibition protects mice from vascular dysfunction induced by septic shock

We studied the effect of LR12 on vascular reactivity during CLP-induced septic shock. The contractile responses to phenylephrine in aortic and mesenteric vessels as well as the endothelium-dependent relaxation induced by acetylcholine were significantly altered in septic mice as compared to control animals (*Figure 4A*). This vascular dysfunction was abrogated in LR12-treated mice.

Septic shock was responsible for a massive vascular inflammatory response as witnessed by the overexpression of both pro- and anti-inflammatory genes (*Tnfα*, *Il-10*, and *Il-6*) in aortas and mesenteric

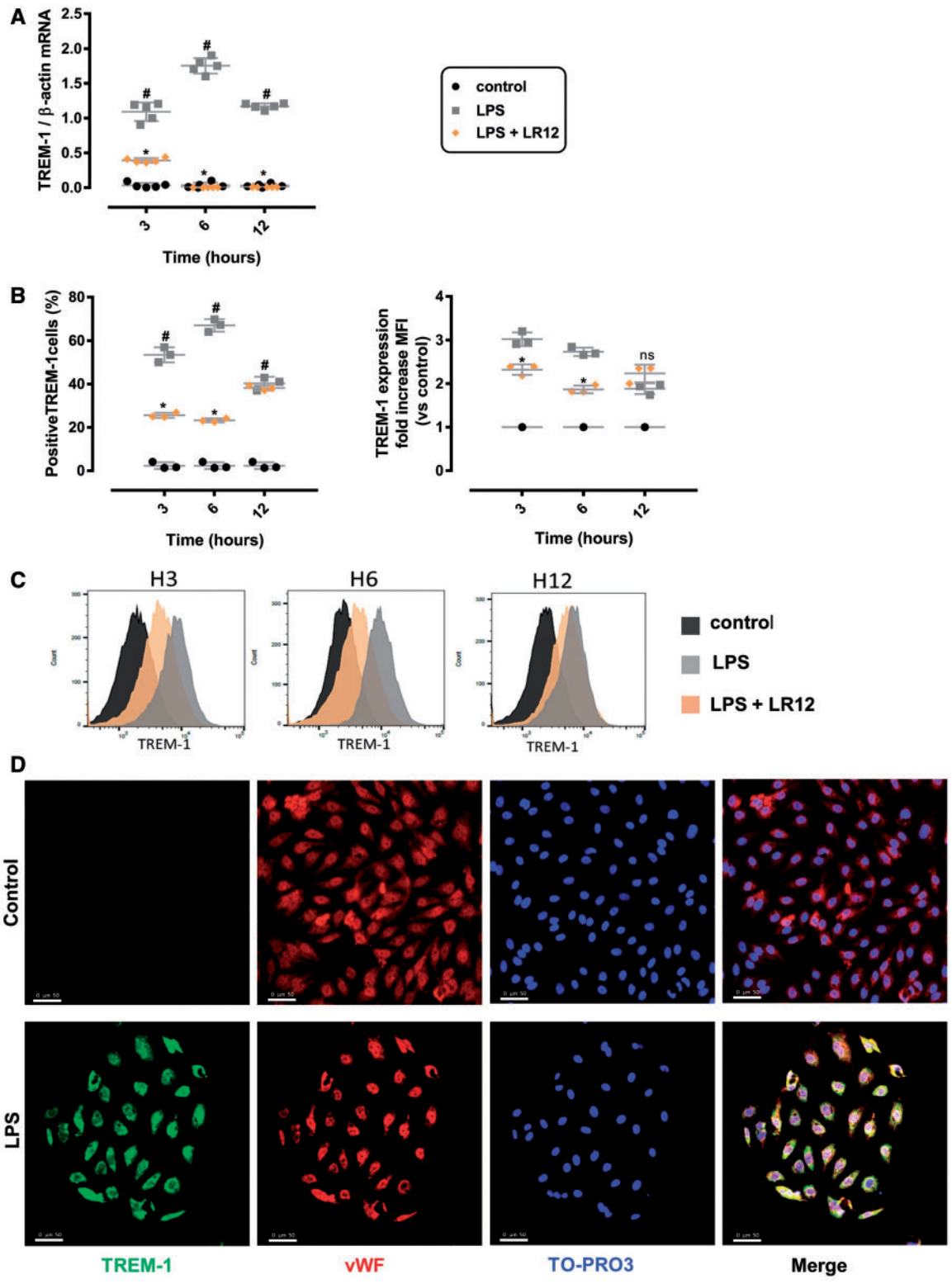

Figure 1 TREM-1 is expressed by HPMEC. (*A*) Time course of *Trem-1* mRNA expression determined by real-time qPCR in HPMEC upon LPS or LPS+LR12 stimulation. #$P < 0.001$ control vs. LPS; *$P < 0.001$ LPS vs. LPS + LR12, $n = 5$ per group (two-way ANOVA). (*B*) Flow cytometry analysis of TREM-1 expression in HPMEC upon LPS or LPS+LR12 stimulation at the indicated times. Percentage of positive TREM-1 cells (left panel) or TREM-1 expression (in mean fluorescence intensity) fold increase vs control (right panel). #$P < 0.001$ control vs. LPS; *$P < 0.005$ LPS vs. LPS + LR12, $n = 3$ per group (two-way ANOVA). (*C*) Histograms of TREM-1 expression in HPMEC after 3, 6, or 12 h stimulation or not with LPS or LPS+LR12. Images are representative from three different experiments. (D) Confocal fluorescent images of resting HPMEC (top panels) or LPS (10 μg/ml, 24 h) stimulated HPMEC (bottom panels). Cells were stained with anti-human TREM-1 (green), vWF (red), and TO-PRO3 (blue) mAbs, Scale bars: 50 μm. Images are representative from 3 different experiments.

