A Critical Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis by Modulating Intestinal Injury and Repair

Cynthia L. Leaphart, Jaime Cavallo, Steven C. Gribar, Selma Cetin, Jun Li, Maria F. Branca, Theresa D. Dubowski, Chhinder P. Sodhi and David J. Hackam

*J Immunol* 2007; 179:4808-4820; doi: 10.4049/jimmunol.179.7.4808
http://www.jimmunol.org/content/179/7/4808

**References**

This article cites 85 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/179/7/4808.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Critical Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis by Modulating Intestinal Injury and Repair

Cynthia L. Leaphart, Jaime Cavallo, Steven C. Gribar, Selma Cetin, Jun Li, Maria F. Branca, Theresa D. Dubowski, Chhinder P. Sodhi, and David J. Hackam

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in preterm infants and is characterized by translocation of LPS across the inflamed intestine. We hypothesized that the LPS receptor (TLR4) plays a critical role in NEC development, and we sought to determine the mechanisms involved. We now demonstrate that NEC in mice and humans is associated with increased expression of TLR4 in the intestinal mucosa and that physiological stressors associated with NEC development, namely, exposure to LPS and hypoxia, sensitize the murine intestinal epithelium to LPS through up-regulation of TLR4. In support of a critical role for TLR4 in NEC development, TLR4-mutant C3H/HeJ mice were protected from the development of NEC compared with wild-type C3H/HeOuJ littermates. TLR4 activation in vitro led to increased enterocyte apoptosis and reduced enterocyte migration and proliferation, suggesting a role for TLR4 in intestinal repair. In support of this possibility, increased NEC severity in C3H/HeOuJ mice resulted from increased enterocyte apoptosis and reduced enterocyte restitution and proliferation after mucosal injury compared with mutant mice. TLR4 signaling also led to increased serine phosphorylation of intestinal focal adhesion kinase (FAK). Remarkably, TLR4 coimmunoprecipitated with FAK, and small interfering RNA-mediated FAK inhibition restored enterocyte migration after TLR4 activation, demonstrating that the FAK-TLR4 association regulates intestinal healing. These findings demonstrate a critical role for TLR4 in the development of NEC through effects on enterocyte injury and repair, identify a novel TLR4-FAK association in regulating enterocyte migration, and suggest TLR4/FAK as a therapeutic target in this disease. The Journal of Immunology, 2007, 179: 4808–4820.

Received for publication May 4, 2007. Accepted for publication July 19, 2007.

Division of Pediatric Surgery, Department of Surgery, Children’s Hospital of Pittsburgh and the University of Pittsburgh School of Medicine, Pittsburgh, PA 15260

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 D.J.H. is supported by RO1GM078238-01 from the National Institutes of Health and the State of Pennsylvania Tobacco Settlement Fund. C.L.L. is supported in part by the Loan Repayment Program for Pediatric Research of the National Institutes of Health.

2 Address correspondence and reprint requests to Dr. David J. Hackam, Division of Pediatric Surgery, Room 4A-486 DeSoto Wing, Children’s Hospital of Pittsburgh, Pittsburgh, PA 15213. E-mail address: david.hackam@chp.edu

3 Abbreviations used in this paper: NEC, necrotizing enterocolitis; FAK, focal adhesion kinase; ATRA, all-trans-retinoic acid; siRNA, small interfering RNA; XTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Copyright © 2007 by The American Association of Immunologists, Inc. 0002-1767/07/$2.00

www.jimmunol.org
from uninjured sites to areas of mucosal disruption, and after re-
stripping cell-cell and cell-matrix contacts, lead to a restoration of
barrier integrity (29, 30). At the same time, immature enterocyte
precursors that are located within the intestinal crypts divide and
migrate into mature enterocytes through the process of enteroc-
yte proliferation to replace the necrotic enterocytes (31). We
have recently shown that intestinal restitutio is significantly reduced in
animals with experimental NEC compared with healthy animals
(11, 32, 33) and that exposure of enterocytes to endotoxin leads to
a significant decrease in the rate of enterocyte migration due to an
increase in focal adhesion kinase (FAK)-dependent cell-matrix ad-
hesiveness (32). Others have shown that the rate of enterocyte
proliferation is significantly reduced upon exposure to LPS (34). 
These findings raise the intriguing possibility that LPS signaling at
the intestinal epithelial surface may lead to a disruption of the
healing response to intestinal injury and thus facilitate the ongoing
tissue damage that occurs in the pathogenesis of NEC. However,
the mechanism(s) whereby LPS signaling may alter healing of the
injured intestine in vivo and the precise role of TLR4 in this pro-
cess, if any, remain largely unexplored.

We now demonstrate that NEC is associated with an increase in
the expression of TLR4 in the intestinal mucosa and that physio-
logical stressors associated with the development of NEC, namely,
exposure to LPS and hypoxia, sensitize the intestinal epithelium to
LPS through the up-regulation of TLR4. Furthermore, the severity
of experimental NEC was found to be significantly reduced in
C3H/HeJ mice that bear a mutation in TLR4 (15), as compared with
C3H/HeOUJ mice that express functional TLR4. Strikingly,
the reduction in NEC severity in C3H/HeJ mice was found to be due to
a significant increase in the healing capacity of the injured
intestinal epithelium as compared with C3H/HeOUJ counterparts,
in association with reduced phosphorylation of FAK. These find-
ings shed light on a novel link between enterocyte TLR4 activation
and reduced intestinal healing, and they suggest a mechanism to
explain the injurious effects of LPS on the intestinal epithelium in
the pathogenesis of NEC.

