Supporting Information

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SI Materials and Methods

Cells, Mice, Materials, and Reagents. Mouse intestinal microvascular endothelial cells (MIMECs) were isolated as described (1). Briefly, freshly harvested ileum from newborn mice was minced into 1-mm pieces and washed five times in DMEM (Fisher Scientific) containing 10% (100μL FBS/mL DMEM) FBS and 1% Dithioerythritol (Sigma-Aldrich) to remove the epithelial lining. The remaining tissue was then digested with collagenase (Sigma-Aldrich) for 1 h and subsequently filtered by using a 70-μm filter and resuspended as a single-cell suspension. Cells were then incubated for 45 min with allophycocyanin–labeled platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody (BD Biosciences) and e450-labeled CD45 antibody (eBioscience). Next, cells were fixed in 4% paraformaldehyde (40μL paraformaldehyde/mL phosphate buffered saline) for 45 min, permeabilized in 1% saponin (Sigma-Aldrich) in PBS for 45 min, and incubated with phycoerythrin (PE)-labeled endothelial NO synthase (eNOS) antibody (BD Biosciences) and/or PE–cy7-labeled toll-like receptor 4 (TLR4) antibody (BioLegend; 117609) for 45 min. Isolated cell suspensions were then assessed by using a BD LSR II flow cytometer to identify MIMECs as the PECAM+ CD45− cells from the pool. Expression of eNOS and TLR4 in MIMECs was analyzed by using FlowJo software (Treestar). MIMECs were isolated from wild-type, $Tlr4^{-/-}$, $Tlr4^{\Delta endoth}$, *Myeloid differentiation primary response gene 88* $(Myd88)^{-/-}$ targeted mutation 1.1, Anthony L DeFranco [B6.129P2(SJL)–*Myd88tm1.1Defr*/J; Jackson Labs], or *Toll–IL-1 receptor domain-containing adapter-inducing interferon-β*1; lipopolysaccharide 2 $(Trif)^{-/-(Lps2)}$ mice (C57BL/6J–*Ticam1Lps2*/J; Jackson Labs).

Gene trap ROSA 26, Philippe Soriano; targeted mutation 4, Liqun Luo $Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)}$, Angiopoietin receptor, Tie2–cre (*B6.Cg–Tg*[endothelial specific receptor tyrosine kinase (*Tek*)–*cre*]*12Flv*/*J*), villin–cre [*B6.SJL–Tg*(*Vil–cre*)*997Gum*/*J*], and $eNOS^{-/-}$ nitric oxide synthase 3, endothelial cell; targeted mutation 1, University of North Carolina (*B6.129P2–Nos3tm1Unc*/*J*) were from Jackson Laboratories. Before tissue collection, animals were treated with either saline or LPS at a concentration of 2.5 mg/kg i.p. for 6 h. Where indicated, (E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile (BAY 11) (EMD Millipore Chemicals) was also injected i.p. at a concentration of 10 mg/kg 1 h before treatment with either saline or LPS. Sildenafil (80 mg/kg; Pfizer) was administered dissolved in the infant formula where indicated on each day of the model.

Peritoneal macrophages were harvested from mice of the indicated strain by peritoneal lavage with 10 mL of ice-cold PBS per animal. Cells were then spun down, resuspended in DMEM/F-12 medium (Life Technologies), and subsequently plated on glass coverslips for 2 h to allow for adherence. The macrophages were then treated with LPS at a concentration of 100 ng/mL for 6 h and assessed for the expression of TLR4 by RT-PCR.

SDS/PAGE and immunoprecipitation were performed and quantified as described in ref. 2.

Nitrite and nitrate were measured by using tri-iodide–based and vanadium HCL-based chemiluminescence, respectively, as described (3).

Induction of Endotoxemia and Necrotizing Enterocolitis in Neonatal Mice. All experiments were approved by the Animal Care and Use Committee and the Institutional Review Board of the University of Pittsburgh. $Tlr4^{-/-}$ mice were generated in our laboratory as described (4). Mice specifically lacking TLR4 within the endothelium ($Tlr4^{\Delta endoth}$) were generated by generating $Tlr4^{loxP}$ floxed mice as described (5), which were then crossed with transgenic mice bearing cre-recombinase expressed under the control of the endothelial-specific Tie2 promoter, transgene insertion 12, Richard A Flavell [*B6.Cg–Tg*(*Tek–cre*)*12Flv*/*J*; Jackson Laboratory] or the enterocyte-specific *villin* promoter (5). Where indicated, newborn mice were injected with LPS (Sigma-Aldrich) at a concentration of 2.5 mg/kg for 6 h. Experimental necrotizing enterocolitis (NEC) was induced in 5- to 10-d-old mice as we have described (5) by using formula gavage [Similac Advance infant formula (Abbott Nutrition):Esbilac (PetAg) canine milk replacer 2:1] five times per day and hypoxia (5% O_2, 95% N_2) for 10 min in a hypoxic chamber (Billups-Rothenberg) twice daily for 4 d. Where indicated, sodium nitrite (Sigma-Aldrich) was added to the formula to make up a 10-μM concentration, and sodium nitrate was added to make a either a 50- or 100-μM concentration.