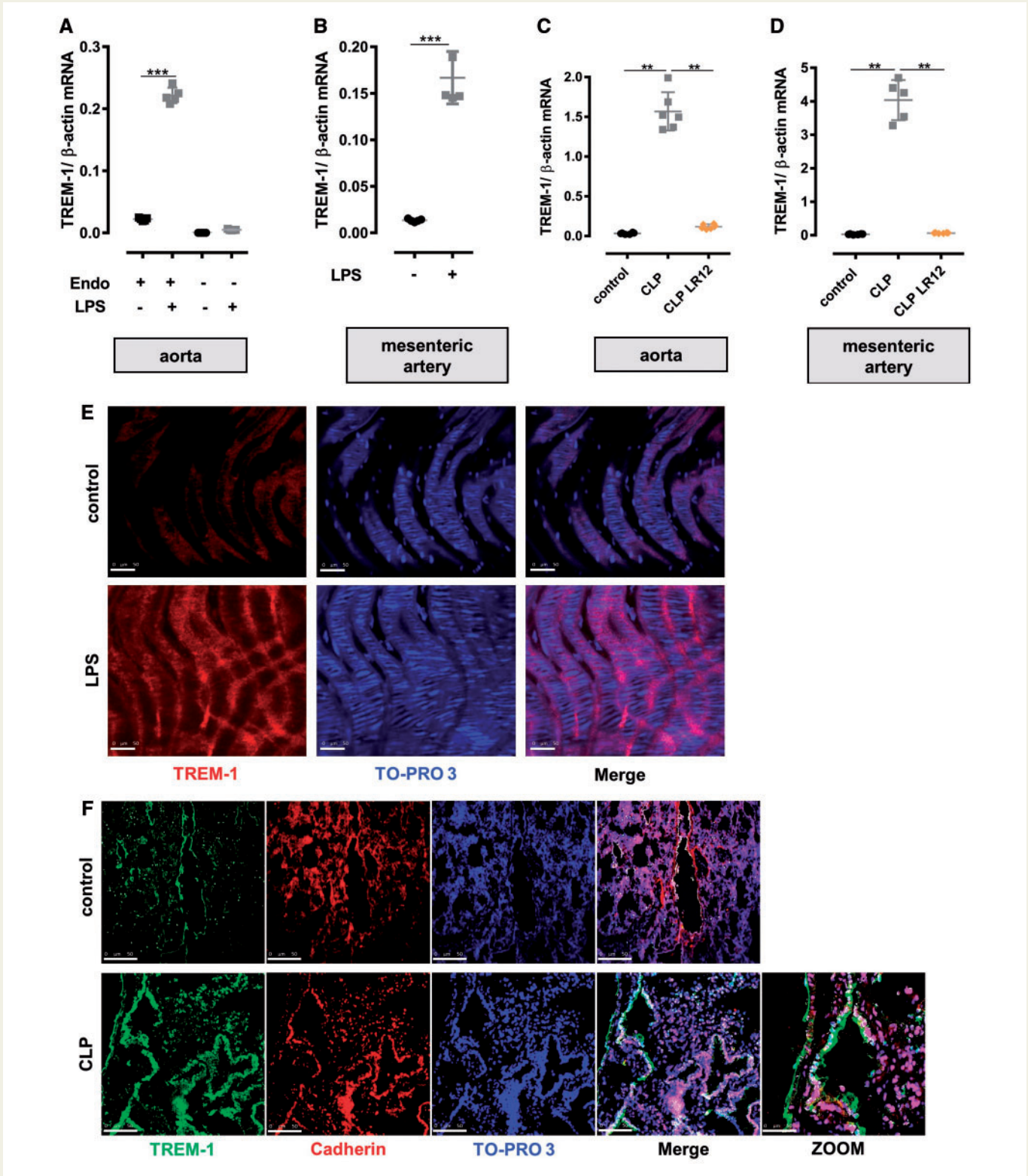

Figure 2 TREM-1 is expressed by vascular endothelium. Aortic (*A*) or mesenteric (*B*) rings from healthy mice were sampled, cleaned and stimulated with LPS (10 μg/ml for 24 h). Endothelium was removed by scrapping and flushing (-Endo) or not (+Endo) in aorta and *Trem-1* mRNA expression was determined by real-time qPCR. ***$P < 0.005$, $n = 5$ per group (*t*-test). Quantification of *Trem-1* gene expression in aortas (*C*) or mesenteric arteries (*D*) from control (black), septic (CLP) (grey) and LR12-treated (5mg/kg i.p. 1 h after CLP) septic mice (CLP LR12) (orange). Vessels were harvested 24 h after the surgery. **$P < 0.0001$, $n = 6$ per group (one-way ANOVA). (*E*) Aorta from healthy mice were sampled, cleaned and stimulated with LPS (10 μg/ml for 24 h). Confocal fluorescent images of unstimulated (control) (top panels) or LPS-stimulated (bottom panels) aortas stained with anti-human TREM-1 (red) and TO-PRO3 (blue) mAbs. Scale bars: 50 μm. Images are representative from 3 different experiments. (*F*) Confocal fluorescent images of the lungs from control (top panels) or septic (CLP) mice (bottom panels). Lungs were harvested 24 h after surgery and stained with anti-human TREM-1 (green), Cadherin5 (red), and TO-PRO3 (blue) mAbs. Scale bars: 50 μm.