Materials and Methods

Cell culture and reagents

Cultured small intestinal cells (IEC-6) were obtained from the American
Type Culture Collection (ATCC) and maintained as described (35, 36).
J774 macrophages and HEK cells were obtained from ATCC and main-
tained as described (37, 38). Where indicated, cells were treated with LPS
(Enterohcrinia coli 011:B4 purified by gel filtration chromatography (>99% pure; Sigma-Aldrich) at concentrations of 100 ng/ml to 50 μg/ml for
6 h or 24 h were exposed to hypoxia (5% oxygen, 95% nitrogen) using
a modular hypoxic chamber (Billups-Rothenberg), or were exposed to
hypoxia (5% oxygen, 95% nitrogen) using a modular hypoxic chamber (Billups-Rothenberg) for 0 –18 h in serum-free
IEC-6 medium that was determined to be endotoxin free using the
Limulus amebocyte assay (Charles River Laboratories). Abs were obtained as
follows: TLR4, Santa Cruz Biotechnology; phosphorylated and total FAK (Biosource). C3H/HeJ and
C3H/HeOUJ mice were obtained from the Jackson Laboratory and housed in
accordance with University of Pittsburgh animal care guidelines. All ani-
mal studies were approved by the Institutional Review Board at the Uni-
versity of Pittsburgh. Discarded human tissue was obtained via waiver of
consent in accordance with University of Pittsburgh anatomical tissue proc-
curement guidelines with approval from the University of Pittsburgh In-
stitutional Review Board. Specifically, tissue was obtained from human
infants undergoing surgical resection for the management of severe NEC.
For comparison purposes, tissue was also obtained at the time of ostomy
closure during which all significant intestinal inflammation would have
resolved.

Where indicated, LPS concentration in medium or serum was deter-
mined using the kinetic colorimetric Limulus amebocyte assay (Charles
River Laboratories) in pyrogen-free tubes, according to the manufacturer’s
instructions, and expressed as endotoxin units per ml (EU/ml) of fluid (39).
For the purpose of the in vitro experiments, a concentration of 50 μg/ml
LPS was used, given that this corresponds to ~15–20 EU/ml as determined
by Limulus assay, which is within the range of serum LPS that we measure
in experimental NEC.

Induction of experimental NEC

To induce NEC, the following experimental protocol was approved by the
Animal Research and Care Committee of the Children’s Hospital of Pitts-
burgh (protocol 0805). NEC was induced in 10-day-old mice that ex-
prescribed either wild-type TLR4 (C3H/HeOUJ) or a nonfunctioning muta-
tion in TLR4 (C3H/HeJ) by the administration of 15 g of Similac 60/40
(Ross Pediatrics) in 75 ml of Esbilac canine milk replacer (Pet-Ag) and
the induction of hypoxia (5% oxygen for 2 min before each feeding) twice
daily for 4 days. Animals are fed 200 μl/5 g of mouse body weight by gavage
every 2–3 min, using a French angiocatheter which is placed into the
mouse esophagus under direct vision. Samples were harvested at day 4
for analysis. We and others have demonstrated that this experimental pro-
tocol induces intestinal inflammation in animals that resembles human
NEC (11, 32, 40, 41). Control (i.e., non-NEC) animals of both strains
remained with their mothers and received breast milk. Where indicated,
breast-fed animals of both strains were injected with LPS (5 mg/kg) i.p. for
12 h before sacrifice or were exposed to hypoxia alone. The severity of
experimental NEC was graded using a previously validated scoring system
from 0 (normal) to 3 (severe) as previously described (41).

SDS-PAGE and immunohistochemistry

For SDS-PAGE, lysates were purified from cultured cells or mucosal
scrapings were obtained from fresh samples of terminal ileum that were
obtained immediately after mice were sacrificed (see animal model,
above). After irrigation of the bowel to remove luminal contents, the
mucosal layer was microdissected from the underlying connective tissue on
the stage of an Olympus SZX7 microscope and placed in cold lysis buffer
containing the protease inhibitors as previously described (11). Mucosal
scrapings were subjected to SDS-PAGE using specific Abs against TLR4 (Santa
Cruz Biotechnology), phosphorylated and total FAK (Biosource). The
determination of band density from radiographic film was performed using a
Bio-Rad GS700 densitometer and QuantityOne analysis software.

To assess for the biochemical interaction of FAK with TLR4, IEC-6,
J774 cells (105 cells/plate) were cultured on 6-cm dishes, washed with
PBS, and solubilized in detergent solution containing 50 mM Tris (pH 8.0), 1% Non-
ident P-40, 0.4% deoxycholate, 62.5 mM EDTA, and 1 μg/ml aprotinin.
The extract was centrifuged for 5 min in an Eppendorf (USA) model 5414
microfuge (10,000 × g) at 4°C to remove insoluble material and nuclei,
and the supernatant was recovered. Where noted in the text, an aliquot of
30 μl of lysate was added to Laemmli sample buffer, heated for 2 min
at 90°C, and subjected to SDS-PAGE. Alternatively, the entire detec-
tion was immunoprecipitated with anti-TLR4 or -FAK Abs, and Ab-Ag
complexes were collected using protein G-coupled Sepharose (Sigma-
Aldrich) as described (42). An equivalent amount (30 μl lysate, 105 cells/
well starting material) of lysates of J774 macrophages and HEK cells were
prepared as positive controls for the TLR4 and FAK Abs. In parallel,
immunoprecipitation experiments were performed with irrelevant Abs at
equimolar concentrations and with uncoated beads. Samples were then
electrophoresed on 8% SDS-PAGE gels and analyzed using Scion Image
software. For immunohistochemistry, cells were processed as described (32)
and fluorescent images were captured using an Olympus Fluoview 1000
confocal microscope under a ×60 oil immersion objective using standard filter
sets. The nuclear stain Draq5 was purchased from AXXORA Platform.
Digital images were prepared and labeled using Adobe Photoshop 7.0
software.

