Supplementary methods:

Antibodies and reagents:

Monoclonal HMGB1 - Sigma-Aldrich (St. Louis, MO); polyclonal anti-HMGB1 - Upstate (Millipore, Billerica, MA); cofilin and phospho-cofilin - Cell Signaling (Danvers, MA); focal adhesion kinase (FAK) and phospho-FAK - Invitrogen (Carlsbad, CA). The Rho kinase inhibitor Y-27632 - EMD Chemicals (Gibbstown, NJ). Ultrapure (>99.6 % pure) LPS and IFNγ were from Sigma-Aldrich. Appropriate secondary antibodies for imunohistochemistry and SDS-PAGE were obtained from Molecular Probes, Eugene, OR and Jackson Immunoresearch, West Grove, PA respectively including Alexa488 (green) and Cy3 (red). GFP-actin was from Invitrogen. Draq5 - Cell Signaling Technologies. The serum concentration of HMGB1 was measured in mice with experimental NEC using an HMGB1 ELISA kit, Shino-Test Corporation, Kanagawa, Japan, according to the manufacturer's instructions.

Determination of macrophage chemotaxis in vitro and in vivo.

Macrophage chemotaxis was measured in vitro using a transmigration chamber with an 8 μm pore size insert (Falcon, BD Biosciences). Briefly, the inserts were placed into 24-well plates containing DMEM-F12 media in the presence or absence of 4μg/ml HMGB1 and/or 100 ng/ml Next-formyl-methyl-leucyl-phenylalanin (fMLP) as described (28). RAW264.7 macrophages (~500,000) were then loaded onto the inserts and incubated at 37°C for 18hrs. Chemotaxis was quantified by counting the number of cells that migrated through the insert using a hemocytometer under phase contrast microscopy.

For assessment of the effects of HMGB1 on macrophage chemotaxis *in vivo*, 3 weeks old mice (TLR4 wild-type C3H/HeOUJ or TLR4 mutant C3H/HeJ, Jackson Labs) were injected with HMGB1 (2 µg/gram body weight) intraperitoneally as described (14). The cells that migrated into the peritoneal cavity were isolated by peritoneal lavage in 5 ml ice cold PBS 3 hours later. Macrophages were then counted directly by plating on coverslips and evaluating by morphology under phase contrast optics after 3 hours of adherence (thereby eliminating the non-adherent erythrocytes and lymphocytes). In parallel, macrophages were quantified by flow cytometry as described (29). In brief, cells were labeled with the macrophage marker FITC-conjugated anti-mouse F4-80, and characterized using an LSRII analyzer (Beckton Dickinson Biosciences, San Jose, CA). Cells were gated using forward versus side scatter initially to gate on single cell suspensions, and eliminate debris, red blood cells and cell clumps. A background level was then set using a sample of cells prepared without antibody. In all cases, a minimum of 10,000 gated events was collected. Events were then analyzed using FACSDiva software, and two parameter histograms were created with F4-80 on the x-axis and an irrelevant channel on the y-axis. Positive cells were located (in Q4) which were significantly above background signal and negative for the irrelevant channel, thereby eliminating autofluorescence.

Determination of enterocyte migration.

Enterocyte migration was measured *in vitro* and *in vivo* as we have performed previously (3, 30, 31). For *in vitro* experiments, a single layer of IEC-6 enterocytes were plated on cover slips, cultured to 90-100% confluence overnight, then serum-starved for

an additional 12 hours. A wound was created with a pipette tip, and the movement of cells into the wound was continuously using either live cell microscopic imaging on an Olympus 1X71 inverted microscope (Melville, NY), or were observed at various time points after being removed from the incubator in six-well plates on the same microscope. Where indicated, cells were treated with LPS (50ug/ml), varying doses of HMGB1 (10 to 1000ng/ml), or Y-27632 (5µM) during the migration assay.

To measure enterocytes migration *in vivo*, 2-3 week old C3H/HeOuJ and C3H/HeJ mice were injected with HMGB1 (1µg per gram of body weight) or an equal volume of PBS intraperitoneally twice a day for two days, followed by the intraperitoneal injection of BrdU (10ul/g, Invitrogen), then sacrificed 18 hours later. The administration of 1 µg/kg of HMGB1 yields serum concentrations of approximately 12.5 ng/ml, which approximates the concentration of HMGB1 that we detect in the serum of mice with experimental NEC (10-15 ng/ml). Formalin-fixed paraffin-embedded sections of the terminal ileum were then immunostained using the BrdU In-Situ Detection Kit (BD Biosciences) according to the manufacturer's protocol. The migration rate was measured by the distance from the bottom of the crypts to the farthest BrdU positive cells along the villi in at least 100 separate villi, as we have shown (3, 4, 30).