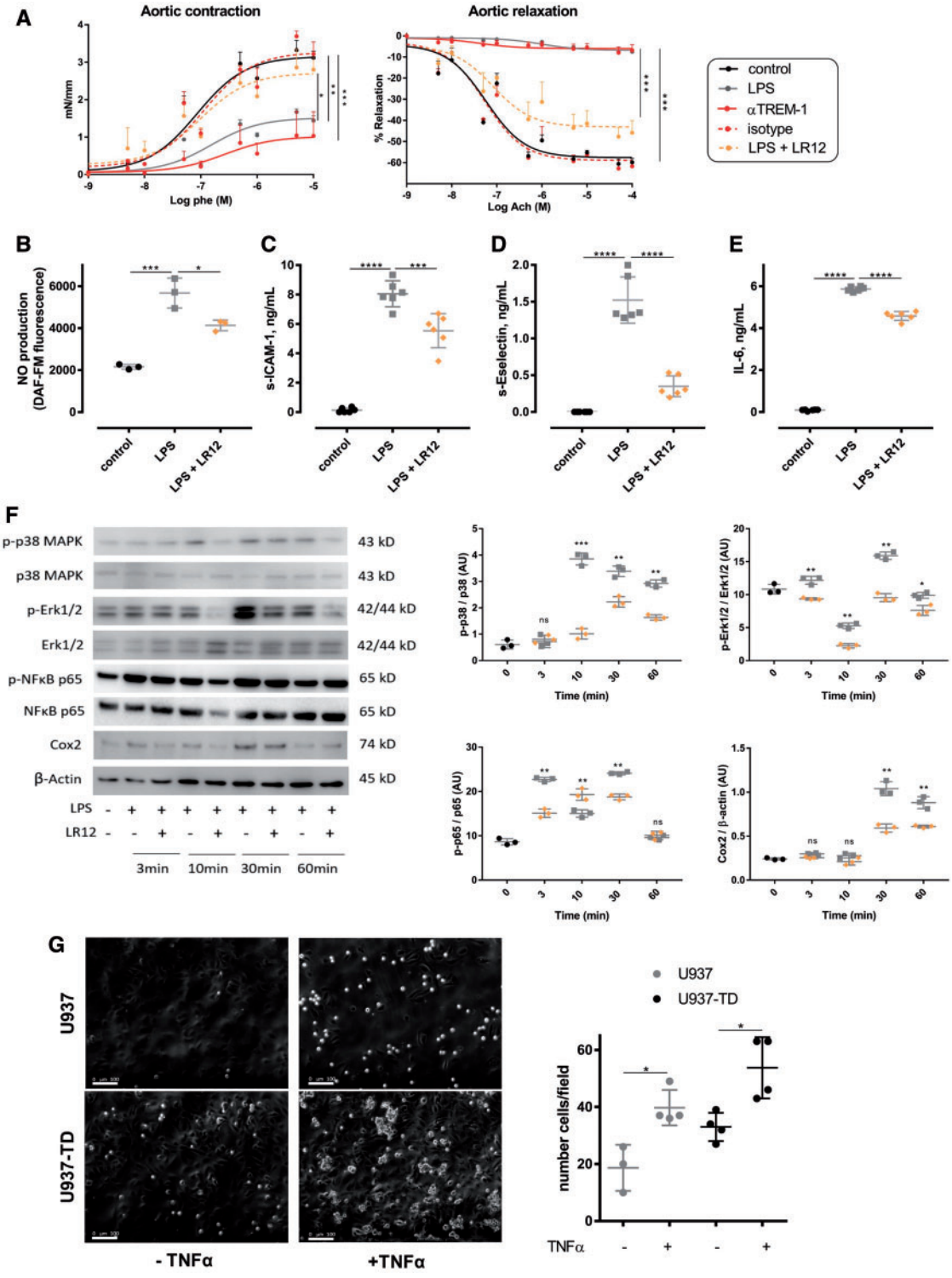

Figure 3 TREM-1 modulation inhibits LPS-induced vascular dysfunction. (*A*) Aortic rings from healthy mice were sampled, cleaned and stimulated with LPS (10 μg/ml), agonistic anti-TREM-1 antibody (αTREM-1) (5 μg/ml), or LR12 (20 μg/ml) during 4h. Concentration response curves to phenylephrine (Phe) (left panel) and to acetylcholine (Ach) (right panel) in aortas from the different group. *$P < 0.05$, **$P < 0.01$, *** $P < 0.005$, $n = 5$ per group (two-way ANOVA). (*B*) The levels of intracellular NO production was determined in HPMEC after 24 h of stimulation (LPS 10 μg/ml ± LR12 20 μg/ml) by FACS using DAF-FM diacetate. *$P < 0.05$, ***$P = 0.0002$, $n = 3$ per condition (one-way ANOVA). ELISA quantifications of s-ICAM-1 (*C*), s-Eselectin (*D*), and IL-6 (*E*) after 24 h of stimulation in HPMEC supernatants. ***$P < 0.005$, ****$P < 0.0001$, $n = 6$ per conditions (*t*-test). (*F*) Western blot of lysates of HPMEC treated for 3, 10, 30, and 60 min with LPS 10 ± LR12 20 μg/ml and their densitometric quantification (AU, arbitrary units). *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, $n = 3$ per condition (two-way ANOVA). (G) U937 or U937-TD cells adhesion to HPMEC under flux at a constant shear stress of 0.5 dyne·cm^{-2} for 3min. HPMEC were stimulated with TNFα (10 ng/ml) or not for 2 h. Cell images of 3 microscopic fields from each channel were captured, and and number of adhering cells analysed. *$P < 0.05$, $n = 3$ per condition (t-test).