The degree of mucosal injury was determined on histological sections of the terminal ileum by a pediatric pathologist who was blinded to the study condition according to our previously published scoring system from 0 (normal) to 3 (severe injury) (6), by expression of the proinflammatory cytokine IL-6 by qRT-PCR, and by the extent of apoptosis in the terminal ileum, which was assessed as the expression by SDS/PAGE of cleaved caspase-3 to total caspase-3 (Cell Signaling Technology; 9664L and 9662, respectively).

Mouse breast milk was extracted on postpartum days 8–12 from lactating mothers by using an electric, piston-driven breast pump device modified from a standard human breast pump to fit to the mouse teats (Lansinoh Laboratories). Before obtaining milk, mothers were separated from their pups for 6 h, anesthetized with isoflurane, and administered a s.c. injection of oxytocin (Sigma-Aldrich) at 0.15 IU per kg of body weight. Three minutes later, milk was collected, yielding ∼1 mL of breast milk per lactating mother. Human breast milk was donated by deidentified donors and collected without any clinical identifiers via a waiver of informed consent by the Director of the University of Pittsburgh Institutional Review Board.

Quantitative Real-Time PCR. Quantitative real-time PCR was performed as described by using the Bio-Rad CFX96 Real-Time System (7) using the primers listed below relative to the housekeeping gene ribosomal protein L15 (RPLO):

eNOS: 155-bp mouse/rat:

Forward, AGGACATATGTTTGTCTGCGGCGA;

Reverse, AAATGTCCTCGTGGTAGCGTTGCT.

IL6: 182-bp mouse/rat: forward, CCAATTTCCAATGCTCTCCT;

Mouse/rat: reverse. ACCACAGTGAGGAATGTCCA.

RPLO: 143-bp mouse/rat/human: forward, GGCGACCTGGAAGTCCAACT;

Reverse, CCATCAGCACCACAGCCTTC.

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3. Pelletier MM, et al. (2006) The measurement of blood and plasma nitrite by chemiluminescence: Pitfalls and solutions. *Free Radic Biol Med* 41(4):541–548.
4. Sodhi CP, et al. (2010) Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology* 138(1):185–196.
5. Sodhi CP, et al. (2012) Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology* 143(3):708–718.
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7. Leaphart CL, et al. (2007) A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J Immunol* 179(7):4808–4820.