Quantitative real-time PCR

Total RNA was isolated from the ileal mucosal scrapings of mice that had
been breast-fed (control) or induced to develop experimental NEC, as well
human control and NEC tissues using the RNeasy kit (Qiagen) and reverse
transcribed (1 μg of RNA) using the Quantitect Reverse Transcription Kit
(Qiagen). Gene-specific cDNA was amplified and quantified in a real-time
thermal cycler system (SYBR Green I; Cycler IQ Real-Time PCR De-
tection System). PCR amplification was then performed in triplicate. In all
cases, water was used instead of cDNA to serve as a nontemplate control.
The reaction protocol included preincubation at 95°C for 15 min to activate
AmpliTaq Gold DNA Polymerase (Applied Biosystems) and amplification
for 40 cycles (15 s at 95°C, 30 s at 56°C, and 60 s at 72°C). The results
were normalized using the housekeeping gene β-actin.

The specific primer sequences encoding transcripts for mouse and hu-
man TLR4 are as follows: mouse TLR4 [sense, 5′-TTTATCAGACGC
CGTTGGTG-3′; antisense, 5′-CAGAGGTGTTGCTCCCATC-3′] (186

The Journal of Immunology 4809

Downloaded from http://www.jimmunol.org/ by guest on April 16, 2017
FIGURE 1. The expression of the LPS receptor TLR4 is increased in the intestinal mucosa in experimental NEC. A–D, The combination of twice daily hypoxia and gavage feeding with enteric formula every 3 h results in the gross (A vs C) and histological development of NEC in newborn mice (B vs D). Bar, 100 μm. Control refers to breast-fed mice; NEC refers to mice that were induced to develop NEC. E, SDS-PAGE of purified mucosal scrapings that were obtained from the terminal ilea of breast-fed mice without NEC (control, lanes 2 and 3) and mice subjected to formula gavage/hypoxia (NEC, lanes 4–7) that were immunoblotted using Abs against TLR4; blots were then stripped and reprobed with Abs against F-actin. Lane 1, +ve, J774 macrophage-positive control. F, Quantification of the relative expression of TLR4 to β-actin in intestinal mucosal scrapings of control and NEC mice. Shown are mean ± SEM of three separate experiments with three animals per experiment. *, p < 0.05 by Student’s t test vs control. G, Quantitative RT (qRT)-PCR demonstrating the ratio of mRNA expression of TLR4 to β-actin in intestinal mucosal scrapings of control and NEC mice. Values means ± SEM of three separate experiments with three animals per experiment. *, p < 0.05 by Student’s t test.

Measurement of enterocyte apoptosis

Two separate techniques were used to measure enterocyte apoptosis. In the first, IEC-6 cells were immunostained with affinity-purified Abs against cleaved caspase-3 (Cell Signaling) then imaged using an Olympus Fluoview 1000 confocal microscope. The percent of enterocytes undergoing apoptosis was determined by quantifying the number of cells that expressed caspase-3 per high power field. To measure apoptosis in the intestine, ileal samples were freshly obtained after induction of NEC and from control animals, then were assessed using TUNEL technology to detect DNA fragmentation. To do so, samples were fixed in 4% formalin in PBS and embedded in paraffin. Sections were deparaffinized and rehydrated with PBS before pretreatment with 20 μg/ml proteinase K (Mili- pore) for 20 min at room temperature. Strand breaks of DNA (occurring during apoptosis) were detected using the ApopTag In Situ Apoptosis Detection Kit (Millipore) per the manufacturer’s protocol. Negative control sections of mouse spleen were incubated with labeling solution without enzymatic solution. Diaminobenzidine substrate (Vector Laboratories) was applied at room temperature for 20 min before counterstaining with aqueous hematoxylin. Mounting medium was applied to coverslips and allowed to dry overnight at room temperature before microscopic evaluation. TUNEL-stained slides were examined using an upright Imager.Z1 microscope with AxioCam MRc5 (Carl Zeiss), and TUNEL-positive cells were quantified using Metamorph software (Universal Imaging Corp.).

Measurement of enterocyte migration and proliferation

In vitro studies

To measure enterocyte migration, IEC-6 cells were grown in serum-free antibiotic-free medium in 12-well plates. Where indicated, cells were treated with all-trans-retinoic acid (ATRA, 10 μm; Sigma-Aldrich) or LPS (50 μg/ml) for 1 h before scraping. Cells were transfected with 5 nM nonpooled FAK small interfering RNA (siRNA; Dharmacon) or nontargeting siRNA as a control using Lipofectamine 2000 (Invitrogen Life Technologies) as a carrier. In preliminary experiments to verify the protocol for siRNA, the reduction of expression of cyclophilin B siRNA (Invitrogen Life Technologies). In all cases, the specificity of siRNA against FAK was verified by assessing the lack of reactivity against other proteins to eliminate the possibility of an off-target result.
For kinetic measurements of enterocyte migration, the following approach was utilized: After transfection, a wound was created within the confluent monolayer by scraping a layer of confluent IEC-6 cells with a pipet tip. Cells were then observed as they moved into the wound on the x-y plane of the individual regions of interest (corresponding to individual cells) across a ruler that had been superimposed onto the indi-
with BrdU (50 mg/kg), and samples of the terminal ileum were immuno-
stained using anti-BrdU Abs. The number of BrdU-positive cells in the
crypts and the intensity of BrdU staining were assessed using Metamorph.