Induction of necrotizing enterocolitis.

All mice were housed and cared for at the Rangos Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA. All experiments were approved by the Children's Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh. Swiss-Webster (CfW) were obtained from Jackson Laboratories

(Jackson Laboratory, Bar Harbor, ME). Experimental NEC was induced in mice as we have previously described and validated (8, 30). Briefly, 10-14 day-old mice were fed Similac Advanced infant formula (Ross Pediatrics:Esbilac canine milk replacer at a ratio of 2:1) five times daily, and exposed to intermittent hypoxia (5% O_2, 95% N_2) for 10 minutes using a modular hypoxic chamber (Billups-Rothenberg, DelMar, CA) twice daily for 4 days. Animals were fed 200 microliters per 5 grams of mouse body weight by gavage over 2-3 minutes, using a 24-French angio-catheter which was placed into the mouse esophagus under direct vision. We and others have demonstrated that this experimental protocol induces intestinal inflammation and the release of pro-inflammatory cytokines in a pattern that closely resembles human NEC (3, 10, 30, 31, 38, 39). Control (i.e. non NEC) animals remained with their mothers and received breast milk. The severity of experimental NEC was graded by a pathologist blinded to the study groups and an additional blinded observer, using a previously validated scoring system from 0 (normal) to 3 (severe) as previously described (30, 38). Immediately after sacrifice, the terminal ileum was harvested 1 cm proximal to the ileocecal valve in 10% neutral buffered formalin or frozen in liquid nitrogen after embedding in Cryo-Gel (Cancer Diagnostics, Inc., Birmingham, MI). Where indicated, mucosal scrapings were obtained by microdissection under 20X power, and collected in RNAlater (Qiagen, Valencia, CA).

Intestinal samples were obtained from human neonates undergoing intestinal resection for NEC or for unrelated indications (control). The intestinal mucosa was micro-dissected from the underlying submucosal tissue and placed in ice-cold tissue lysis buffer (10% Glycerol, 62.5mM Tris (pH6.6), 7.5% SDS) containing protease inhibitors (1mM sodium

pyrophosphate, 20mM sodium fluoride, 2μg/ml aprotinin, 5μg/mL pepstatin, 0.5mM PMSF, and 50μM leupeptin) or placed in RNAlater solution. In parallel, samples obtained at laparotomy from preterm human infants undergoing intestinal resection for management of NEC or at the time of stoma closure was either prepared for biochemical analysis (see below) or placed in 2% paraformaldehyde overnight, transferred to 30% sucrose, and then frozen in Cryo-Gel (Cancer Diagnostics, Inc, Birmingham, MI) for immunohisotochemical analysis (please see below). All human tissue was obtained and processed as discarded tissue via waiver of consent with approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines.

Generation of wild-type and dominant negative TLR4 adenoviruses.

Adenoviruses expressing wild-type (WT) or dominant negative (P712H) TLR4 with the C-terminal fusion of GFP were constructed using the Adeno-X Expression System 2 (Clontech) according to the manufacturer's protocol, as we have recently described (9). TLR4 mutant P712H was constructed from wild-type mouse TLR4 cDNA from pUNO-mTLR4 (Invivogen) by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Both mouse TLR4 wild-type and mutant coding sequences were cloned into pDNR-CMV and integrated into Adeno-X acceptor vectors by Cre-loxP recombination. The resulting plasmids were linearized and transfected into the packaging cell line HEK293. Adenoviruses were further amplified in HEK293 cells. Where indicated, Adenoviruses were added to IEC-6 cells at an MOI of 20 to assure an infection rate of more than 90%, and incubated for 48 hours before migration, cell contraction force or the

Rho activation assays. We have demonstrated previously that the dominant negative TLR4 – but not wild-type TLR4 - inhibits LPS-mediated activation of p38 MAPK and NF-kB (9).

*Statistical Analysis***.**

Statistical analysis was performed using SPSS 13.0 software. ANOVA was used for comparisons for experiments involving more that two experimental groups. Two-tailed student's t-test was used for comparison for experiments consisting of two experimental groups. For analysis of the incidence of NEC, chi-square analysis was performed.