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The Roles of Bacteria and TLR4 in Rat and Murine Models of Necrotizing Enterocolitis[1]

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Bacteria are thought to contribute to the pathogenesis of necrotizing enterocolitis (NEC), but it is unknown whether their interaction with the epithelium can participate in the initiation of mucosal injury or they can act only following translocation across a damaged intestinal barrier. Our aims were to determine whether bacteria and intestinal epithelial TLR4 play roles in a well-established neonatal rat model and a novel neonatal murine model of NEC. Neonatal rats, C57BL/6J, C3HeB/FeJ (TLR4 wild type), and C3H/HeJ (TLR4 mutant) mice were delivered by Cesarean section and were subjected to formula feeding and cold asphyxia stress or were delivered naturally and were mother-fed. NEC incidence was evaluated by histological scoring, and gene expression was quantified using quantitative real-time PCR from cDNA generated from intestinal total RNA or from RNA obtained by laser capture microdissection. Spontaneous feeding catheter colonization or supplementation of cultured bacterial isolates to formula increased the incidence of experimental NEC. During the first 72 h of life, i.e., the time frame of NEC development in this model, intestinal TLR4 mRNA gradually decreases in mother-fed but increases in formula feeding and cold asphyxia stress, correlating with induced inducible NO synthase. TLR4, inducible NO synthase, and inflammatory cytokine induction occurred in the intestinal epithelium but not in the submucosa. NEC incidence was diminished in C3H/HeJ mice, compared with C3HeB/FeJ mice. In summary, bacteria and TLR4 play significant roles in experimental NEC, likely via an interaction of intraluminal bacteria and aberrantly overexpressed TLR4 in enterocytes. *The Journal of Immunology,* 2006, 177: 3273–3282.

N ecrotizing enterocolitis (NEC)[3] is one of the leading causes of death among premature infants. Although the underlying pathophysiology is poorly understood, several contributing factors, such as undeveloped regulation of intestinal microcirculation, a propensity toward apoptosis of intestinal epithelial cells, an exaggerated inflammatory response, and abnormal bacterial colonization have all been identified as potential flaws in the physiology of the premature intestinal mucosa (1–6). In experimental animal models and some human epidemiologic analyses, formula feeding, intestinal ischemia, and bacterial overgrowth are associated with an increased risk of bowel necrosis (5–7). Furthermore, i.v. platelet-activating factor (PAF) causes severe bowel injury in a well-established acute model of NEC in adult rats, but PAF failed to elicit injury in the same model in germfree rats (8) and NEC does not occur in a sterile environment in utero. All of these data suggest that bacteria might be implicated in NEC pathogenesis, but their exact role and the mechanisms of their effects in NEC are not known.

Microbial products bind to and activate a family of pattern recognition molecules called human TLR, resulting in signal transduction and an inflammatory response. There are more than 10 identified members of the TLR family, with well-defined specificities to various components of bacteria, viruses, or fungi (9). The best characterized member of this family is TLR4, the receptor for LPS, which is the best known and first discovered bacterial cell wall component that can elicit cellular responses. In the healthy adult intestinal epithelium and in intestinal epithelial cell lines, along with TLR2 and TLR6, TLR4 is expressed at very low levels, and their ligands elicit minimal to no cellular responses, thereby reducing the risk of a local inflammatory response resulting from commensal bacterial flora or from negligible amounts of pathogens (10–13). This suppressed TLR expression later in development is likely to be the result of a postnatal adaptive mechanism because there is abundant expression of TLR2 and TLR4 in an enterocyte cell line developed from fetal intestine and on basolateral surfaces of the crypt epithelium in fetal intestinal tissue sections (14). As a functional correlate to these findings, oral administration of LPS to fetal and newborn rats elicits high lethality, whereas 1-mo-old rats are highly resistant to even higher doses of LPS (15). In germfree animals, the adult intestine remains responsive to bacterial products as the introduction of a prototypical commensal bacterium, *Bacteroides thetaiotaomicron*, results in activation of a gene program leading to expression of junctional proteins, nutrient- and transport-related genes, and antimicrobial peptides (16, 17).

Contrary to healthy intestinal development, there is an apparent grossly increased expression of TLR4 in the epithelium of biopsy specimens from ulcerative colitis (UC), Crohn's disease (CD), and in the dextran sodium sulfate (DSS) model of murine colitis (18–20). This change appears to be relatively specific as TLR3 and

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[3] Abbreviations used in this paper: NEC, necrotizing enterocolitis; CD, Crohn's disease; CT, cycle threshold; DSS, dextran sodium sulfate; FFCAS, formula-feeding and cold/asphyxia stress; iNOS, inducible NO synthase; LCM, laser capture microdissection; MF, mother fed; PAF, platelet-activating factor; QRT-PCR, quantitative real-time RT-PCR; UC, ulcerative colitis.

TLR5 remain unchanged or decreased in the inflamed gut epithelium (13, 18–20). These observations suggest that TLRs participate in the pathogenesis in adult inflammatory bowel disease, but their exact roles are not well understood. In the DSS model of colitis, TLR4 knockout mice exhibit increased susceptibility to colitis (21–23), but in mice where the myeloid cell-specific deletion of Stat3 results in spontaneous colitis, the introduction of TLR4$^{-/-}$ genotype protected the animals from colitis (24) and a synthetic TLR4 antagonist reduced inflammation and tissue injury in the DSS model of colitis and in colitis of MDR1a-deficient mice (25). Additionally, although multiple studies analyzing genetic polymorphism drew a correlation between UC and CD incidence and/or severity and certain alleles of TLR4, CD14, and NOD2/CARD15 (26–36), there are considerable discrepancies among these studies regarding the precise association of various alleles with disease phenotype. At least one study analyzed TLR4, CD14, and CARD15 polymorphisms in NEC and found no correlation between disease incidence and/or severity and genotype (37). However, the unusually high incidence of NEC in combination with the very low frequency of mutant alleles in both the control and NEC populations makes it difficult to draw solid conclusions from this study regarding the role of TLR4 polymorphism in NEC.

To test the hypothesis that inappropriate host pathogen interactions and consequent innate immune signaling and inflammation contribute to the pathogenesis of NEC, we performed experiments in a well-established neonatal rat model and a novel neonatal murine model of NEC. Herein, we demonstrate the role of exogenous bacteria on the incidence of NEC in a neonatal rat model, the normal developmental regulation of intestinal TLR4 gene expression, and the effects of formula feeding and asphyxial stress on TLR4, inducible NO synthase (iNOS), and Gro/Cinc (i.e., the rat equivalent of human IL-8) expression. Furthermore, to investigate the specific role of TLR4 in experimental NEC, we developed a novel neonatal murine model of NEC, which allowed us to demonstrate a dramatic decrease of inflammation and NEC incidence in animals carrying a function-limiting mutation in TLR4. Our data strongly suggest that formula feeding and hypoxia stress in combination with exposure to exogenous bacteria are prerequisites for the activation of inflammation that causes NEC and that an abnormal increase of intestinal epithelial TLR4 expression plays a significant role in this response.

