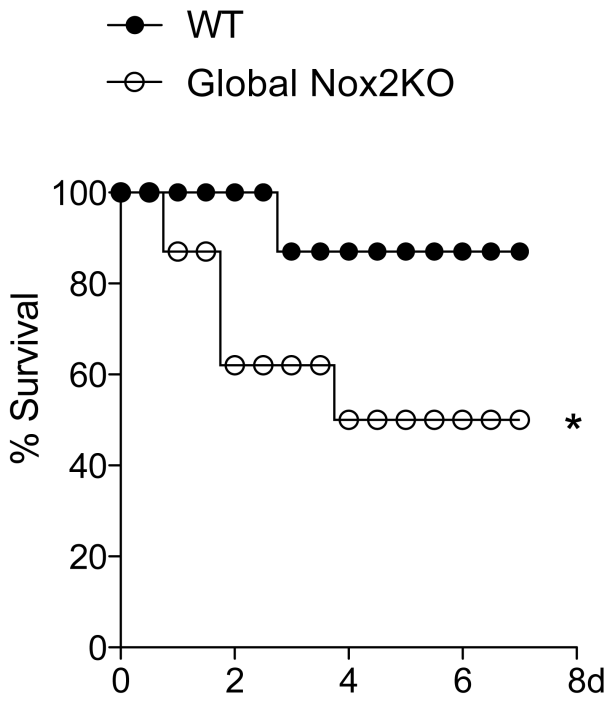
SUPPLEMENTAL DATA

Endothelial Nox2 limits systemic inflammation and hypotension in endotoxemia by controlling expression of toll-like receptor 4

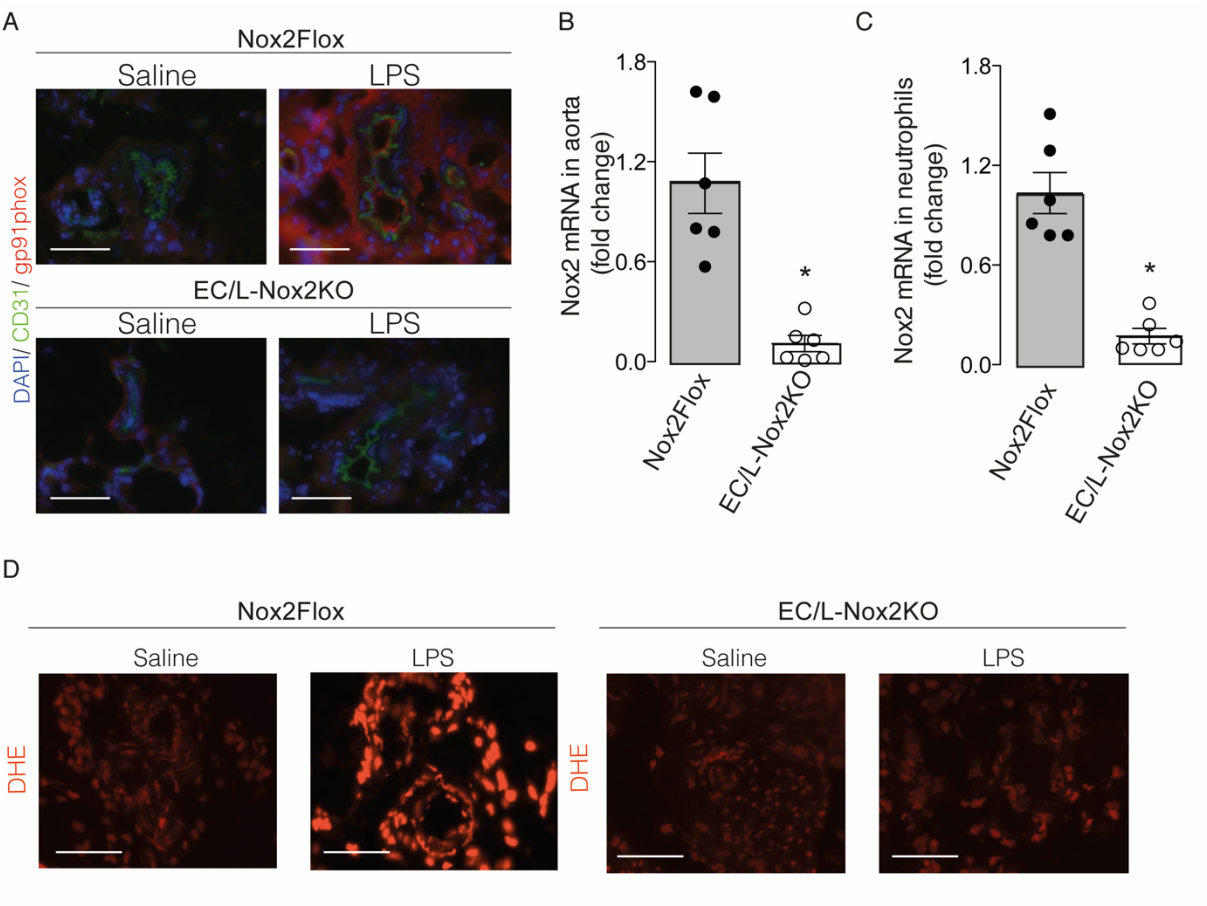
Silvia Cellone Trevelin[1], Can Martin Sag[2], Min Zhang[1], José Carlos Alves-Filho[3], Thiago Mattar Cunha[3], Célio Xavier dos Santos[1], Greta Sawyer[1], Thomas Murray[1], Alison Brewer[1], Francisco Rafael Martins Laurindo[4], Andrea Protti[1], Lucia Rossetti Lopes[5], Aleksandar Ivetic[1], Fernando Queiroz Cunha[2], Ajay M. Shah[1*].

[1]King's College London, British Heart Foundation Centre for Research Excellence, School of Cardiovascular Medicine & Sciences, London, United Kingdom; [2]Department of Internal Medicine II, University Hospital of Regensburg, Germany; [3]Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, Brazil; [4]Heart Institute, School of Medicine, Sao Paulo, Brazil;[5]Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil.