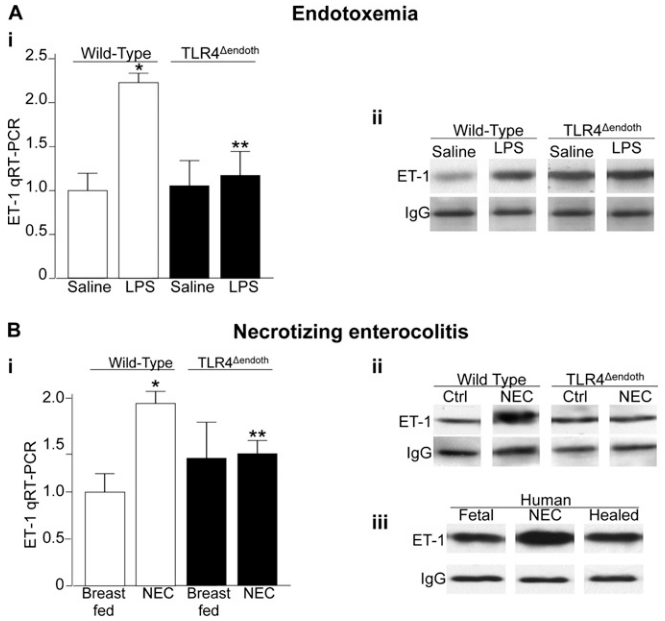

Fig. S1. TLR4 activation in the endothelium increases the expression of endothelin-1 (ET-1). (*A*, *i*) qRT-PCR (mean ± SEM) showing the expression of ET-1 in wild-type or TLR4$^{\Delta endoth}$ mice treated with saline or LPS as indicated. *$P < 0.05$ LPS vs. saline; **$P < 0.005$ LPS treated wild-type vs. TLR4$^{\Delta endoth}$ mice. (*ii*) SDS/PAGE for ET-1 expression in immunoprecipitates that had been pulled down with antibodies to ET-1 from the terminal ileum of wild-type or TLR4$^{\Delta endoth}$ mice that were treated with saline or LPS; IgG is shown as a loading control. (*B*, *i*) qRT-PCR (mean ± SEM) showing the expression of ET-1 in wild-type or TLR4$^{\Delta endoth}$ mice that were either breast-fed controls or induced to develop NEC as indicated. *$P < 0.05$ NEC vs. control; **$P < 0.005$ NEC in wild-type vs. TLR4$^{\Delta endoth}$ mice. (*ii* and *iii*) SDS/PAGE of immunoprecipitates of ET-1 that had been first pulled down with antibodies to ET-1 from the terminal ileum of wild-type or TLR4$^{\Delta endoth}$ mice that were either breast-fed controls or subjected to experimental NEC (*ii*) or from the resected ileum from a fetus, from an infant with NEC, and a neonate 6 wk after the resolution of NEC at the time of stoma reversal (*iii*); IgG is shown as a loading control. Results are representative of three separate experiments.

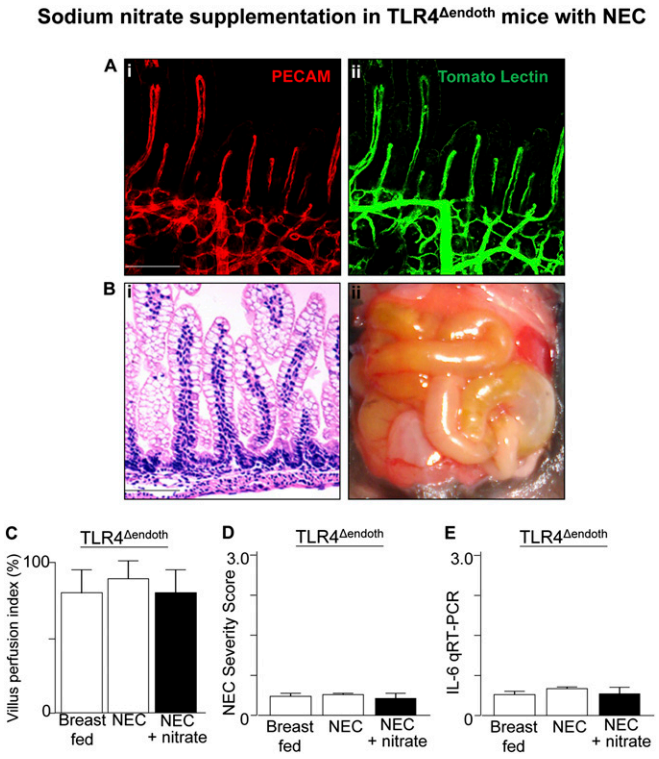

Fig. S2. Sodium nitrate supplementation does not further improve perfusion or reduce severity of experimental NEC in TLR4$^{\Delta endoth}$ mice. (*A*) Representative confocal micrographs from whole-mount sections of terminal ileum of TLR4$^{\Delta endoth}$ mice that were induced to develop NEC and administered sodium nitrate (50 μM) daily for each day of the 4-d model. Mice were then subjected to intracardiac injection with the fluorescent tracer tomato lectin 5 min before they were killed. Whole mounts were immunostained with antibodies to PECAM-1 to assess the microvasculature of the indicated organ (red images); the corresponding blood flow (Lucifer yellow) appears in green. (*B*) Representative histomicrographs of the terminal ileum (*i*) and photograph (*ii*) of the intestine after fresh autopsy of newborn TLR4$^{\delta endoth}$ mice who were induced to develop NEC. (*C*–*E*) Villus perfusion index (mean ± SEM) as described in *Materials and Methods* (*C*), NEC severity score (*D*), and qRT-PCR showing the expression of IL-6 in the intestinal mucosa (*E*) of TLR4$^{\delta endoth}$ mice induced to develop NEC along with the addition of sodium nitrate. Shown for comparison purposes are the values for mice that were breast-fed and for TLR4$^{\delta endoth}$ mice subjected to experimental NEC. There are no significant differences between groups. Data are mean ± SEM. Results are representative of three separate experiments. (Scale bar: 100 μm.)