Materials and Methods

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Neonatal rat model

Neonatal rats were delivered from time-dated pregnant Sprague-Dawley dams by Cesarean section at E21 following isoflurane anesthesia to prevent any exposure to maternal milk feeding. The pups were stabilized, dried, and maintained in an incubator at 37°C, and bowel/bladder function was stimulated by a soft cotton-tip applicator every 3 h. Asphyxia stress was accomplished by exposure to 100% nitrogen for 60 s, followed by exposure to cold (4°C) for 10 min twice daily, as described previously (7). Neonatal rats were fed Esbilac puppy formula (~200 kcal/kg/day) every 3 h via an orogastric feeding catheter. The feeding volume began at 0.1 cc every 3 h and was increased incrementally to account for somatic growth. Animals were randomized to feeds with sanitized vs colonized feeding catheters. Sanitized catheters were washed thoroughly before and after each feed using a solution of equal parts 0.5% Triton X-100 and normal saline, rinsed with sterile water, followed by 70% ethanol, then were dried. Colonized catheters were not washed, but rather left in standing water between feeds throughout the protocol. An additional set of animals were treated with exogenous bacteria via sanitized feeding catheter as described below. Animals were euthanized at 24, 48, and 72 h of life, then intestines were collected, linearized, and cut into pieces representing duodenum, ileum, jejunum, and colon. Animals that died before 24 h of life were excluded from our analysis, because based on our extensive experience with this

model, NEC-like histology does not develop before 24 h of life. On this basis, we excluded 4 of 69 animals in the clean catheter group, 1 of 75 animals in the contaminated catheter group, 3 of 27 in the *Serratia marcescens* group, 1 of 27 in the *Klebsiella pneumoniae* group, and 2 of 26 in the viridans streptococci group. From some experiments, the sections were snap frozen for RNA isolation; from others the sections were further cut up in shorter pieces and frozen in OCT in a manner that resulted in a series of intestinal cross-sections along the intestinal tract.

Neonatal mouse model

The neonatal mouse model was developed to directly evaluate the effect of specific genes on the incidence of NEC. For this study, we evaluated the effect of TLR dysfunction by using the mutant strain C3H/HeJ (TLR4 mutant) and comparing to C3HeB/FeJ (control) wild-type mice. Cesarean section was performed between E20–21 using isoflurane anesthesia. Animals were recovered for 2 h and then fed every 2 h starting with 0.03 ml, increasing to 0.04 ml in the subsequent 24 h (to deliver ~200 kcal/kg/day). All animals were fed with feeding catheters that were not sanitized and stressed with the same protocol as the neonatal rats (100% nitrogen and cold stress). Animals were euthanized at 72 h, or earlier if demonstrating signs of distress, and the intestines were collected in 10% formalin and processed for H&E staining or were embedded in OCT compound for cryosectioning.

Microbiologic analyses

Bacterial colonization of feeding catheters was analyzed via standard techniques in the microbiology laboratory at our institution. Routinely, the catheters we called contaminated were soaked between feedings in 10 ml of deionized water during the 72-h duration of the experiment. By 24 h of the experiment, the water became turbid. The water that was used to store contaminated catheters was streaked onto Blood Agar, MacConkey, and Chocolate Agar plates (Remel) using a standard bacteriological loop (~10 μl). Colony density was evaluated on the 0–4+ quadrant scale, where 0 represents no growth and 1–4+ represents >10 colonies present in the highest quadrant showing growth. Three predominant bacterial species were identified by standard bacteriological methods (38) (Table I). Isolated colonies were aseptically transferred to Luria-Bertani growth medium without antibiotics and incubated at 37°C overnight. Standard suspensions of organisms in Luria-Bertani medium were prepared using OD readings. Quantities of organisms were confirmed by hemocytometer counting and by plating dilutions on blood agar followed by colony counting. For animals challenged with exogenous bacterial isolates (viridans streptococci, *Klebsiella pneumoniae*, or *Serratia marcescens*), fresh bacteria were cultured daily before feeding, were washed, quantified by plating dilutions, and given via the orogastric feeding catheter at 10^8 CFU/feeding/animal.

Sample collection and tissue processing

Intestine was processed immediately: half for H&E histology and tissue injury scoring or with OCT for slide preparation and hematoxylin staining for laser capture microdissection (LCM), and the other half were prepared for RNA extraction. H&E-stained histological sections were analyzed, and tissue injury scores were assigned as stage 0–4 by a blinded investigator based on a scoring system that we described previously (3). Based on our earlier studies, a score of >1 is clearly associated with NEC. Data shown are the number of specimens with a score < 2 as –NEC and a score ≥ 2

Table I. *Dominant bacterial isolates from contaminated catheters^a*

Animal Study No.	K. pneumoniae	S. marcescens	Viridans Streptococci
419		4+	3+
420	2+	1+	3+
421	1+	2+	4+
422		3+	4+
423		1+	2+
424	1+	1+	2+
425	1+	1+	2+

^{a} Data shown are colony growth evaluations of agar plate cultures from individual experiments. Catheters in the colonized catheter group were soaking in water between feedings. At the end of experiment (72 h), a sample of the water was collected and plated on agar plates as described in *Materials and Methods*. Colonization was evaluated by subjective evaluation of colony density on plates by a qualified blinded observer on a 0–4 quadrant scale. Colonies were then subjected to secondary evaluation to identify bacterial species.

Table II. *The incidence of NEC in formula-fed, stressed neonatal rats fed with sanitized and colonized catheters[a]*

	+NEC	−NEC	No. of Animals (n)	Rate per 100 (%)
Sanitized catheter	13	51	64	20.3
Colonized catheter	39	35	74	52.7

[a] Data shown are the number of animals that were identified (+NEC) and (−NEC) in each group, followed by group totals and the percent (+NEC) incidence. Formula-fed neonatal rats were cold/asphyxia stressed twice daily and were assigned into groups as indicated by line. NEC was evaluated based on previously published histological criteria. Groups were statistically significantly different at $p < 0.001$ (χ^2 test).

as +NEC. For LCM, all specimens were frozen in cryomold in ethanol dry ice bath and stored at −80°C until slicing. Following cryosectioning to 8 μm, sections were captured on RNase-free slides and placed immediately on dry ice. Staining and dehydration are performed using LCM-certified solutions provided in the Histogene frozen section staining kit (Arcturus). Briefly, slides are fixed and dehydrated with solutions of 75% ethanol, 95% ethanol, 100% ethanol, and xylene and stained with RNase-free staining solution that contained hematoxylin only. The tissue for RNA analysis was processed after separation into four intestinal segments. By embedding carefully arranged segments looped over a string, we were able to obtain 20–30 cross-sections along the intestinal tract, allowing further localization of gene expression and injury.

Laser capture microdissection

The LCM was done using PixCell system and CapSure LCM caps (Arcturus). Cells were isolated from villi, crypts, and submucosa immediately after staining of tissue sections. Approximately 500 cells were isolated from each specimen, then RNA was extracted through a series of steps according to PicoPure RNA isolation kit protocol (Arcturus).