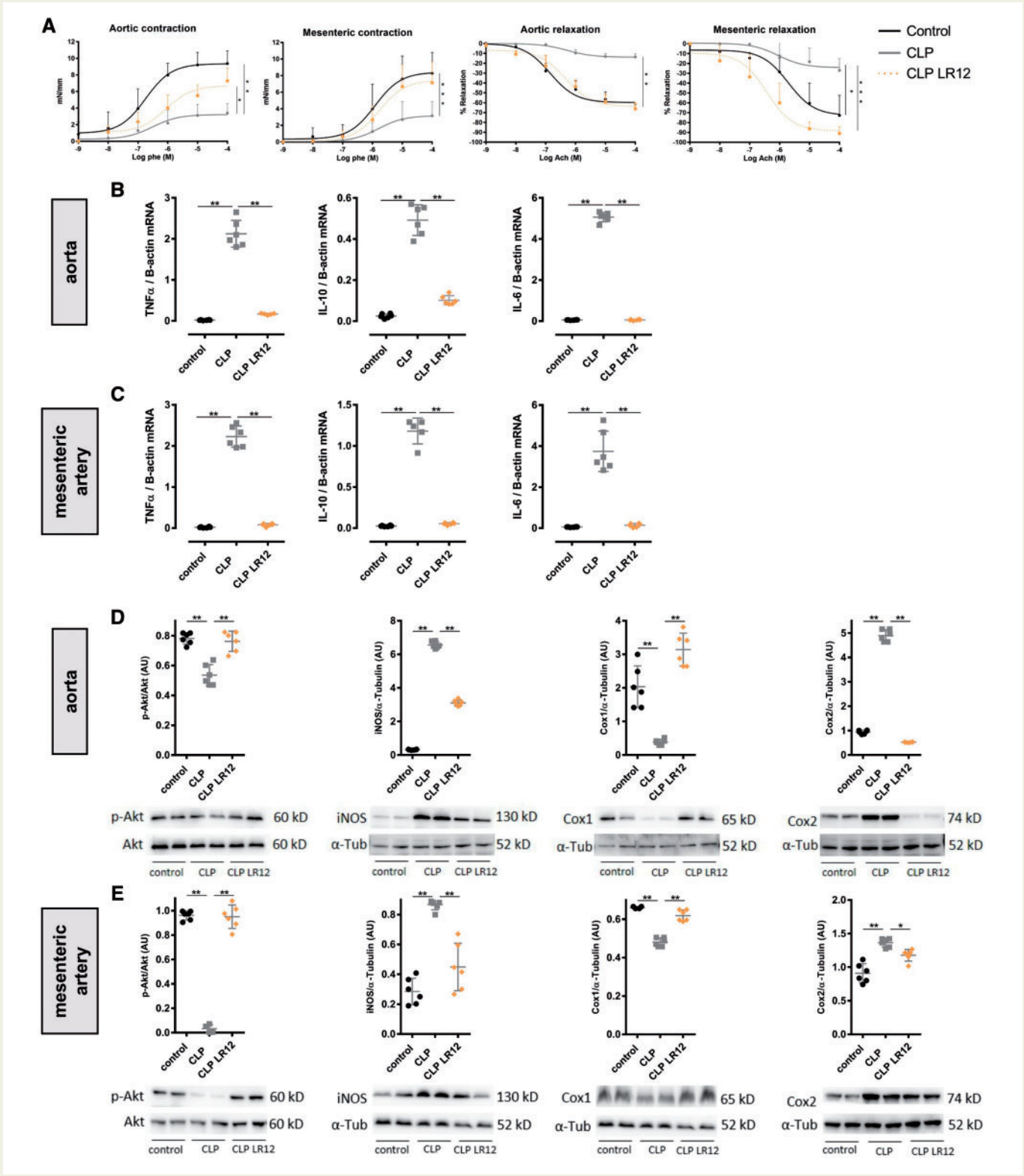

Figure 4 TREM-1 inhibition protects mice from vascular dysfunction induced by septic shock. (*A*) Concentration response curves to phenylephrine (Phe) (top panels) and to acetylcholine (Ach) (bottom panels) in aortas and mesenteric arteries from control (black), septic (CLP) (grey), and LR12-treated (5 mg/kg i.p. 1 h after CLP) septic mice (CLP LR12) (orange). Vessels were harvested 24 h after the surgery. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $n = 7–9$ per group (two-way ANOVA analysis). Quantification of *Tnfα*, *Il-10* and *Il-6* gene expression in aortas (*B*) or mesenteric arteries (*C*) from the three groups of mice. $^{**}P < 0.0001$, $n = 6$ per group (one-way ANOVA). Western blots of (p)-AKT, iNOS, COX-1, and COX-2 of aorta (*D*) and mesenteric arteries (E) from the three groups of mice and their densitometric quantification (AU, arbitrary units). $^{*}P = 0.001$, $^{**}P < 0.0001$, $n = 6$ per group (one-way ANOVA).