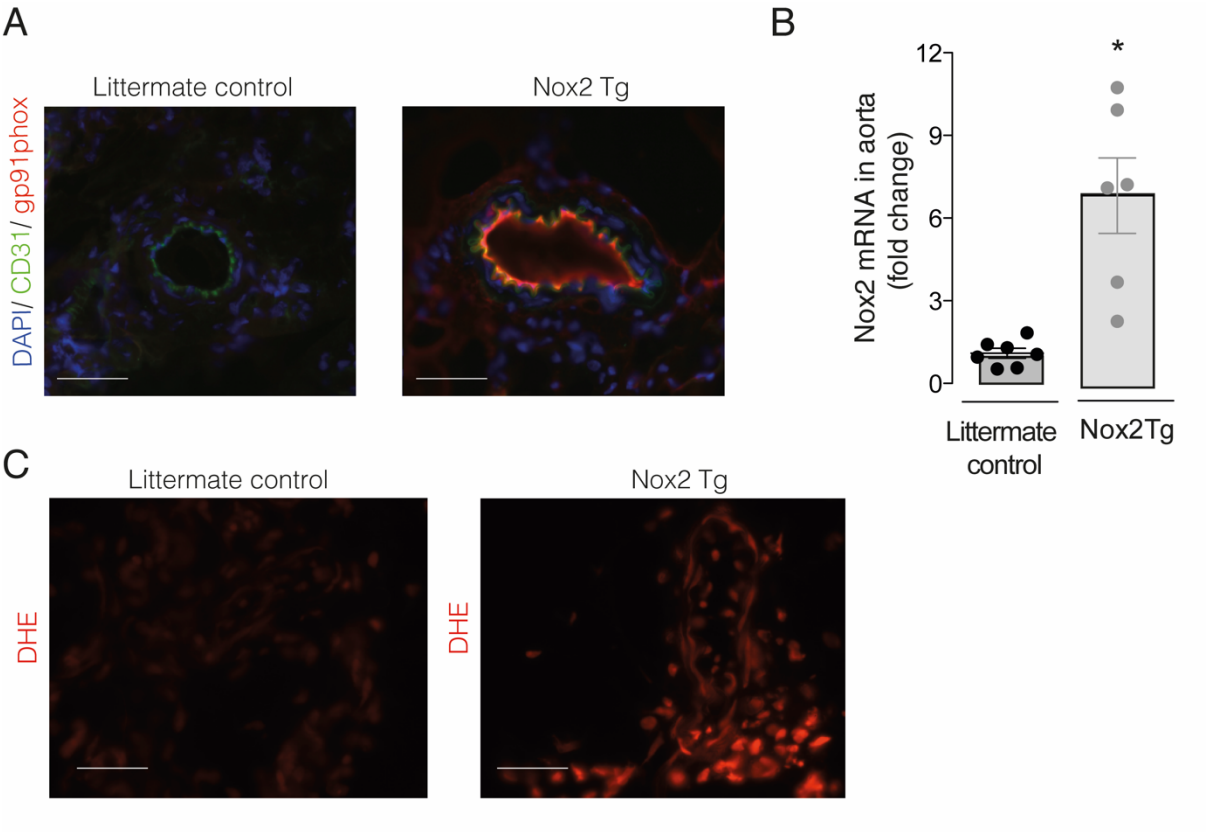
***Correspondence:** Prof. Ajay M Shah, M.D. King's British Heart Foundation Centre for Research Excellence, School of Cardiovascular Medicine and Sciences, King's College London, 125 Coldharbour Lane SE5 9NU, London, United Kingdom. Phone: +44 (0) 20 7848 5189. email: ajay.shah@kcl.ac.uk.



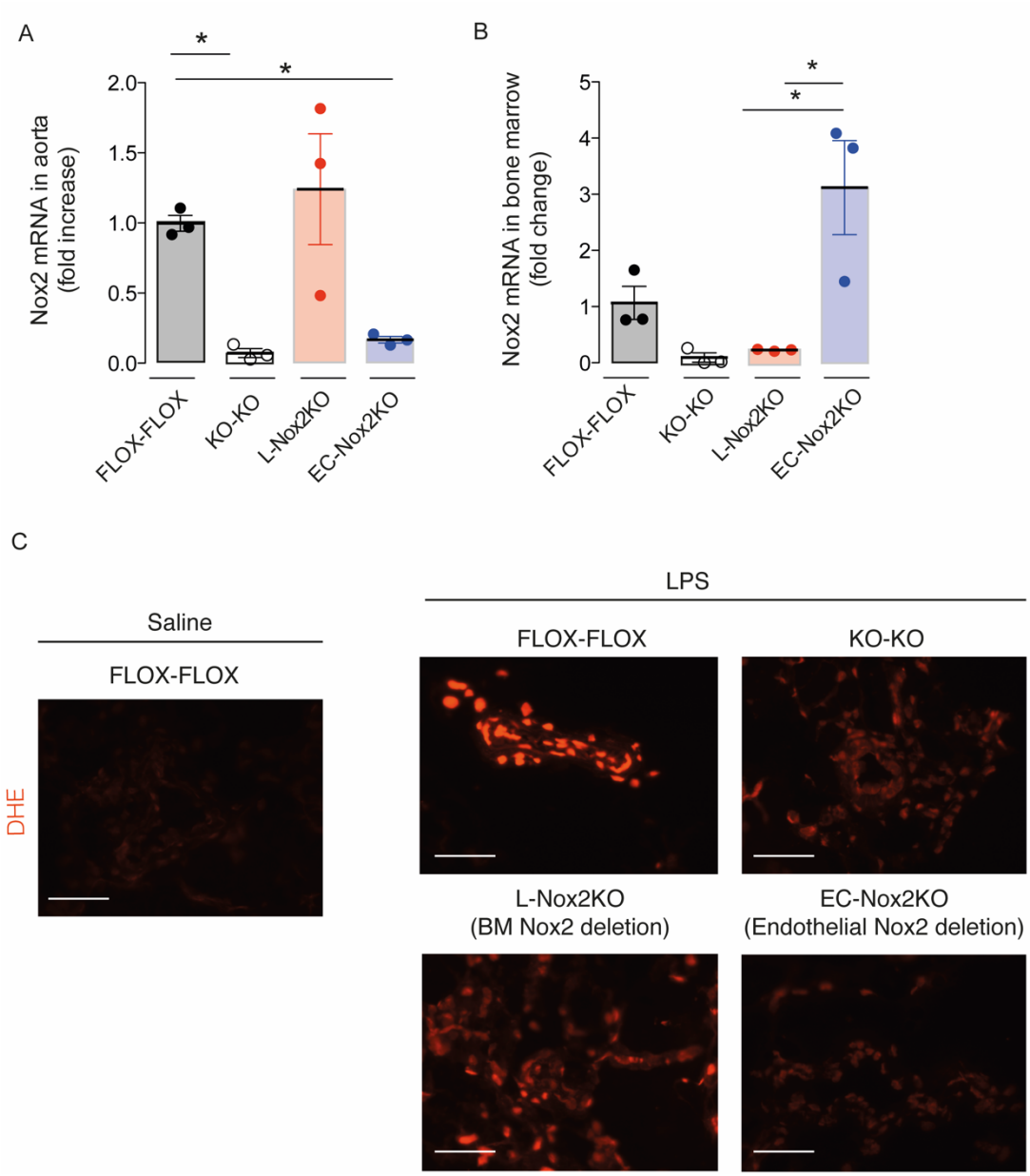
Supplemental digital content 1. Wild type (WT) and Nox2 deficient (Global Nox2KO) mice were injected with LPS (10 mg/kg i.v.). The survival rates were evaluated up to 7 days after challenge with LPS. Log-rank (Mantel-Cox) test.*P<0.05 (n=8 per goup).



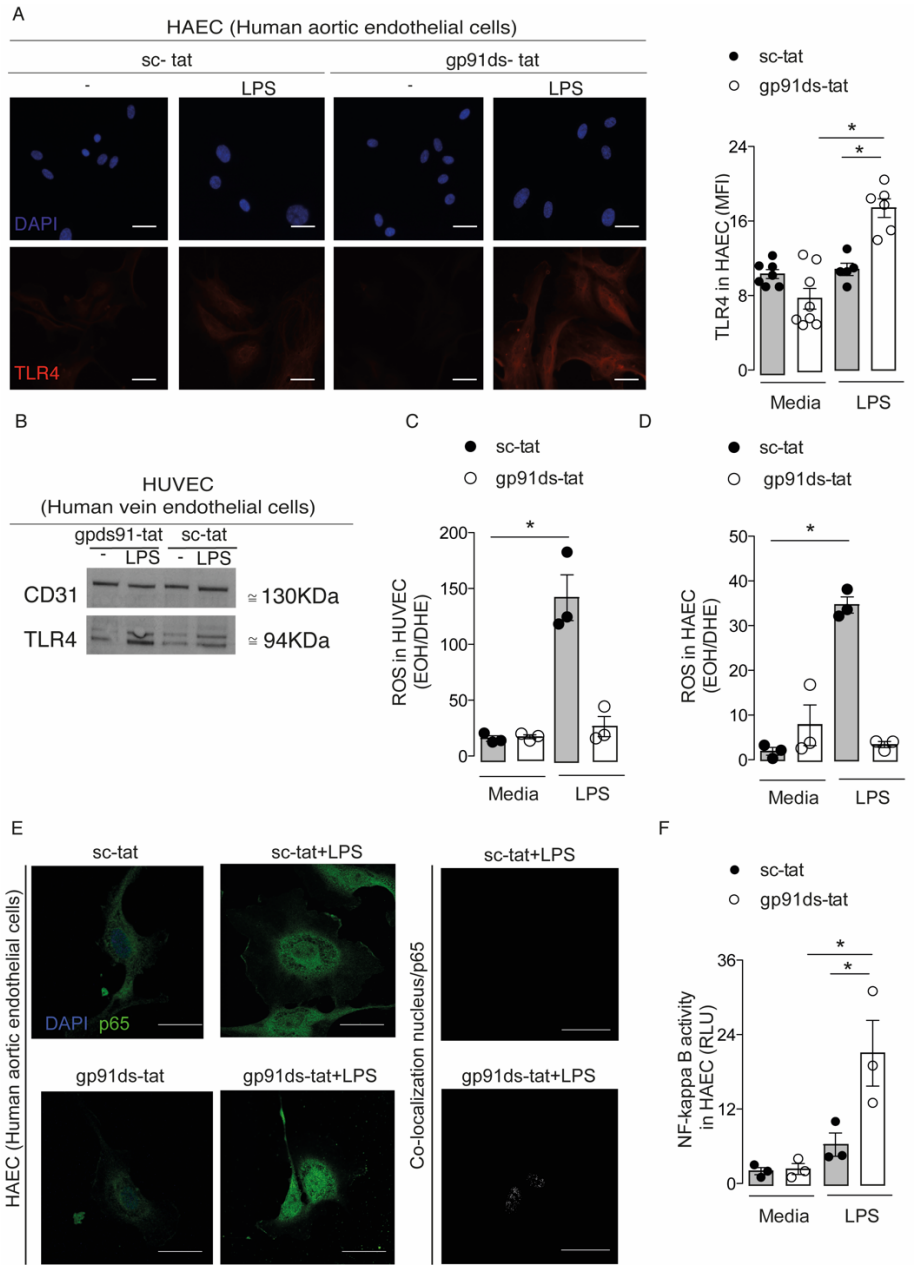
Supplemental digital content 2. Tie2-targeted deletion of Nox2 reduces its expression in neutrophils and blood vessels. (A-C) EC/L-Nox2KO mice and Nox2Flox (Nox2-replete) littermate controls mice were treated intravenously with lipopolysaccharide (LPS, 10mg/kg) or phosphate buffered saline. (A) Nox2 (gp91phox, red) and CD31 (green) staining in mesenteric vessels, conducted 12 hours after LPS injection. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, blue). Images were acquired in epifluorescence microscope. Scale bars, 50µm. Nox2 mRNA levels in aorta (B) and neutrophils purified from bone marrow (C). Data are mean ± SEM. *P<0.05 for highlighted comparisons (n=6 per group). Mann-Whitney test. (D) Dihydroethidium (DHE) staining in mesenteric vessels, 12 hours after LPS injection. Scale bars, 50µm. Images represent the results obtained from 3 mice per group.



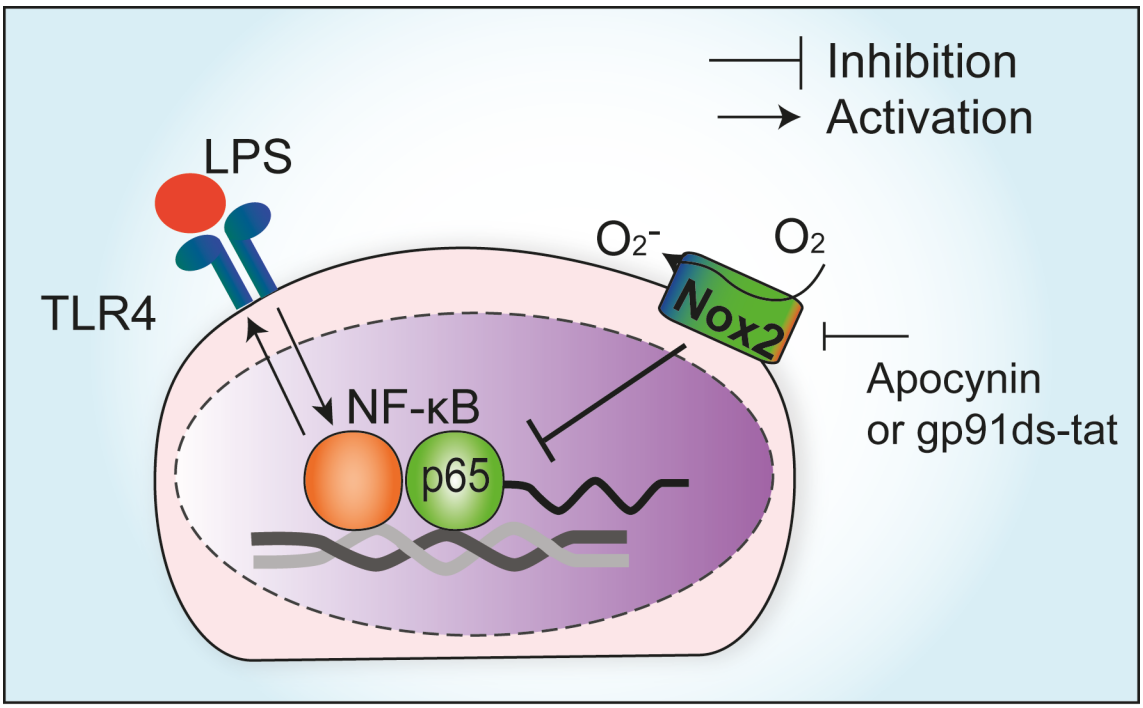
Supplemental digital content 3. Tie2-targeting Nox2 overexpression enhances reactive oxygen species in mesenteric vessels. (A) Nox2 expression in meseteric vessels of Tie-2 targeted transgenic Nox2 mice (Nox2 Tg) and littermate control. Nox2 (gp91phox, red) and CD31 (green) staining in mesenteric vessels. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, blue). Images were acquired in epifluorescence microscope. Scale bars, 50µm. Images represent the results obtained from 3 mice per group. (B) Levels of mRNA for Nox2 in aorta by qPCR. Graph represents mean ± SEM. **P*<0.05 (n=6-7 per group). Mann-Whitney test. (C) Dihydroethidium (DHE) staining in mesenteric vessels. Scale bars, 50µm.



Supplemental digital content 4. Generation of bone marrow (BM) chimeric mice. BM chimeric mice with Nox2 deletion either in EC cells (EC-Nox2KO) or in BM cells (L-Nox2KO). KO-KO (Nox2 deficiency in EC and BM cells) and FLOX-FLOX (Nox2-replete) were used as negative and positive controls, respectively. Nox2 mRNA levels in aorta (A) and BM cells (B). *P<0.05 (n=3 per group). (C) Dihydroethidium (DHE) staining, 12 hours after LPS iv injection (10mg/kg). Images were acquired in epifluorescence microscope. Scale bars, 50μm. Images represent the results obtained from 3 mice per group.



Supplemental digital content 5. Nox2 inhibition by gp91ds-tat increases NF-κB activation and TLR4 expression in endothelial cells. Human aortic or vein endothelial cells (HAEC and HUVEC, respectively) were pre-incubated with gp91ds-tat (30 μM) or scrambled-tat (sc-tat, 30 μM) for one hour and stimulated with lipopolysaccharide (LPS, 200 ng/ml). (A) Immunostaining for TLR4 (red stain), after 24 hours of LPS stimulation. Images were acquired by fluorescence microscopy. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Scale bars, 25μm. MFI: mean fluorescence intensity. Representative images on the left and mean data±SEM on the right. (B) Immunoblotting for TLR4 in HUVEC after 24 hours of LPS stimulation. (C-D) Analyses of high performance liquid chromatography (HPLC) for superoxide-mediated dihydroethidium (DHE) oxidation product 2-hydroxyethidium (EOH). Cells were pre-incubated with the peptides gp91ds-tat (30μM) or scrambled-tat (sc-tat, 30μM) one hour before being stimulated 4 fours with LPS (200ng/ml). Graph is mean±SEM. (E) Co-localization of p65 NF-κB subunit (green) in the nucleus (blue) of HAEC, 30 minutes after LPS stimulation. Images were acquired in a confocal microscopy. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Colocalization of nucleus and p65 is represented in white/black panels. Scale bars, 25μm. (F) Luciferase reporter assays for NF-κB activation after 24 hours of LPS stimulation. RLU: relative lumen units. Graphs represents mean±SEM. **P*<0.05 for highlighted comparisons. Kruskal-Wallis followed by Dunn's Multiple Comparison test (A, right panel; C, D, F).



Supplemental digital content 6. Nox2 in endothelial cells (EC) regulates systemic inflammation and hypotension in sepsis. Schematic showing downregulation of NF-κB and TLR4 expression by Nox2 in endothelial cells, which may account for reduced multi-organ failure and death induced by lipopolysaccharide (LPS).