Quantitative real-time RT-PCR (QRT-PCR)

Transcript levels were determined using QRT-PCR and *GAPDH* as a housekeeping gene reference. Primers and probes used: rat TLR4 primer and probe sets were designed using the Primer Express software from ABI (sense: ATCATCCAGGAAGGCTTCCA; antisense: GCTGCCTCAGCAAGGACTTCT, probe: 6FAM-AGAGCCGGAAAGTTAT). All other primers and probes were ABI TaqMan gene expression assays. All probes were 6FAM-labeled MGB probes, except the GAPDH probes, which were VIC labeled. In initial experiments, we used both actin and GAPDH as housekeeping controls until we reassured ourselves that both housekeeping genes provided identical results. When using intestinal total RNA where RNA quantification was very accurate, for both housekeeping genes the CT values showed very small variability. The various treatment groups were compared with respect to GAPDH copy numbers and there were no consistent patterns of changes observed among groups. For LCM samples, where input RNA quantities cannot be quantified readily, we observed a greater variability of housekeeping gene cycle threshold (CT) values, but we did not find any specific pattern in the changes that would have indicated a condition-dependent change. For subsequent experiments, GAPDH

Table III. *The incidence of NEC in neonatal rats fed with sanitized feeding catheters or sanitized feeding catheters and formula supplemented with cultured isolates from contaminated catheters[a]*

	+NEC	−NEC	No. of Animals (n)	Rate per 100 (%)
Sanitized	13	51	64	20.3
S. marcescens	14	10	24	58.3
K. pneumoniae	11	15	26	42.3
Viridans streptococci	8	16	24	33.3

[a] Data shown are the number of animals that were identified (+NEC) and (−NEC) in each group, followed by group totals and the percent (+NEC) incidence. Formula-fed neonatal rats were cold/asphyxia stressed twice daily and were assigned into groups as indicated by line. Rows 2–4 represent animals fed using sanitized catheters and formula supplemented with the indicated isolates (10^8 CFU/feeding/animal). NEC was evaluated based on previously published histological criteria. Groups were statistically significantly different as follows: overall $p < 0.05$ (χ^2). Pairwise post analysis vs sanitized: *S. marcescens*, $p < 0.005$; *K. pneumoniae*, $p < 0.05$; viridans streptococci, n/s.
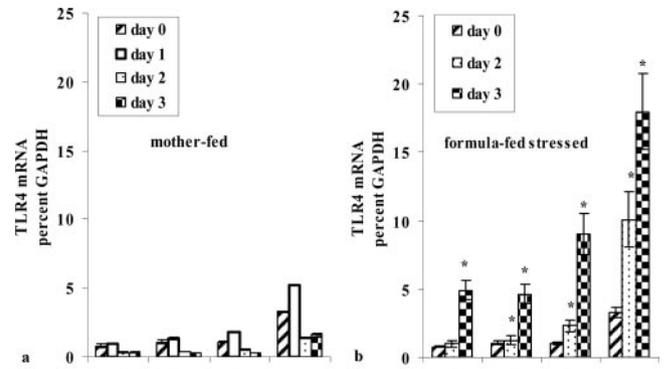

FIGURE 1. TLR4 mRNA expression levels in intestinal segments of MF control (*a*) and formula-fed hypoxia-stressed (*b*) neonatal rats. Following total RNA isolation from duodenum (d), jejunum (j), ileum (i), and colon (c) at days 0, 1, 2, and 3 in MF animals and on days 0, 2, and 3 in FFCAS animals, then cDNA was synthesized, and copy numbers of TLR4 and GAPDH were determined using QRT-PCR. Data shown are mean ± SEM TLR4 mRNA copy numbers expressed as percent GAPDH; each column representing at least $n = 5$ animals and duplicate measurements. ∗ denotes statistically significant difference from MF control at $p < 0.01$.

was used as reference. RNA was isolated from tissue samples, using RNA STAT-60 protocol (Tel-Test), or from laser-captured cells, using the PicoPure RNA Isolation kit. RNA was quantified, and integrity was verified by agarose gel electrophoresis, except for the LCM samples, where RNA integrity was judged by a maximum CT value of <34 for GAPDH. cDNA was prepared by reverse transcription from 1 μg of RNA in a 40-μl reaction from tissue RNA and from the entire 10-μl elution volume from LCM RNA, followed by PCR. Each QRT-PCR contained 1 μl of cDNA, 100 nM each of forward and reverse primers, 300 nM sequence detection (MGB) probes, and 12.5 μl of universal PCR master mix (Applied Biosystems) in a reaction volume of 25 μl. Real-time PCR from tissue samples were preformed in a Prism 9600 SDS (Applied Biosystems) and from LCM samples in a SmartCycler (Cepheid) or a Roror-Gene 3000 (Corbett Robotics). Fluorescence intensity was monitored and recorded in real time, and CT values were determined using the same background subtraction and threshold values for all reactions. For each primer and probe set, we generated 10-fold dilutions of a pooled cDNA sample that was abundant in the respective cDNA, and we performed real-time PCR. The CT values were plotted vs the dilution factor, and we have found that there was a perfect linear relationship between 2^{-T} and the dilution factor for all of our reactions at CT values < 38, above which CT the detection lost linearity. For TLR4 and GAPDH reactions, a standard curve was prepared by 10-fold
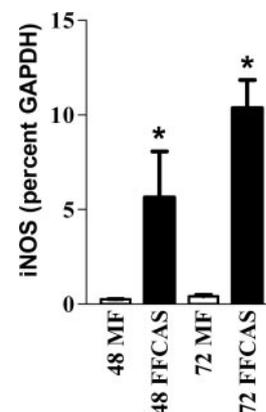
FIGURE 2. Increased iNOS expression in the ileum of formula-fed, stressed (FFCAS) neonatal rats. Following total RNA isolation from the ileum at days 2 and 3, cDNA was synthesized, and copy numbers of iNOS and GAPDH were determined using QRT-PCR. Data shown are mean ± SEM. iNOS mRNA copy numbers expressed as percent GAPDH; each column representing at least $n = 5$ animals and duplicate measurements. ∗ indicates statistically significant difference from MF control at $p < 0.01$.

FIGURE 3. LCM from ileal sections. From a string of intestinal cross sections we selected areas immediately adjacent to the first section that showed morphology of the colon and collected ~500 cells each from villi, crypts and submucosa. Panels shown are section before LCM, remaining section after LCM and LCM samples in caps. Each sample was subjected to micro RNA isolation, cDNA synthesis and QRT-PCR analysis of TLR4 and GAPDH.
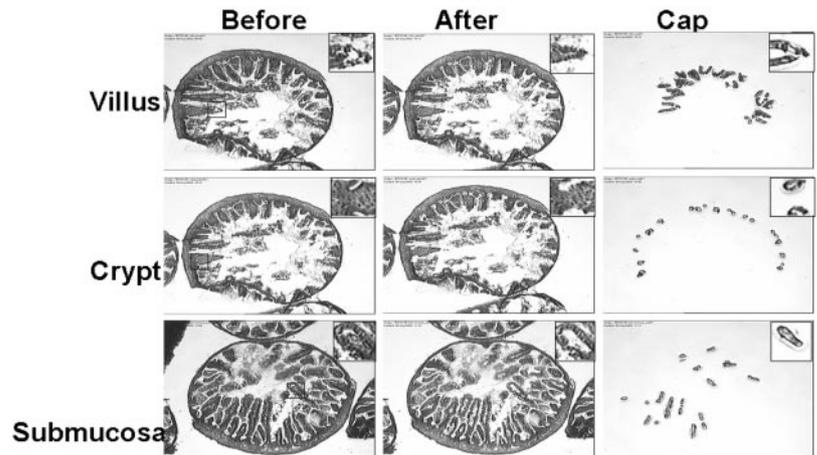
dilutions of cDNA with known copy numbers. Copy numbers were calculated for each PCR target from the same cDNA, and genes of interest were normalized to copy numbers of GAPDH. For iNOS, copy numbers were not determined, but expression values were expressed as percent GAPDH using the formula: $2^{-CT\ iNOS}/2^{-CT\ GAPDH} \times 100$.