Statistical analysis

Data are means ± SEM, and comparisons are by two-tailed Student’s t test
or ANOVA, with statistical significance accepted for \( p < 0.05 \). Additional
statistical information regarding specific comparisons is provided in the
figure legends.

Results

The expression of TLR4 is increased in the intestinal mucosa of
animals with experimental NEC

NEC typically occurs after the newborn intestine has been colo-
nized with Gram-negative, enteric flora and develops after a hy-
poxic insult in the formula-fed newborn \((44, 45)\). To define the
molecular mechanisms that contribute to the development of NEC,
we first sought to test the hypothesis that the LPS receptor TLR4
plays a central role in its pathogenesis. To test this hypothesis, we
utilized a mouse model of NEC that involves the enteral admin-
istration of formula every 3 h to newborn mice, along with expo-
sure to 2 min of hypoxia twice daily. As shown in Fig. 1, A–D, this
treatment leads to the development of patchy necrosis of the in-
testine, which bears similarity to the human disease (see Fig. 2). To
investigate a possible role for TLR4 in the pathogenesis of NEC,
mucosal scrapings were prepared from the terminal ileum of new-
born mice with and without NEC and subjected to SDS-PAGE and
real-time PCR. As is shown in Fig. 1, E–G, the development of
experimental NEC was associated with a significant increase in the
expression of TLR4 protein (Fig. 1, E and F) and mRNA (Fig. 1G)
compared with control animals. To assess for the potential signif-
ificance of these findings to the human disease, specimens of intesti-
tine were obtained from human infants undergoing intestinal re-
section for severe NEC (see Fig. 2, A and B). As shown in Fig. 2,
amarked increase in the mucosal expression of TLR4 protein (Fig.
2, C and D) and mRNA (Fig. 2E) was detected in the small intesti-
ne of human infants who underwent surgical resection in the
management of severe NEC as compared with the expression in the
intestine obtained at the time of subsequent surgery for stoma
closure, at which the extent of inflammation would have com-
pletely resolved. These findings suggest the possibility that the
expression of TLR4 may be increased in the intestinal mucosa (or
on inflammatory cells within the intestinal mucosa) in response to
factors that contribute to the development of NEC.

Exposure to endotoxin and hypoxia leads to an increase in the
expression of TLR4 in enterocytes in vitro and in vivo

We next sought to investigate further the mechanisms that could
contribute to the increase in TLR4 expression in enterocytes ob-
served in NEC. The development of NEC is associated with ex-
posure to high levels of circulating LPS (Refs. 6, 7, and 46 and Fig.
3A), raising the possibility that exposure of enterocytes to LPS
could lead to increased TLR4 expression. The concentration of
LPS in the sera of infant mice with NEC was used to determine the
concentration of LPS for subsequent in vitro and in vivo studies

FIGURE 5. TLR4 inactivation protects against the
development of enterocyte apoptosis in experimental
NEC. A and B, Merged confocal micrographs of IEC-6
cells that were either untreated (A, control) or treated
with LPS (50 μg/ml, 14 h) and then immunostained
with Abs against the apoptotic marker cleaved
caspase-3 (green), rhodamine phalloidin (red), and the
nuclear marker Draq-5 (blue). Bar, 10 μm. Arrows,
Apoptotic cells. C, Quantification of apoptosis of IEC-6
cells (■) or of enterocytes in vivo (□); ctrl, Untreated
animals, “NEC” = animals with experimental NEC.
Values are means ± SEM of three separate experiments
with >100 cells per experiment enumerated. *, \( p < 
0.05 \) by Student’s t test vs control; **, \( p < 0.05 \) vs
wild-type animals with NEC by ANOVA. D–G, Micro-
graphs showing TUNEL-stained terminal ileum of wild-
type mice (D and E) and TLR4-mutant mice (F and G)
that were either breast fed (D and F) or induced to
develop NEC (E and G). Arrows, TUNEL-positive entero-
cytes. Higher magnification of the region of interest in E
and G are shown (E’ and G’, respectively). Bar, 10 μm.
Representative of at least three separate experiments
with more than three animals per group.
and is within the range of LPS detected clinically as described (46). As shown in Fig. 3B, LPS treatment led to a profound increase in the expression of TLR4 as compared with untreated cells. This effect was also observed in vivo, given that the expression of TLR4 was significantly increased in the intestinal mucosa of newborn animals injected with LPS as compared with mice injected with saline (Fig. 3C). In addition to a requirement for colonization of the intestine with Gram-negative bacteria, the development of NEC is most often observed in the setting of a systemic hypoxic insult (47). To assess whether

**FIGURE 6.** TLR4 activation reduces enterocyte proliferation in IEC-6 cells and in experimental NEC. A, XTT proliferation assay in IEC-6 enterocytes in the absence (Ctrl) or presence (LPS) of LPS (50 μg/ml). Shown are ODAbs units, mean ± SEM of four separate experiments. *, p < 0.05 by Student’s t test vs control cells. B, Quantification of enterocyte proliferation in the intestinal mucosa of wild-type and TLR4-mutant mice under breast-fed conditions (Ctrl) or after the induction of experimental NEC. To measure proliferation in vivo, animals were injected with BrdU 1 h before sacrifice, and tissues were immunostained for BrdU expression as described in Materials and Methods. Values are means ± SEM of 4 separate experiments with 3 animals per group with a minimum of 50 crypts enumerated per high power field; *, p < 0.05 vs NEC-treated mutant mice by ANOVA. C–F, Representative images showing enterocyte proliferation within the intestinal mucosa of wild-type (C and D) and TLR4-mutant animals (E and F) under control conditions (C and E) or after the induction of experimental NEC (D and F).