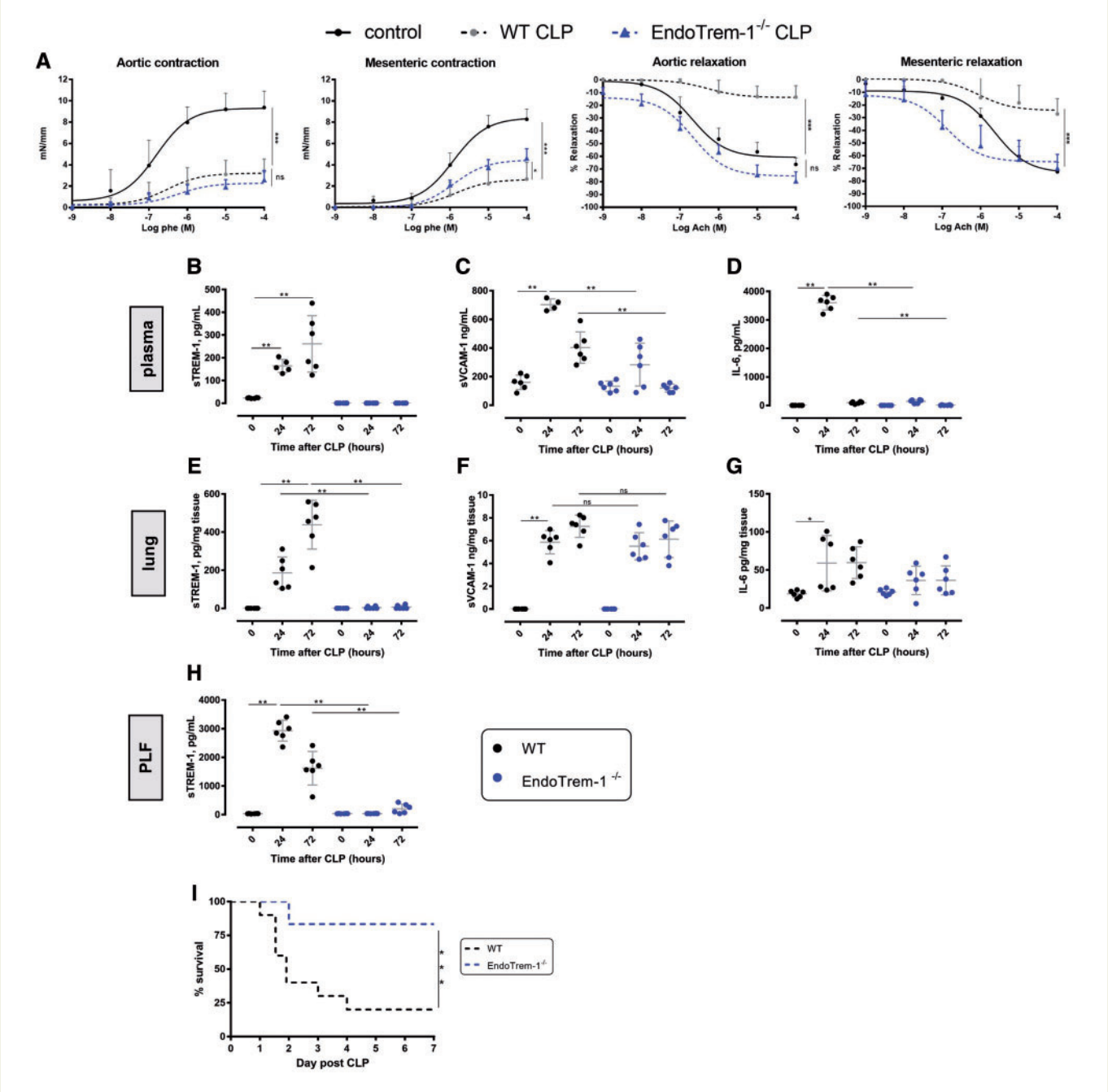

Figure 5 Targeted endothelial *Trem-1* deletion ('EndoTrem-1$^{-/-}$') protects mice from septic shock. (*A*) Concentration response curves to phenylephrine (Phe) (top panels) and to acetylcholine (Ach) (bottom panels) in aortas and mesenteric arteries from control (black), WT CLP (grey) and EndoTrem-1$^{-/-}$ CLP (blue) mice. Vessels were harvested 24 h after the surgery. *$P < 0.05$, **$P < 0.01$, $n = 7$–9 per group (two-way ANOVA analysis). (*B*–*H*) ELISA quantifications of sTREM-1, sVCAM-1 and IL-6 in plasma, lung and PLF in WT CLP and EndoTrem-1$^{-/-}$ CLP mice at the indicated times. *$P < 0.05$, **$P < 0.001$, $n = 6$ per group (one-way ANOVA). (*I*) Kaplan–Meier estimate of survival after CLP in WT and EndoTrem-1$^{-/-}$ mice ($n = 12$–15 per group). Survival curves are compared using the log-rank test. ***$P = 0.0006$ WT vs. EndoTrem-1$^{-/-}$.

arteries (*Figure 4B* and *C*). This inflammation was largely reduced in LR12-treated mice.