Indirect immunohistochemistry

Frozen sections (4–6 μm thick) of OCT-embedded intestines were generated as described above before application of standard indirect immunohistochemistry procedures. All procedures were done at room temperature unless otherwise specified. In brief, cells were fixed with mixed methanol and acetone (1/1) at $-20°C$ for 10 min and subsequently permeabilized with PBST (PBS with 0.1% Tween 20) for 20 min. For detection of TLR4, samples were incubated for 1 h with a blocking solution (PBST with 2%BSA and 5% chicken serum), followed by incubation with goat polyclonal IgG against TLR4 (1/25 dilution; Santa Cruz Biotechnology) or with nonimmune goat IgG. Samples were then incubated for 1 h with Alexa Fluor 488-conjugated chicken-anti-goat IgG (H+L) (1/100; Molecular Probes). Slides were washed three times with PBST between each procedure. They were finally incubated with Hoechst solution (1 μg/ml) for 5 min and mounted with coverglass using anti-fade mounting medium (Invitrogen Life Technologies). Preparations were examined on the stage of an Olympus IMT-10 microscope equipped for epifluorescence and the appropriate filters for Alexa Fluor 488 and Hoechst. Images were collected using a cooled charge-coupled device camera and were relayed to IP Lab Spectrum software. Images were taken with the same exposure setting for control and experimental specimens, and both sets of images were subjected to the same background correction and contrast enhancement settings. Corresponding Hoechst and Alexa Fluor images were pseudocolored and overlaid using IPLab Spectrum standard functions.

Statistical analysis

In studies measuring gene expression in vivo that demonstrate parametric statistics, ANOVA is used with Tukey's posthoc to analyze intergroup differences. In studies that evaluate different conditions on incidence of NEC, χ^2 was performed. For all studies, $p < 0.05$ is considered significant.

Results

Orogastric feeding tube contamination increases the incidence of NEC in rats

To begin investigating the role of bacteria in experimental NEC, we performed experiments in a neonatal rat model of NEC under different sanitary conditions. For one set of animals, the feeding catheters were not sanitized between feedings, while for another set of animals, the feeding catheters were extensively washed and rinsed with 70% ethanol between feedings. We have found significantly different incidences of NEC in these two groups of animals. In animals that were fed with unsanitized catheters, the incidence of NEC was similar to the incidence that was described for this model previously (53%; Table II). In animals that were fed with sanitized catheters, the incidence of experimental NEC was dra-

matically decreased (20%; Table II, $p < 0.001$, χ^2). To further identify specific bacteria involved in catheter contamination and NEC, we analyzed the catheter water in which the feeding catheters of the nonsanitized group were maintained between feedings. The quantification of bacteria found in the catheter water is shown in Table I. The predominant organisms were identified from seven independent experiments as *Klebsiella pneumoniae*, *Serratia marcescens*, and viridans streptococci. These bacteria were then isolated, cultured, and quantified, and the individual species were fed to various groups to distinguish their independent contribution to NEC and to allow exact dosing of bacteria. As shown in Table III, there was an independent and statistically significant increase in NEC scores for Gram-negative organisms as compared with the group that was fed using the sanitized catheters. *S. marcescens* and *K. pneumoniae* had 58 and 42% incidence of histologic NEC, respectively, when compared with the sanitized group of 20% under equal conditions (χ^2, $p < 0.05$). In case of the Gram-positive viridans streptococci, the proportion of animals exhibiting pathological histology was between the proportions in the clean catheter group and the proportion of pathology observed in animals fed
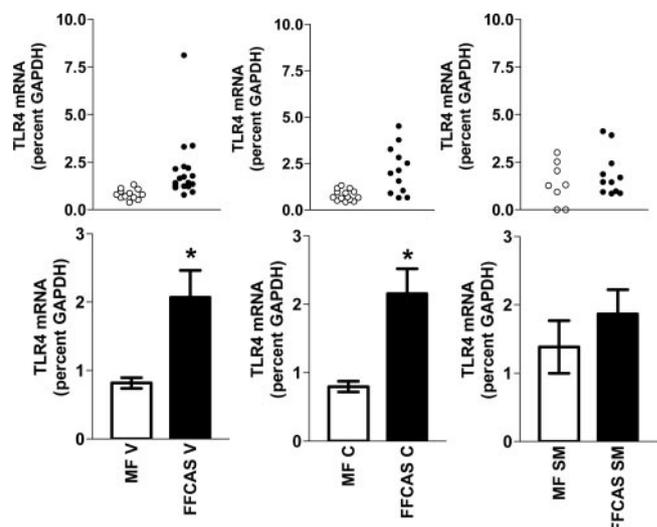
FIGURE 4. QRT-PCR analysis of TLR4 mRNA levels from LCM specimen. RNA was obtained from LCM samples as shown in Fig. 3. Following cDNA synthesis, TLR4 and GAPDH copy numbers were determined and TLR4 expression was expressed as percent GAPDH. *Top panels*, Scattergrams of independent measurements; *bottom panels*, mean ± SEM in villi (V), submucosa (SM), and crypts (C). *, Significant difference at $p < 0.05$

with Gram-negative organisms. Since there was no statistical significance observed between the Gram-positive group and either of the other extremes, we cannot prove or exclude some contribution by Gram-positive organisms in NEC pathology.

Formula-feeding and cold/asphyxia stress (FFCAS) results in increased TLR4 expression in the intestine

Since formula feeding using contaminated catheters does not result in experimental NEC without cold asphyxia stressing in this model (data not shown), we hypothesized that cold asphyxia stressing sensitizes the intestine to bacterial challenge, perhaps by stimulating the expression of TLR. To test this hypothesis, we analyzed mRNA levels of TLR4, the receptor for LPS, in mother-fed (MF) control vs FFCAS animals in different segments of the small and large intestine (duodenum, ileum, jejunum, and colon) and at different ages postpartum. Analysis of gene expression along the intestinal tract revealed a general trend to increased TLR4 expression levels toward the distal part of the gastrointestinal tract (Fig. 1). Our developmental analysis has shown a transient rise of TLR4 mRNA at 24 h in our MF animals and then a decline in expression over time (Fig. 1a). Conversely, FFCAS animals demonstrated a sustained increase in TLR expression from birth through 72 h in all segments and exhibited greater values in the distal gut (Fig. 1b).

iNOS is up-regulated in FFCAS

One of the potential downstream effectors of TLR4 activation is iNOS. Given the very low baseline expression and high-level induction of iNOS upon stimulation with LPS, iNOS mRNA is a very sensitive marker and a well-recognized mediator of injury. Analysis of mRNA from the ileal samples of MF control and FFCAS animals revealed a high level induction of iNOS upon formula-feeding and cold/asphyxia stressing (Fig. 2), consistent with the notion that the increased TLR4 expression in the presence of bacteria results in increased downstream gene expression.

LCM identified the epithelial layer as the site of FFCAS-induced TLR4 expression

Since gene expression data from whole intestinal homogenates reflect a compound expression level across the epithelium, submucosa in-

flammatory cells, and the muscle wall, from this data the specific site of change cannot be identified. To test the hypothesis that an interaction of bacteria with TLR expressed on the epithelial surface is an important contributor to pathogenesis, we performed LCM to separately evaluate gene expression in the epithelial layer and in the submucosa of MF control and FFCAS intestine. This analysis was performed with great care to ensure collection of samples only from the terminal loop of the ileum, given the segment-specific differences in gene expression that we observed upon analysis of TLR4 expression from intestinal homogenates, and because the terminal ileum is the predominant site of injury in NEC. To document the area of analysis, Fig. 3 depicts images before and after collection of samples via LCM and the collected tissue samples in the LCM cap. The caveat with LCM analysis in this model is that we were able to obtain good quality RNA from all three target areas (i.e., villi, crypts, and submucosa) only from animals with intact or almost intact morphology. Consequently, it is likely that our analysis grossly underestimates the maximum changes of gene expression that may occur during or immediately prior to the gross morphological changes. Analysis of villus and crypt enterocytes using LCM showed that TLR4 mRNA significantly increased from MF control animals to FFCAS neonates (Fig. 4). Unlike in the epithelial layer, TLR4 gene expression remained unchanged in the submucosa of FFCAS ileum when compared with MF.