**FIGURE 7.** TLR4-mutant mice show enhanced intestinal restitution after the development of NEC compared with wild-type counterparts. A–D, Micrographs showing the migration of BrdU-labeled enterocytes in the terminal ileum of wild-type (A and B) and TLR4-mutant mice (C and D) that were either breast fed (control, A and C) or induced to develop NEC (B and D). Arrows, Position of BrdU-labeled enterocytes. E, Rate of enterocyte migration as quantified by measuring the ascent of BrdU-labeled enterocytes from the crypt to the villus. Values are means ± SEM of 4 separate experiments with 3 animals per group where at least 100 villi were examined per group. *, p < 0.05 by ANOVA vs mutant mice with NEC. F, Kinetic analysis of the migration of enterocytes in vivo. Wild-type (left bars) and TLR4-mutant (right bars) mice were either breast fed ([]) or induced to develop NEC (■) and then sacrificed 4, 14, or 24 h after BrdU injection; and migration was assessed as the percentage of the maximum villus height reached by the leading BrdU-labeled enterocyte in each group at each time point. Values are means ± SEM of three separate experiments at each time point with three mice per group. p < 0.05 vs wild-type mice with NEC at 14 h (+) or 24 h (++) of migration by ANOVA.
hypoxia could affect TLR4 expression levels in enterocytes, IEC-6 cells were placed in a hypoxic chamber for varying durations, and the expression of TLR4 was assessed. As shown in Fig. 3D, hypoxic treatment resulted in a marked increase in the expression of TLR4 in IEC-6 enterocytes. This effect was also observed in vivo, because the expression of TLR4 in the intestinal mucosa was significantly increased in newborn mice that were exposed to hypoxia as compared with those that remained under normoxic conditions (Fig. 3E). These findings together indicate that factors that are important in the pathogenesis of NEC, namely, LPS colonization of the intestine and exposure to hypoxia, lead to an increase in the expression of TLR4 and suggest that TLR4 signaling may contribute to the pathogenesis of NEC.

The severity of NEC is significantly reduced in TLR4-mutant mice compared with wild-type counterparts

The pathogenesis of NEC involves the presence of high concentrations of LPS in the setting of a hypoxic injury, circumstances that were observed to lead to an increase in TLR4 expression. To determine the effects, if any, of TLR4 expression in the pathogenesis of experimental NEC, we next examined the extent and severity of NEC that could be induced in animals with and without mutations in TLR4. After the administration of enteral feeds and hypoxic treatment, newborn C3H/HeOUJ mice that express functional TLR4 (hereafter called TLR4-wild-type mice), were found to develop intestinal inflammation and systemic sepsis typical of NEC (Fig. 4, A and D; quantification in Fig. 4G). By contrast, C3H/HeJ mice that express an inhibitory mutation in TLR4 (hereafter called TLR4-mutant mice) demonstrated a marked reduction in the extent and severity of NEC (Fig. 4, B and F; quantification in Fig. 4G). There were no differences between strains after necrotic conditions (Fig. 4, G). By contrast, in TLR4-mutant mice, the incidence of apoptosis was significantly decreased as compared with wild-type mice (compare Fig. 5, D). These findings indicate that functional TLR4 signaling plays a critical role in the pathogenesis of NEC.

TLR4 signaling leads to increased enterocyte apoptosis in vitro and in vivo

To define the mechanisms by which TLR4-mutant mice are protected from the development of NEC, we now hypothesize that TLR4 signaling leads to enhanced small intestinal injury and a loss of mucosal repair mechanisms. Previous authors have demonstrated that the earliest events leading to mucosal injury in experimental NEC involve an increase in enterocyte apoptosis (41). We therefore sought to determine the effects of TLR4 activation on the rates of apoptosis of enterocytes and to assess whether the rate of enterocyte apoptosis was decreased in TLR4-mutant mice. As shown in Fig. 5, A–C, LPS exposure caused an increase in the rate of apoptosis of IEC-6 cells. In TLR4-wild-type mice that were induced to develop NEC, a significant number of enterocytes were found to demonstrate features of apoptosis, as determined by the expression of activated caspase-3, at greater rates than that observed after exposure to control, breast-fed conditions (compare Fig. 5, D and E). By contrast, in TLR4-mutant C3H/HeJ mice, the incidence of apoptosis was significantly decreased as compared with wild-type mice (compare Fig. 5, F and G), and approached that of untreated animals (see Fig. 5C). These findings suggest that TLR4 signaling leads to increased apoptosis of enterocytes in vitro and in vivo, worsening the degree of intestinal injury in the pathogenesis of NEC.