During septic shock, the existence of an impaired synthesis and production of some constitutive proteins and an overexpression of inducible proteins has been demonstrated. The two most important signaling pathways are the NOS and COX pathways.[28] In the CLP group, a decreased expression of p-Akt and COX-1 was observed relative to the control animals. LR12 administration restored the expression of these two proteins (*Figure 4D* and *E*). In parallel, we quantified the expression of inducible proteins (iNOS, COX-2). CLP induced an increase expression of these proteins, and this phenomenon was attenuated by LR12.

Pharmacological inhibition of TREM-1 is thus able to prevent from septic shock induced vascular inflammation and dysfunction.

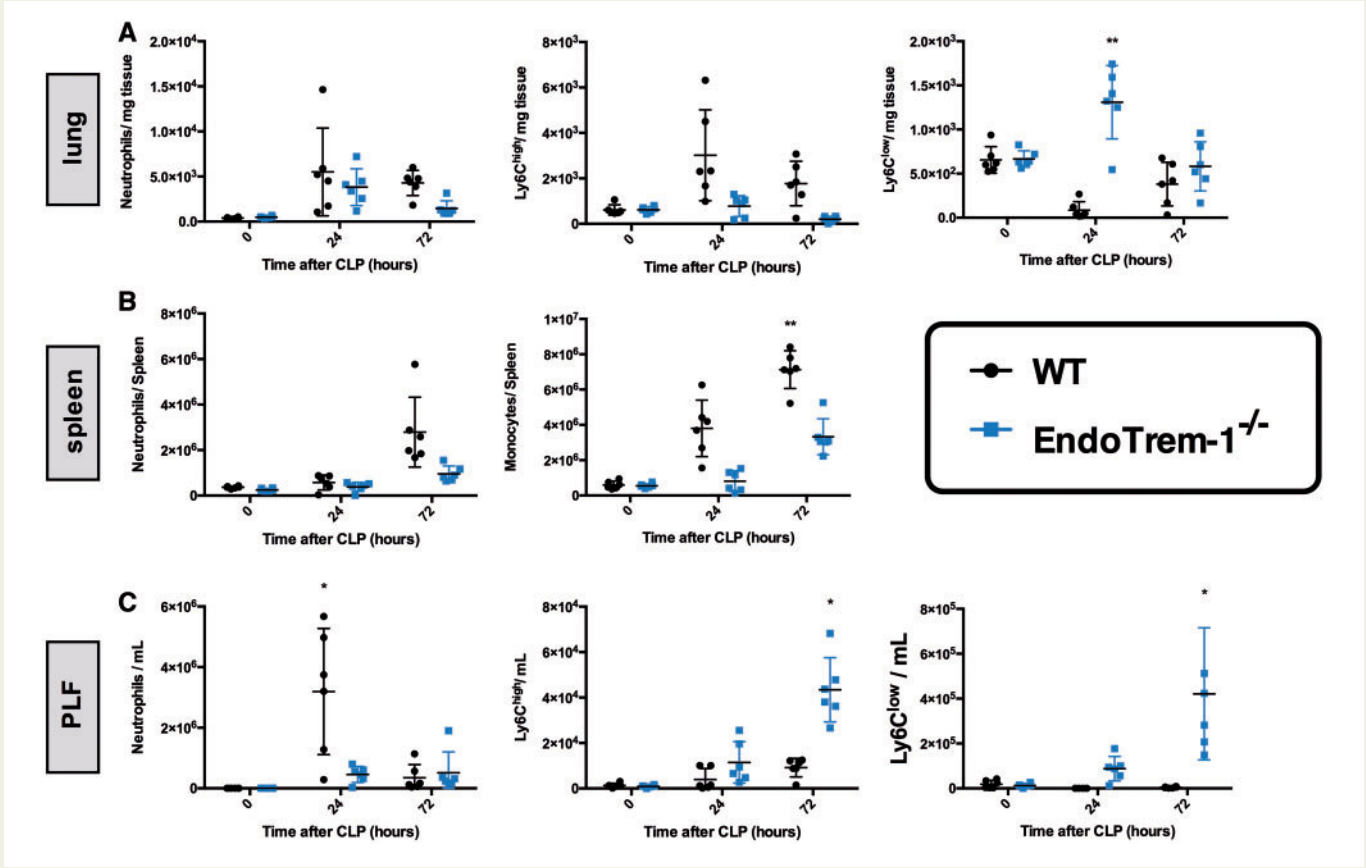

Figure 6 TREM-1 regulates leukocytes mobilization from remote compartments. Flow-cytometric quantification of monocytes (Ly6C^{High} and Ly6C^{Low}) and neutrophils in the lung (*A*), spleen (*B*), and PLF (*C*) at different time points after CLP. *$P < 0.05$, **$P < 0.01$ WT vs. EndoTrem-1$^{-/-}$, $n = 6$ per group (two-way ANOVA analysis).

3.5 Targeted endothelial *Trem-1* deletion protects mice from septic shock

Septic shock was induced by CLP in endothelium-conditional *Trem-1* deficient mice (EndoTREM-1$^{-/-}$) and WT littermates. As expected, sepsis induced a profound vascular hyporeactivity in WT animals, both in terms of contractility and endothelium-dependent relaxation (*Figure 5A*). Although contractility was still impaired in EndoTREM-1$^{-/-}$ mice, vasorelaxation was completely restored, pointing to a role of endothelial TREM-1 in vascular relaxation.