Increased iNOS and Gro/CINC expression correlates with increased TLR4 expression in the villus epithelium

Similar to our studies on TLR4, we used LCM to quantify iNOS mRNA from villi, crypts, and submucosa to collect information regarding the prevalent area of iNOS production in this model (Fig. 5) and to correlate the sites of TLR4 and iNOS expression. Since iNOS is a gene that is induced downstream of TLR4 activation, we expected to find elevated iNOS levels in the epithelium, i.e., the site of increased TLR4 expression. As we expected, we found a significant increase of iNOS expression in the ileal villus epithelium but not in the submucosa. Interestingly, iNOS expression remained unchanged in the crypt epithelium, perhaps due to a limited access of bacteria to these cells. Gro/CINC, a rat functional
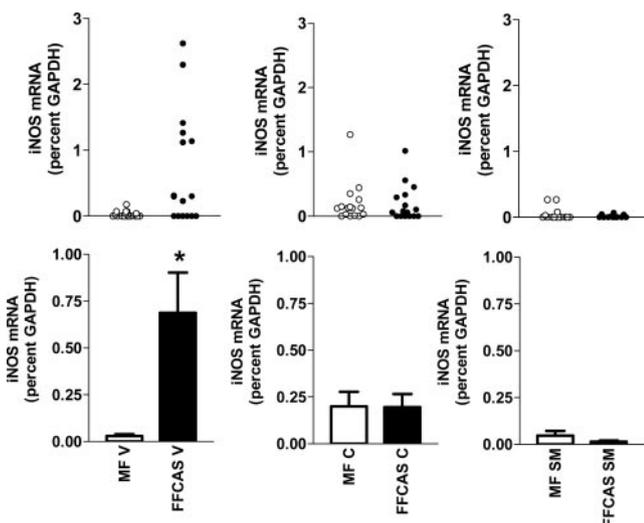
FIGURE 5. Analysis of iNOS expression in rat intestinal villus and crypt epithelium and in the submucosa using LCM. RNA was obtained from LCM samples as shown in Fig. 3. Following cDNA synthesis, iNOS and GAPDH copy numbers were determined and iNOS expression was expressed as percent GAPDH. *Top panels*, Scattergrams of independent measurements; *bottom panels*, mean ± SEM in villi (V), submucosa (SM), and crypts (C) ∗ depicts statistically significant difference at $p < 0.01$.
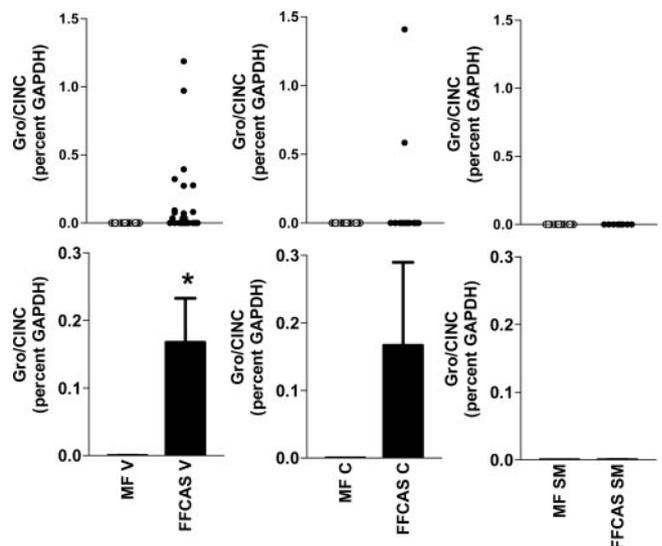
FIGURE 6. Analysis of Gro/CINC expression in rat intestinal villus and crypt epithelium and in the submucosa using LCM. RNA was obtained from LCM samples as shown in Fig. 3. Following cDNA synthesis, Gro/CINC and GAPDH copy numbers were determined, and Gro/CINC expression was expressed as percent GAPDH. *Top panels*, Scattergrams of independent measurements; *bottom panels*, mean ± SEM in villi (V), submucosa (SM), and crypts (C). ∗ depicts statistically significant difference at $p < 0.05$.

FIGURE 7. Influence of TLR4 genotype on murine experimental NEC and localization of TLR4 in murine intestine. Mouse pups were either MF or were formula-fed and cold asphyxia stressed, then intestines were collected and fixed in formaldehyde as described in *Materials and Methods*. Following embedding in paraffin and sectioning, sections were processed for H&E staining (*a–c*), or following embedding in OCT, frozen sections were prepared then processed of anti-TLR4 immunohistochemistry (*d–f*). To illustrate the effect of TLR4 genotype on NEC, shown are representative areas of the ileum from sections of samples collected at 72 h from MF C3HeB/FeJ (TLR4 wild-type) mice (*a*), formula-fed, stressed C3HeB/FeJ mice (*b*), and formula-fed, stressed C3H/HeJ (TLR4 mutant) mice (*c*). Quantitative assessment of a large number of such preparations is shown in Table IV. *d–f*, Results of indirect immunohistochemistry staining of TLR4 in C3HeB/FeJ mice using a goat polyclonal Ab to murine TLR4 (*a* and *b*) or using nonimmune goat IgG (*c*). Nuclei were stained with Hoechst dye. Black-and-white gray-scale images were collected, then were merged into a pseudocolor overlay: blue = nuclei, green = TLR4. C indicates the area of crypts, V marks villi, and arrowheads point to white = solitary epithelial cell apical membrane staining, red = intensely stained cells in the submucosa, and yellow = continuous apical membrane staining.
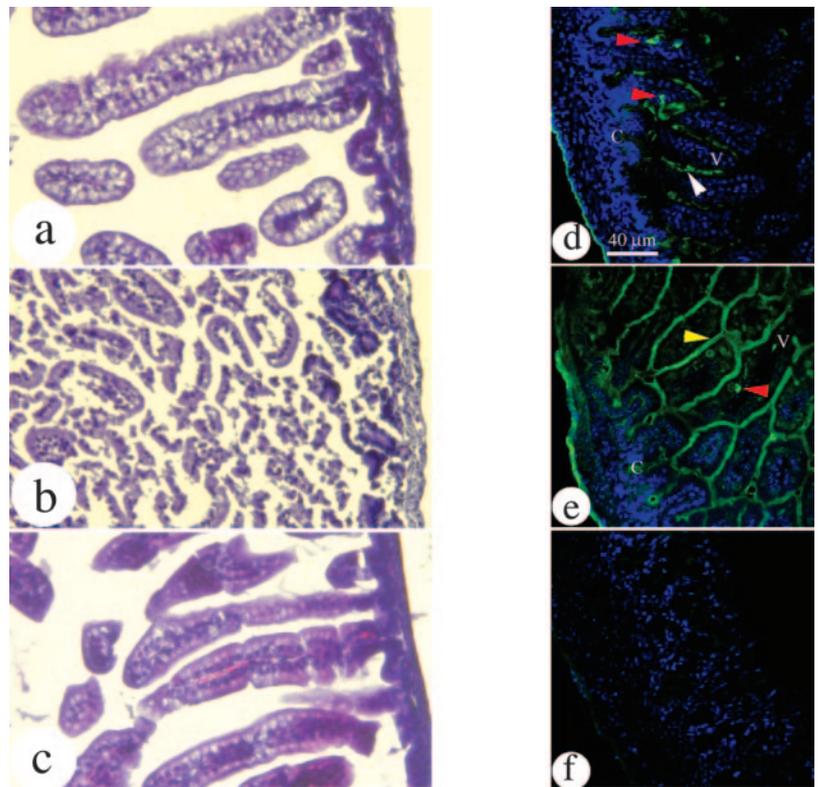
equivalent of human IL-8, is regulated similarly to iNOS upon TLR4 activation and has been implicated in inflammatory bowel disease and NEC. Analysis of Gro/CINC mRNA levels from LCM samples revealed changes closely resembling changes in iNOS expression, showing a marked increase of Gro/CINC expression in the villus epithelium in formula-fed, stressed rat pups (Fig. 6). Similar to our data on TLR4, our data on iNOS and Gro/CINC are likely to underestimate maximum levels of gene expression changes due to our inability to collect high-quality RNA from the more severely affected animals using LCM.