TLR4 signaling leads to a reduction in enterocyte proliferation and migration in vitro and in vivo

Having shown that LPS treatment leads to an increase in enterocyte apoptosis, we next sought to determine whether TLR4 activation affects mucosal repair processes, which could account in part for the increased severity of NEC that develops in TLR4-wild-type mice compared with mutant counterparts. Repair from epithelial damage requires the precise synchronization of enterocyte migration and proliferation (48). As such, disruptions in either migration or proliferation could markedly reduce the capacity for intestinal repair and regeneration. In view of this, we next examined the effects of TLR4 activation on enterocyte proliferation in vitro and in vivo. As shown in Fig. 6A, the exposure of IEC-6 cells to LPS led to significant decrease in enterocyte proliferation. The induction of experimental NEC in TLR4-wild-type mice led to a marked reduction in enterocyte proliferation that was restored in

FIGURE 8. TLR4 activation leads to phosphorylation of FAK in enterocytes. A, SDS-PAGE showing the expression of phospho-FAK (pFAK), total FAK (tFAK), and actin in IEC-6 cells that were exposed to LPS (50 μg/ml) for the time points indicated. Blots were first probed for pFAK and then stripped and reprobed for tFAK and actin. Values are the means ± SEM of pFAK:tFAK band density ratios; *, p < 0.05 vs 0 h by ANOVA of four separate experiments. B–C, SDS-PAGE showing the expression of pFAK and FAK in mucosal scrapings obtained from wild-type mice (B) or TLR4-mutant mice (C) under control conditions (B and C, lanes 1–4) or after the induction of NEC (B, lanes 5–8; C, lanes 5–9). D–E, In vitro interaction of TLR4 and FAK as shown by immunoprecipitation. Cells (1 × 10⁶) were processed in each group. D, SDS-PAGE showing the expression of TLR4 in the following samples: IP-TLR4, IEC6 cell lysate immunoprecipitated with an anti-TLR4 Ab; IP-FAK, IEC6 cell lysate immunoprecipitated with an anti-FAK Ab; −ve, IEC-6 whole-cell lysates immunoprecipitated with irrelevant IgG. Shown also is the expression of TLR4 in an equivalent amount of lysates of J774 cells (macrophages (Mo) as a positive control) and IEC-6 cells. E, SDS-PAGE showing the expression of FAK in the following samples: IP-FAK, IEC6 cell lysate immunoprecipitated with an anti-FAK Ab; IP-TLR4, IEC6 cell lysate immunoprecipitated with an anti-TLR4 Ab; −ve, IEC-6 cell lysate immunoprecipitated with irrelevant IgG. Shown also is the expression of FAK in an equivalent amount of lysates of HEK cells (as a positive control) and IEC-6 cells. Representative of four separate experiments.
TLR4-mutant animals (Fig. 6B; see also Fig. 6, C–F, for representative images). These findings demonstrate that TLR4-dependent signaling leads to an inhibition of one of the major tissue repair processes, namely, enterocyte proliferation, suggesting a mechanism whereby TLR4-mutant animals may be protected from the development of NEC.

Because mucosal healing requires efficient migration of enterocytes from uninjured mucosa to sites of injury, a process termed intestinal restitution, we next sought to assess whether TLR4 could inhibit enterocyte migration. In support of a possible role for TLR4 signaling in the inhibition of intestinal restitution, we have previously shown that the exposure of enterocytes to LPS leads to a dose-dependent inhibition of migration through increased focal adhesion formation leading to enhanced cell-matrix adhesiveness (40). We therefore next sought to assess the in vivo significance of these prior in vitro findings in the context of TLR4 activation. To do so, NEC was induced in both TLR4-wild-type and TLR4-mutant animals, and intestinal restitution was measured as the migration of BrdU enterocytes along the crypt-villus axis, as described in Materials and Methods. As shown in Fig. 7, A, B, and E, intestinal restitution was significantly reduced in TLR4-expressing mice, consistent with the inhibitory effects of TLR4 activation on enterocyte migration. By contrast, the rate and extent of intestinal restitution were significantly increased in TLR4-mutant animals (Fig. 7, C–E) with experimental NEC and reached levels similar to that seen in breast fed controls (Fig. 7E).

To assess the effects of TLR4 signaling on intestinal restitution in greater detail, a kinetic analysis was performed in both TLR4-wild-type and TLR4-mutant animals with and without experimental NEC that were sacrificed 4, 14, or 24 h after the i.p. injection of BrdU, and the percent of maximum villus height attained by the leading BrdU-labeled enterocyte was assessed. As shown in Fig. 7F, wild-type mice demonstrated a steady increase in the degree to which the leading BrdU-labeled enterocyte reached the summit of the villi, which was significantly decreased in wild-type animals with NEC at both 14 and 24 h. By contrast, in TLR4-mutant...
animals with NEC, the extent of enterocyte migration was significantly greater than that of the TLR4-wild-type counterparts and was similar to that of wild-type mice without NEC at each time point. Importantly, the extent of enterocyte migration in TLR4-mutant mice without NEC was similar to that of TLR4-wild-type mice without NEC at each time point studied. Taken in aggregate, these experiments suggest that signaling through TLR4 contributes to the development of NEC in part through enhanced mucosal injury (via apoptosis), and reduced repair capacity (through effects on proliferation and restitution).