Soluble TREM-1 concentrations, a marker of TREM-1 activation, were markedly increased in the plasma, the PLF and the lungs from WT septic compared to sham-operated animals (control). In EndoTREM-1$^{-/-}$ mice, sTREM-1 level was reduced, pointing to ECs as a major source of TREM-1 (*Figure 5B, E*, and *H*). Plasma concentrations of soluble VCAM-1 and IL-6 were also reduced in EndoTREM1$^{-/-}$ animals (*Figure 5C* and *D*). Finally, survival was clearly improved in the EndoTrem1$^{-/-}$ group as compared with the WT group (*Figure 5I*).

Using PCR-arrays, we found that the sepsis-induced alteration of many inflammatory genes in the lungs was dampened in EndoTREM-1$^{-/-}$ mice (see Supplementary material online, *Table S1*), mostly at 24 h after CLP.

We finally examined whether the modulation of inflammation could be explained by a reduction of the number of inflammatory infiltrating cells in the lungs, spleen, and PLF (*Figure 6*). In the lung, the most commonly organ reported to be involved during sepsis,[29] we observed an accumulation of neutrophils and inflammatory Ly6C^{high} monocytes at 24 h in WT septic mice. This accumulation was dampened in EndoTrem1$^{-/-}$ mice suggesting that blocking endothelial *Trem-1* reduced monocytes and neutrophil mobilization to the lung. In contrast, endothelial *Trem-1* deletion favoured the accumulation of reparative cells (Ly6C^{low} monocytes). Although spleen infiltration by myeloid cells was reduced in EndoTrem1$^{-/-}$ mice, the peritoneal cavity was rapidly populated with monocytes, suggesting a role of endothelial TREM-1 in regulating leukocytes trafficking.

All in all, targeted endothelial *Trem-1* deletion confers protection during septic shock in modulating inflammatory cells mobilization and activation, in restoring vasoreactivity, and in improving the survival.

4. Discussion

The TREM family encompasses at least 5 different receptors sharing low sequence homology with each other. Among them, TREM-1 is expressed on the surface of neutrophils, mature monocytes, macrophages and hypoxic dendritic cells, and plays a major role in the pathophysiology of inflammation-associated disorders.[12] During various models of sepsis, we observed that the TREM-1 inhibition, in addition to reducing inflammation and improving survival, prevented from hemodynamic instability.[19,20]