Reduced incidence of NEC in TLR4 mutant mice in a novel murine model of NEC

To evaluate the role of specific gene products in neonatal NEC and because of the poor availability of knockout/mutant rats, we developed a neonatal mouse model of NEC based on our well-described neonatal rat model. Despite the small size of neonatal mice, we were able to pass an orogastric feeding tube (1.9 F percutaneous central line tubing, NeoPICC) into the stomach and feed animals puppy formula to approximate reasonable nutrition. Using the asphyxia/formula-feeding stress, by 48–72 h of life, some animals developed abdominal distention, cyanosis, respiratory distress, and pallor and exhibited gross and microscopic evidence of intestinal necrosis. In the first series of 50 C57BL/6J mice challenged with this protocol (from 8 separate litters), we found an incidence of NEC of 66% (33 of 50 histological NEC score > 1). Following development of the model with consistent results, we used this model to determine whether TLR4 plays a significant role in experimental NEC, as suggested by the increased TLR4 expression and the downstream effector iNOS expression in the ileal epithelium in the neonatal rat model. We obtained endotoxin-resistant mice that harbor a function-limiting mutation in the *TLR4* gene (C3H/HeJ) and compared their incidence of NEC to controls (C3HeB/FeJ). Groups that were analyzed were of similar mean birth weight; C3HeB/FeJ mean birth weight of 1.33 g and C3H/

HeJ mean birth weight of 1.29 g. Histologically, there was a dramatic decrease in NEC in the TLR4 mutant mice when compared with controls (Fig. 7, *a–c*), 5 of 43 (12%) C3H/HeJ vs 16 of 26 (62%) C3HeB/FeJ controls ($p < 0.01$, see Table IV). To evaluate TLR4 localization, we performed indirect immunohistochemistry in tissue sections from the ileum of MF and formula-fed, stressed neonatal C3HeB/FeJ mice using an Ab to murine TLR4. As shown in Fig. 7, *d–f*, TLR4 is localized predominantly to the apical membranes of enterocytes, and there is an apparent increase of TLR4 immunoreactivity in samples from formula-fed, stressed animals.

Analysis of gene expression in wild-type and TLR4 mutant mice using LCM and QRT-PCR

Although it has proven to be significantly more difficult than in samples from rat, we collected sufficient quantity of RNA from LCM samples of villi, crypts, and submucosa of MF and formula-fed, stressed C3H/HeJ and C3HeB/FeJ mice for analysis with QRT-PCR. We have found that similar to rats, in C3HeB/FeJ mice, there was a significantly increased TLR4 expression in the villous and crypt epithelium but not in the submucosa of formula-fed, stressed pups (Fig. 8, *top panels*). Interestingly, there was no significant change of TLR4 gene expression in C3H/HeJ mice upon

Table IV. *The incidence of NEC in neonatal TLR4 mutant mice compared with mice with wild-type TLR4[a]*

|	+NEC	−NEC	No. of Animals (*n*)	Rate per 100 (%)
C3H/HeJ	5	38	43	12
C3HeB/FeJ	16	10	26	62

[a] Data shown are the number of animals that were identified (+NEC) and (−NEC) in each mouse strain, followed by group totals and the percent (+NEC) incidence. Formula-fed neonatal mice were cold/asphyxia stressed twice daily and were evaluated for NEC based on previously described scoring of H&E-stained sections. Value of $p < 0.0001$ (χ^2).

FIGURE 8. QRT-PCR analysis of murine TLR4 mRNA levels from LCM specimens. RNA was obtained from LCM samples, and following cDNA synthesis, TLR4 and GAPDH copy numbers were determined, then TLR4 expression was expressed as percent GAPDH. *Top panels*, Mean ± SEM in villi (V), crypts (C), and submucosa (SM) from MF and FFCAS C3HeB/FeJ mice; *bottom panels*, mean ± SEM in villi (V), crypts (C), and submucosa (SM) of MF and FFCAS C3H/HeJ, TLR4 mutant mice. *, Significant difference at $p < 0.05$
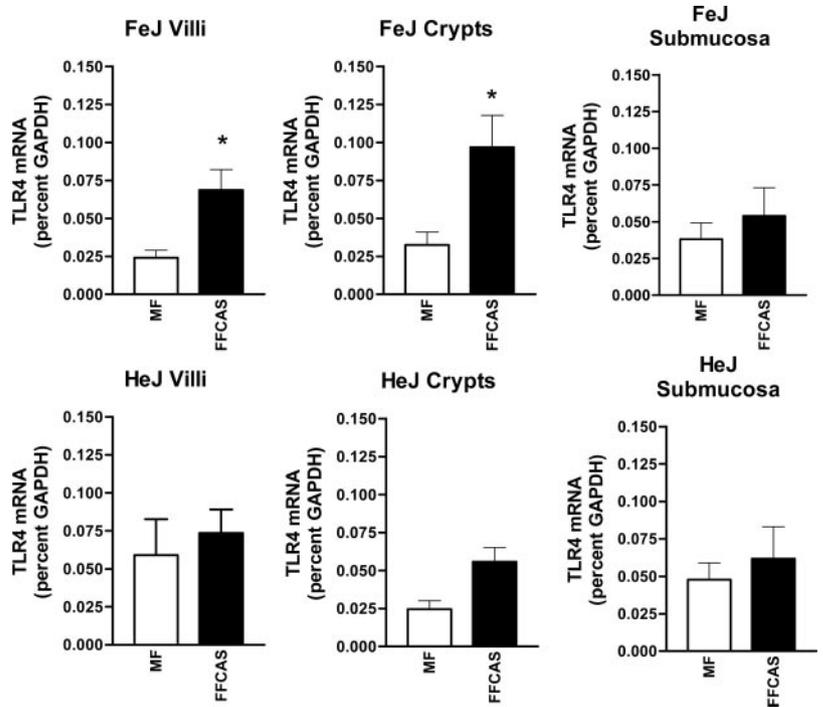
formula-feeding and stress, as compared with MF animals (Fig. 8, *bottom panels*). Since TLR5 has been implicated in the pathogenesis of inflammatory bowel diseases, we evaluated TLR5 expression from murine intestinal LCM samples. We found that TLR5 is expressed at a high constitutive level in all three types of cells (V, C, and SM) that we collected with LCM, its expression level is >2 orders of magnitude higher than TLR4, and that TLR5 mRNA levels did not differ either among mouse strains or as a consequence of formula-feeding and hypoxia stress (Fig. 9). When we analyzed iNOS and CXCL1 (functional analog of human IL-8 and rat Gro/CINC) in murine LCM samples, we found that the expression levels of both were highly variable and exhibited no significant
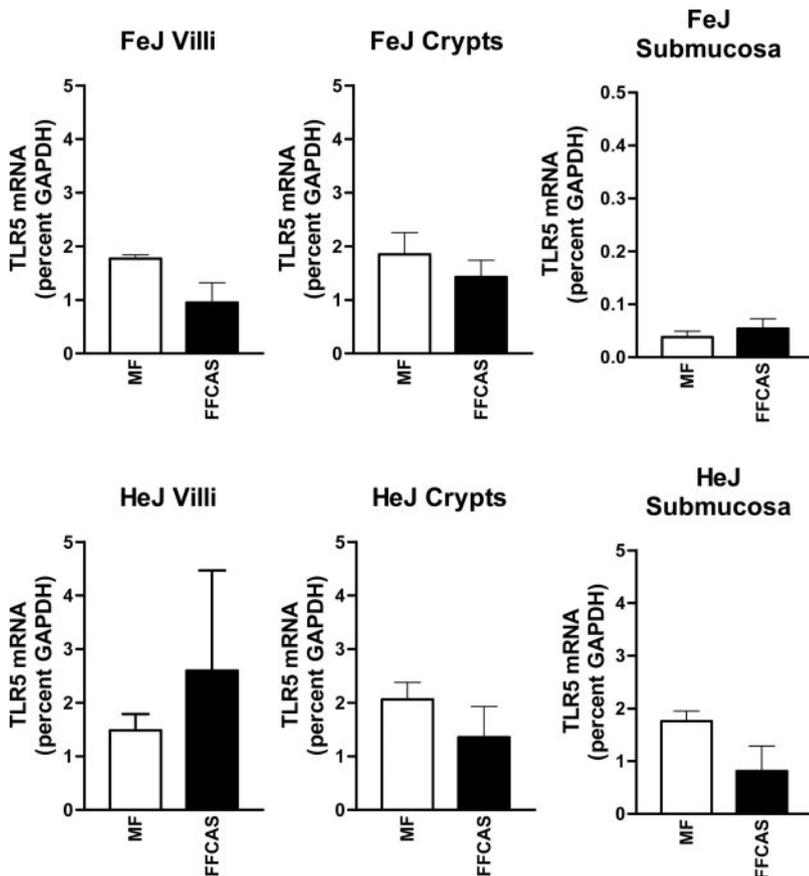
FIGURE 9. QRT-PCR analysis of murine TLR5 mRNA levels from LCM specimens. RNA was obtained from LCM samples, and following cDNA synthesis, TLR5 and GAPDH copy numbers were determined, then TLR5 expression was expressed as percent GAPDH. *Top panels*, Mean ± SEM in villi (V), crypts (C), and submucosa (SM) from MF and FFCAS C3HeB/FeJ mice; *bottom panels*, mean ± SEM in villi (V), crypts (C), and submucosa (SM) of MF and FFCAS C3H/HeJ, TLR4 mutant mice.