**Experimental NEC is associated with TLR-dependent activation of FAK within enterocytes**

In the next series of studies, we sought to investigate the molecular mechanisms that mediate the TLR4-dependent inhibition of intestinal restitution in experimental NEC. Previous studies from our group have demonstrated that the activation of TLR4 in IEC-6 cells by LPS inhibits enterocyte migration in a dose-dependent manner leading to the activation of FAK and a subsequent increase in the formation of focal adhesions (32). We therefore next considered the possibility that TLR4 activation in NEC would lead to an increase in FAK phosphorylation and that FAK activation was required to inhibition enterocyte migration after LPS treatment. As shown in Fig. 8, LPS leads to a time-dependent increase in the phosphorylation of FAK in IEC-6 cells, supporting results from previous studies (32). The induction of experimental NEC led to the phosphorylation of FAK in small intestinal mucosal scrapings of TLR4-expressing mice but not in TLR4-mutant mice (Fig. 8, B and C), suggesting that TLR4 signaling in NEC leads to the phosphorylation of FAK in vivo. This finding raises the intriguing possibility that an interaction between TLR4 and FAK could mediate in part the inhibition of enterocyte migration observed after LPS treatment. In support of this, we detected the expression of TLR4 in IEC-6 cell lysates that had been immunoprecipitated using affinity-purified anti-FAK Abs (Fig. 8D), suggesting an association between TLR4 and FAK. The degree to which this interaction occurred may be inferred from studies in which FAK was detected in IEC-6 cell lysates that had been immunoprecipitated with Abs against TLR4 (Fig. 8E). Neither TLR4 nor FAK was detected in the lysates obtained after immunoprecipitation using an irrelevant IgG (Fig. 8, D and E, lane 3). These findings together now demonstrate a novel link between the expression of TLR4 and FAK, and raise the possibility that these interactions could regulate enterocyte migration.

**TLR4 activation inhibits enterocyte migration in a FAK-dependent manner**

To test directly whether FAK was required for the inhibition of enterocyte migration by LPS, a migration assay was utilized involving a kinetic analysis of IEC-6 cells moving into a scraped wound. This assay allows the tracking of individual migrating cells as opposed to measuring the leading edge of the migrating field. A
The mean migration rate among three individual such experiments is quantified in Fig. 9E, in which the locations at each time point of eight individual cells moving within the x-y plane are identified by the letters a–g. The mean migration rate among three individual such experiments is quantified in Fig. 9H. Pretreatment of cells with ATRA which inhibits the proliferation of several cell types including IEC-6 cells (49–53), and which we found to impair the proliferation of IEC-6 cells by XTT assay (not shown), did not significantly alter the rate of migration compared with untreated cells (see Fig. 9, F and H), suggesting that the effects of LPS on migration are relatively unaffected by effects on proliferation. Treatment of IEC-6 cells with LPS resulted in a significant inhibition of enterocyte migration (see Fig. 9, G and H), consistent with our previous findings (32, 40).

Using this migration assay, we next considered whether inhibition of FAK using siRNA would affect the ability of enterocytes to migrate after treatment with LPS. To do so, IEC-6 cells were transfected with specific siRNA against FAK, which resulted in >80% inhibition of expressed protein, whereas treatment with control siRNA engineered against no known product had no effect (Fig. 10A). As expected, treatment of IEC-6 cells with LPS, or LPS plus control siRNA significantly inhibited enterocyte migration compared with untreated cells (Fig. 10, B, C, and F). Strikingly, treatment of IEC-6 cells that had undergone prior siRNA-mediated knockdown of FAK with LPS significantly reversed the inhibitory effect of LPS on migration (Fig. 10, D and F), whereas treatment of cells with siRNA against FAK alone had no effect on the baseline rate of migration (Fig. 10, E and F). These data now demonstrate a novel link between TLR4 and FAK in the regulation of migration, and they suggest a novel mechanism by which intestinal restitution may be inhibited during conditions of endotoxin exposure such as NEC.

Discussion

NEC is a severe intestinal disorder affecting preterm infants that is characterized by marked destruction of the intestinal mucosa followed by the development of systemic sepsis (4). Although the precise pathways leading to the development of NEC is incompletely understood, evidence points to a clear role for the interaction between the intestinal microbial flora and the host immune system in its pathogenesis. Specifically, the onset of NEC occurs at a time when the intestinal lumen is colonized by Gram-negative flora (54, 55), which usually occurs at ~8–10 days after birth (56–58). Additional evidence for a role for bacteria in the pathogenesis of NEC is found in the fact that NEC outbreaks occur in clusters within neonatal intensive care units in a pattern that is indicative of an infective etiology (59) and that NEC clinically responds to the administration of broad-spectrum antibiotic therapy (60, 61). A specific role for Gram-negative bacterial LPS in the pathogenesis of NEC is supported by the results of studies performed in newborn rats and piglets in which the oral or i.v. administration of LPS in combination with hypoxic treatment was associated with changes in the intestine resembling NEC (62–64), and the finding that high levels of pathogens are detected in the peritoneal cavities of neonates with NEC (65). On the basis of findings, we sought to define a role for the LPS receptor, TLR4, in the pathogenesis of NEC. We now demonstrate that animals expressing wild-type TLR4 developed significantly increased severity of NEC compared with TLR4-mutant counterparts, due to an increase in enterocyte loss by apoptosis and a reduced capacity of the TLR4-wild-type mice to undergo intestinal repair through both decreased proliferation and restitution as compared with TLR4-mutant counterparts. These findings speak to a novel role for TLR4 in regulating the balance between injury and repair in the intestine, and in so doing, in determining the extent of NEC that develops in animals at risk for this disease.