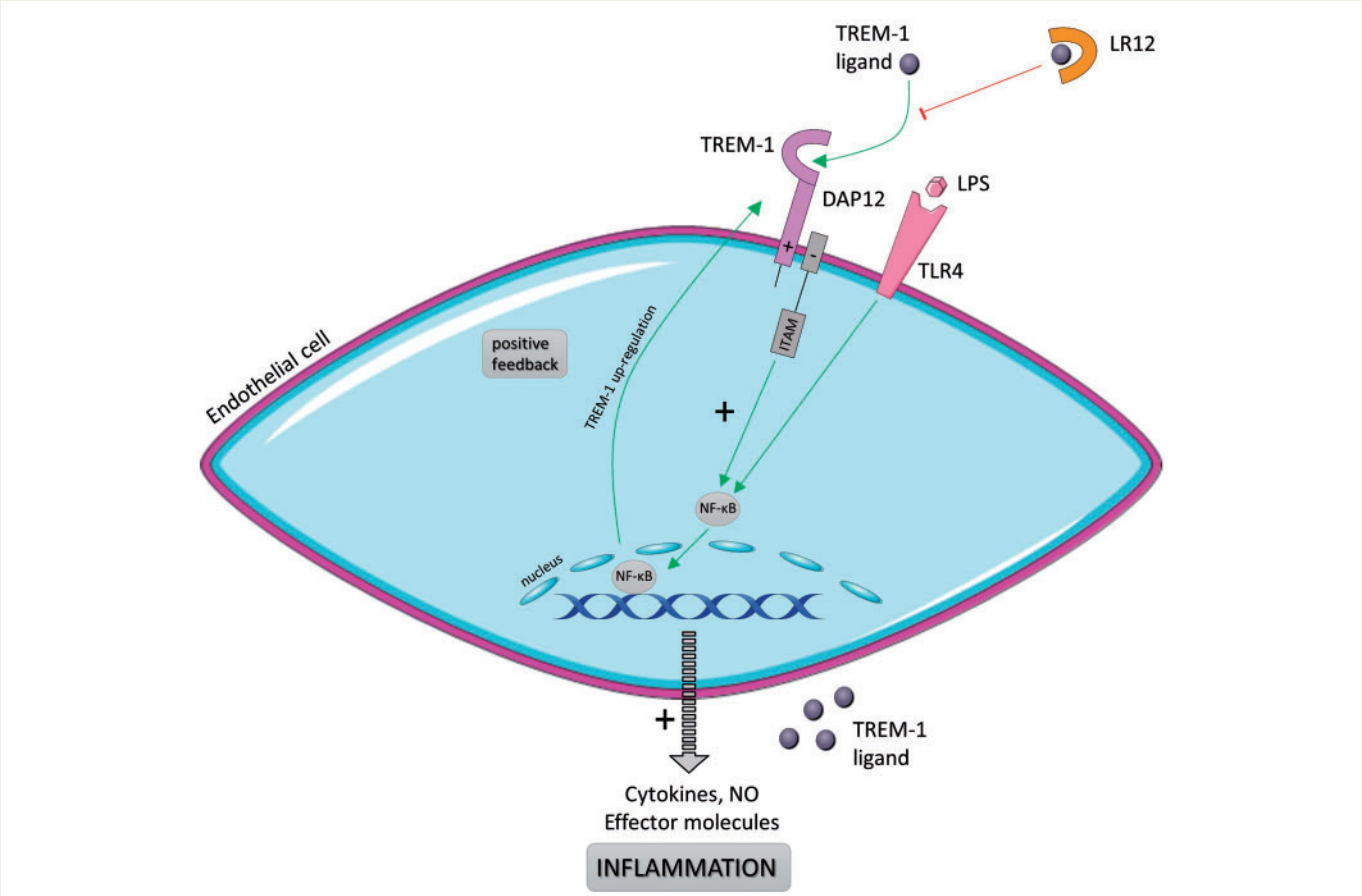

Figure 7 Schematic representation of TLR4–TREM-1 relationship. LPS binds to TLR4 and triggers the activation and translocation of the transcription factor into the nucleus where it induces the transcription of several pro-inflammatory, including *Trem-1*. This yields to a TREM-1 up-regulation, clusterization and multimerization. After ligation of its ligand, TREM-1 associates with the adaptor molecule DAP12 (that possesses an ITAM motif), leading to phosphorylation of a panel of downstream kinases that in turn activate NF-κB and amplify the inflammatory response. The LR12 peptide, by trapping TREM-1 ligand(s), inhibits TREM-1 engagement and therefore modulates this amplification loop.

Indeed, vascular hyporeactivity is the hallmark of septic shock and is attributed to endothelial dysfunction.[4,8] Although TREM-1 has first been described on myeloid cells, several studies support its presence in non-professional immune cells such as epithelial cells or platelets.[10,15,16]

We here showed that TREM-1 is also expressed on ECs especially after LPS stimulation. These results are in line with those of Laskin *et al.*[30] who were the first to suggest a TREM-1 expression in mouse liver ECs, which was upregulated upon stimulation with LPS, TNFα, or IL-1β.[31] We further extended these findings in showing the TREM-1 is fully functional at the endothelial level both in mice and humans.

A crucial role of the endothelium is to regulate the vasomotor tone: we observed that TREM-1 activation compromised both contractility and relaxation and that this alteration was abrogated with LR12, a clinical-range TREM-1 inhibitory peptide,[20,22] 24 both *in vitro* and *in vivo*. NO overproduction by activated ECs has been pointed to be responsible for the vascular hyporeactivity. As such, we found that TREM-1 inhibition reduced NO production by ECs as well as iNOS expression in septic mice. Moreover, the sepsis-induced vascular inflammation was also largely decreased in LR12 treated animals.

The LR12 peptide does not bind to TREM-1 but prevents ligation of the TREM-1 ligand and then specifically inhibits TREM-1.[26] Indeed, LPS activates TLR4 that in turn leads to TREM-1 up-regulation, clustering and multimerization, and the generation of TREM-1 ligand(s) that binds TREM-1, further amplifying the inflammatory response (*Figure* 7). Hence, LR12 by acting as a decoy receptor for TREM-1 ligand(s), abrogates this amplification loop, without interfering with the initial response.

However, despite several candidates, the nature of the TREM-1 ligand remains largely speculative and this precludes precise analysis of LR12-TREM-1 ligand interactions.[18]

A limitation of the use of TREM-1 inhibitory peptides such as LR12 is that the 'endothelio-protective' effect observed *in vivo* may just be the result of a decrease of systemic inflammation and inflammatory cells activation: it has repeatedly been shown that pharmacologic inhibition or genetic deletion of TREM-1 prevented from hyper-responsiveness and death during acute inflammatory diseases.[21–23,31] To get rid of this concern we generated endothelial-conditional *Trem-1* ko (EndoTrem1$^{-/-}$) mice whose non-ECs normally express TREM-1. Using these animals, we clearly found that deletion of *Trem-1* in ECs protected from sepsis-induced vascular dysfunction, inflammation, and modulated leukocyte trafficking. Finally, survival was strikingly improved in septic EndoTrem1$^{-/-}$ mice as compared with WT littermates. This pointed to a clear role of endothelial TREM-1 in mediating many of the deleterious

aspects of septic shock. However, we did not monitor blood pressure, nor investigate organ dysfunction in our septic shock model and thus cannot ascertain whether the beneficial effects conferred by endothelial Trem-1 deletion were mostly related to hemodynamic improvement, organ protection or both. Nevertheless, we previously showed both in septic shock pigs and endotoxemic monkeys that LR12 administration prevented from blood pressure decrease.[19,20]

Beyond adding to the knowledge of sepsis physiology and TREM-1 biology, this study may have clinical implications. Indeed, the LR12 peptide is under clinical development www.clinicaltrials.gov, NCT03158948) for septic shock. A concern that may arise for the broad inhibition of TREM-1 with LR12 is that it could compromise several important functions of TREM-1 such as bacterial clearance or myeloid cells survival. As such, the design of endothelium-specific TREM-1 inhibitors may prove interesting in preventing endothelial dysfunction while preserving myeloid cells functionality.

We reported that TREM-1 is expressed and inducible in ECs and plays a direct role in vascular inflammation and dysfunction. The targeted deletion of endothelial *Trem-1* conferred protection during septic shock in modulating inflammatory cells mobilization and activation, restoring vasoreactivity, and improving survival. Targeting mechanisms responsible for endothelial dysfunction that occurs during acute inflammatory disorders could be a great interest in the management of critically ill patients.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: Marc Derive and Sébastien Gibot are co-founders of INOTREM SA, a company developing TREM-1 inhibitors.

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