FIGURE 10. QRT-PCR analysis of murine TNF-α mRNA levels from LCM specimen. RNA was obtained from LCM samples, and following cDNA synthesis, TNF-α and GAPDH copy numbers were determined, then TNF-α expression was expressed as percent GAPDH. *Top panels*, Mean $\pm$ SEM in villi (V), crypts (C), and submucosa (SM) from MF and FFCAS C3HeB/FeJ mice; *bottom panels*, mean $\pm$ SEM in villi (V), crypts (C), and submucosa (SM) of MF and FFCAS C3H/HeJ, TLR4 mutant mice. $*$, Significant difference at $p < 0.05$; $**$, significant difference at $p < 0.01$.
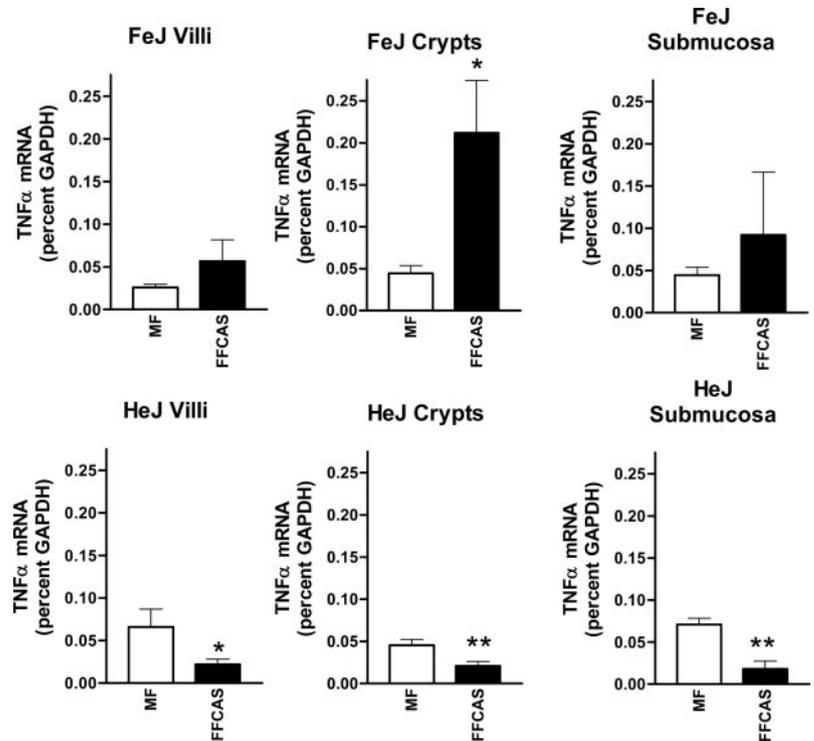
differences either among mouse strains or as a consequence of formula-feeding and hypoxia stress (data not shown). Since there was no change in the expression of these inflammatory markers, we analyzed TNF-α and found a significant increase in the crypt epithelium of C3Hbe/FeJ mice and observed a significant decrease in all three cell types of C3H/HeJ mice as a consequence of formula-feeding and stress (Fig. 10).

Discussion

NEC is a major problem in neonatal intensive care units worldwide. However, prevention and treatment strategies remain inadequate and inconsistent across different centers due to the inability to precisely define NEC pathophysiology. Nonetheless, progress in the last decade defined the roles of premature regulation of intestinal microcirculation and locally produced inflammatory mediators such as PAF, IL-8, TNF-α, iNOS, and others as critical determinants of pathology (for review, see Ref. 39). An altered bacterial colonization pattern (40), the protective role of probiotics (41–43), occasional clustering of NEC in neonatal intensive care units (44), the observation that NEC does not occur in a sterile environment in utero, and the absence of NEC-like pathology in an acute NEC model in germfree rats (8) suggest that bacteria play a role in NEC pathogenesis, but underlying defects in host-pathogen interactions that might lead to NEC are unknown. Our present report elucidates the importance of bacteria and TLR4 on intestinal inflammation and necrosis and shows that 1) exposure to bacteria contribute to NEC in the neonatal rat model, 2) TLR4 gene expression decreases in intestines of healthy, MF pups after birth to very low levels, but increases in villus epithelium in formula-fed/asphyxia-stressed rats, 3) high TLR4 expression correlates with increased iNOS and Gro/CINC (i.e., the functional correlate to human IL-8) expression, 4) the site of increased TLR4 and iNOS expression is the intestinal epithelium, and 5) in a novel neonatal mouse model of NEC, TLR4 mutant mice (C3H/HeJ) have a decreased incidence of NEC compared with control animals (C3HeB/FeJ). These results indicate that host-bacteria interactions play a role in NEC and suggest that bacterial-derived products activate TLR on intestinal epithelial cells that are abnormally upregulated following neonatal stress, resulting in downstream inflammatory gene expression and NEC.