The current study provides novel insights into the role of TLR4 in the pathogenesis of intestinal inflammation and provides a departure from current thinking in this area. Previous authors have demonstrated that TLR4 plays an important role in protecting the host from the development of chemical-induced colonic inflammation through the maintenance of intestinal homeostasis and the production of cytoprotective factors (66–68). However, subsequent studies have demonstrated that TLR4 may play a permissive role in the development of spontaneous colonic inflammation (69), suggesting either that the net effects of TLR4 on colitis are dependent on the specific disease process examined or that the interaction with various downstream effectors influences the extent of intestinal inflammation that develops. The current work would seem to increase the likelihood of these latter possibilities. The inflammation observed in NEC is predominantly localized to the small intestine as opposed to the colon (3, 70), implying that the effects of TLR4 activation within small intestinal epithelial cells may lead to different effects than its role on the colonic epithelium. In support of this, it has previously been demonstrated that small intestinal enterocytes, including IEC-6 cells, are more responsive to LPS than colonic enterocytes, including colonic Caco-2 cells, due in part to differences in TLR4 expression and/or activity (71, 72). Moreover, the increase in expression of TLR4 within the ileum that we have observed after exposure to formula feeding/gavage suggests that TLR4-dependent signaling within the small bowel mucosa may be increased after exposure to these stressors. The combined effects of the enhanced baseline sensitivity of the small intestine to LPS and the up-regulation of TLR4 expression in the intestine on inflammatory cells that migrate to the intestine in response to the various stressors may partially explain the observed effects of TLR4 in the induction of NEC. In support of this possibility, Caplan and colleagues (6) have recently demonstrated that TLR4-expressing mice are more susceptible to the development of NEC in a model of formula feeding and cold asphyxia through a mechanism involving the enhanced interaction with luminal bacteria. The current work provides additional mechanistic insights into these findings.

An important finding of the current study lies in the observation that the cell adhesion protein FAK and the innate immune receptor TLR4 coassociate as determined by immunoprecipitation. At first glance, this finding is rather unexpected, given the apparent disparate roles for these two molecules. However, given the broad roles that FAK exerts within mammalian cells, a FAK-TLR4 association may shed light into the various effects of TLR4 activation in mucosal injury and repair that we now detect. For instance, FAK expression and signaling have been shown to play significant roles in the regulation of apoptosis, migration, and proliferation of a variety of cell types, both under basal conditions and during conditions of inflammatory stress (73–75). Moreover, serine phosphorylation of FAK has been shown to participate in the regulation of migration (76). The finding that activation of enterocyte TLR4 by LPS and in experimental NEC led to an increase in the serine-mediated phosphorylation of FAK (see Fig. 8) is therefore consistent with the novel observation that inhibition of FAK restores the abilities of enterocytes to migrate after TLR4 activation (see Fig. 10). These findings raise the intriguing possibility that the interaction between TLR4 and FAK may also regulate these cellular processes and thereby serve as an important branch point in the signaling events that lead to the development of NEC. In support of this possibility, Zeisel et al. (77) have reported a functional interaction between FAK and MyD88 pathways. The current findings provide additional in vivo relevance to these observations.
Although we now define a role for TLR4 in the pathogenesis of NEC, we are not able to determine precisely whether the effects of TLR4 activation occur at the level of the enterocytes themselves or whether activation of TLR4 on host immune cells or on other cells may be required in the pathogenesis of NEC. We fully acknowledge that the effects of LPS in causing an increase in enterocyte apoptosis and a decrease in enterocyte restitution in vivo may all be indirect effects of TLR4 activation of nonenterocyte populations. And although the current studies provide evidence that levels of LPS are significantly increased in the sera of mice with NEC as compared with control mice (Fig. 3), the possibility exists that the activation of TLR4 within the small intestine occurs through factors other than LPS itself. In this regard, TLR4 has been shown to be activated by a variety of nonbacterial endogenous molecules that are released at inflammatory sites during from dying and injured tissues and therefore may alert the host to the presence of remote injury (78, 79). Such molecules, including fibronectin (80), heat shock proteins (81), and high-mobility group box 1 protein (82), may activate TLR4 to cause the activation of the host immune system and the release of proinflammatory cytokines. Such activation of TLR4 in response to endogenous molecules during stress may explain the observation that the severity of various noninfectious models of critical illness are dependent on the activation of TLR4, including hemorrhagic shock (83–86) and ischemia reperfusion injury (87–88). The relative contribution of endogenous vs exogenous molecules in the activation of TLR4 in the pathogenesis of NEC remains of great scientific interest with respect to unraveling the complex origins of NEC.

In summary, we now provide evidence that TLR4 plays a critical role in the pathogenesis of NEC, by essentially disrupting the balance between mucosal injury and repair within the small intestine. As described in the current findings, we now propose that in response to significant endotoxemic/hypoxic stress, TLR4 expression and signaling are increased in the newborn enterocyte monolayer and/or in immune cells that migrate into the inflamed tissue, rendering the intestine increasingly susceptible to endotoxin upon its subsequent colonization by Gram-negative flora. The resultant activation of TLR4 within the enterocyte tips the balance from intestinal homeostasis toward apoptotic injury, at the same time impairing repair mechanisms through effects on proliferation and migration (Fig. 11). The net effect is the development of intestinal inflammatory changes that characterize NEC. Although further studies are required to pinpoint the precise location at which TLR4 acts and to determine the temporal sequence by which TLR4 activation leads to the development of NEC, these studies provide insights into the development of NEC and provide potentially important therapeutic clues in the management of this devastating disorder.

Disclosures
The authors have no financial conflict of interest.

References