The TLR are a family of pattern recognition receptors with specificity for particular bacterial components and ligands (45), and they play important roles in the proinflammatory response to infection and in the adaptive response that allows probiotic bacterial colonization. Genetic polymorphisms that compromise TLR function or downstream signaling are associated with varying severity and incidence of infectious and atopic diseases (45). Although the TLR family of receptors includes 11 members with specificities for a broad range of microbial-derived products, in this study, we investigated the role of TLR4 in neonatal NEC because TLR4 is the receptor for the most common and best known bacterial-derived product (i.e., LPS) that has been implicated in multiple disease processes, including NEC. Signal transduction of TLR4 involves activation of NFκB, a potent activator of gene transcription, which, upon activation, translocates to the nucleus and initiates synthesis of multiple proinflammatory mediators, including iNOS and IL-8 (46). Human intestinal epithelial cells have been shown to be unresponsive to ligands for TLR4 because in normal health there is little to no evidence of intestinal inflammation (13). Although pronounced TLR2 and TLR4 mRNA expression and corresponding immunolabeling in tissue sections has been reported in fetal human intestine (14), healthy adult human intestine exhibits little to no TLR2 and TLR4 expression (18, 47). Recent evidence indicates that LPS responsiveness rapidly declines after birth in primary murine enterocytes (48). Correlating with these molecular and cellular level findings, enteral administration of even low doses of LPS is lethal in newborn rats, but within a few weeks of age, these animals attain a remarkable resistance to LPS, and oral doses several folds over the lethal dose in newborns result in no pathology (15). Our findings in MF rats correlate with these findings and indicate readily detectable intestinal TLR4 expression at birth, a rapid decline of TLR4 expression after birth, and virtually undetectable levels of iNOS and Gro/CINC in the epithelium of MF rats at 72 h of life. To the contrary, the increase in gene expression of

TLR4 and downstream inflammatory mediators in the intestinal epithelium following formula-feeding and asphyxia stress is a critical finding in this report.

The data suggest that in addition to increased expression, there is increased TLR-dependent signaling in formula-fed, stressed rats, since we found significantly increased iNOS and Gro/CINC expression in intestinal villus epithelium as compared with MF controls. Notably, iNOS and IL-8 are among the inflammatory molecules that have been implicated in NEC pathogenesis (2, 4, 49), and they are downstream genes that are activated following TLR4 activation. These findings are in good correlation with evidence obtained using human specimens, indicating high-level induction of IL-8 mRNA and concomitant-elevated IL-8 secretion from fetal ileal mucosal explants by LPS but a lack of such a response in mucosal explants from infants and older children (4, 40). Interestingly, we did not observe a similar increase of iNOS or Gro/CINC expression in the crypt epithelium, despite the elevated TLR4 expression in these cells. It is yet to be determined whether this dichotomy of response reflects a difference in signaling efficiency between villus and crypt epithelium, or differential access of bacteria, or bacterial-derived products to villus vs crypt epithelium. We have not been able to observe consistent changes of either iNOS or CXCL1 (murine correlate of rat Gro/CINC and human IL-8) in the mouse model, but we demonstrated up-regulation of TNF-α in the intestinal epithelium of mice with wild-type TLR4 and down-regulation of TNF-α expression in mice with mutant TLR4. These data may suggest that there are differences between the relative roles of various inflammatory mediators in the rat and mouse models but are consistent with hypoxia and TLR4-dependent activation of inflammation in both rodent models.

There are both similarities and differences regarding TLR-related observations in UC, CD, the DSS model of colitis, and in our rodent models of NEC. Similar to our findings of increased epithelial TLR4 and unchanged TLR5 expression in experimental NEC, there is increased enterocyte-specific expression of TLR4 and decrease of TLR5 expression in UC and CD (18, 19) and in the DSS model of colitis (20). However, in our experimental NEC model, TLR4 deficiency appears to be clearly protective, while in a murine model of colitis, the role of TLR4 is less clear. In human molecular epidemiological studies, a correlation between the incidences of UC and CD and the frequency of Asp[299]Gly, a function-limiting TLR4 genetic polymorphisms, is less than clear. One study has found a strong association between the Asp[299]Gly allele and both UC and CD (30); in another cohort, there was a correlation between the same allele and CD but not UC (26). Two studies revealed no association between Asp[299]Gly and either UC or CD (27, 35), and the allele appears to be absent in the Japanese and Chinese populations with or without UC or CD (34, 36). In another study, there was no correlation between the Asp[299]Gly allele and NEC, but the small number of NEC patients precludes any strong conclusions (37). Studies in animal models are somewhat more consistent; however, they are not without controversy. Although CH3/HeJ and CH3/HeJBir, TLR4 mutant mice, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice all exhibit increased susceptibility to DSS-induced colitis (21–23), synthetic TLR4 antagonists reduce inflammation and disease score in the same model and in the colitis of MDR1a-deficient mice (25). It is yet to be determined whether different spatial and cell type-specific expression patterns of TLR or the different developmental stages of innate and adaptive immunity are responsible for the dichotomy of the roles these molecules might play in neonatal NEC and adult inflammatory bowel disease. It is plausible that, in neonates where the adaptive immune system is far from being fully developed (50, 51), aberrantly over-expressed epithelial TLR4 might promote bacterial-toxin-induced epithelial injury with ensuing rapid tissue destruction in the absence of adaptive immune protection. In older animals where the adaptive immune system is fully developed, epithelial TLR4 might be involved in epithelial damage, but lymphoid and myelomonocytic TLR4 expression could be involved in innate protective mechanisms and in coordinating an adaptive immune response following the breach of the epithelial barrier.

Bacteria have long been thought to contribute to the initiation of neonatal NEC, although the precise mechanisms have remained elusive. Studies have suggested that bacterial translocation is a prerequisite for disease and that the inflammatory response is dependent on increased mucosal permeability allowing for this response. Neonatal animal studies have suggested that exogenous bacteria are required for NEC and that probiotic organisms can reduce the incidence of NEC (40–42). In this study, we show that the presence of bacteria are critical for the initiation of intestinal inflammation and NEC and that Gram-negative organisms can stimulate the response. Our studies did not intend to define whether bacterial colonization of the small intestine is required for these effects or whether dead bacteria or bacterial cell wall products that reach the jejunum are sufficient to cause pathology. Consequently, it was beyond the scope of the present study to perform quantitative microbiology of intestinal colonization. Nonetheless, our findings may have immediate clinical relevance in the care of premature neonates who are at risk for NEC. In the premature infant cared for in the neonatal intensive care environment, patterns of bacterial colonization are abnormal with decreased species diversity and a paucity of anaerobes (52, 53). It has been suggested previously that an altered colonization pattern of the gastrointestinal tract or exposure to bacteria influences the incidence of NEC in this high-risk population. In fact, in a recent report (5), excessive colonization of feeding catheters was associated with an increased incidence of NEC in preterm infants. Interestingly, in the aforementioned study, one of the main bacterial species associated with NEC was a *Klebsiella* species, i.e., bacteria that have been correlated with NEC in other studies and bacteria that we have shown to associate with experimental NEC in the present study.

In summary, bacteria have been implicated in the pathogenesis of neonatal NEC, but the events at the molecular level have not been well elucidated. Our data from both neonatal rats and TLR mutant mice suggest that bacteria and the activation of aberrantly expressed TLR on intestinal epithelia are pivotal in NEC. A better understanding of early bacterial colonization and characterization of the mechanisms underlying the regulation of epithelial TLR expression might lead to novel methods that may help to reduce the incidence of NEC.

Disclosures

The authors have no financial conflict of interest.

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