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Oral Administration of Surfactant Protein-A Reduces Pathology in an Experimental Model of Necrotizing Enterocolitis

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Abstract

OBJECTIVES—Necrotizing enterocolitis (NEC) frequently results in significant morbidity and mortality in premature infants. Others reported that mice deficient in pulmonary surfactant protein-A (SP-A) born and raised in a nonhygienic environment succumb to significant gastrointestinal tract pathology, and enteral administration of purified SP-A significantly reduced mortality. We hypothesized that oral administration of purified SP-A can ameliorate pathology in an experimental model of neonatal NEC.

METHODS—Experimental NEC was induced in newborn Sprague-Dawley rat pups by daily formula gavage and intermittent exposure to hypoxia. Purified human SP-A (5 µg/day) was administered by oral gavage. After 4 days, surviving pups were sacrificed, and intestinal pathology was assessed by histological examination of distal terminal ileal sections. Intestinal levels of inflammatory cytokines (IL-1β, IFN-γ and TNF-α) were assessed by ELISA and levels of toll-like receptor 4 (TLR4) by western analysis.

RESULTS—61% of the gavaged rat pups that survived to day 4 met the criteria for experimental NEC after hypoxia while treatment with SP-A significantly reduced mortality and assessment of NEC. Intestinal levels of pro-inflammatory cytokines were significantly increased in pups exposed

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to hypoxia. Administration of SP-A to pups exposed to hypoxia significantly reduced IL-1 β and TNF- α levels, but had little effect on elevated levels of IFN- γ . SP-A treatment of hypoxia-exposed pups significantly reduced expression of intestinal TLR4, key in NEC pathogenesis.

CONCLUSIONS—In a rat model of experimental neonatal NEC, oral administration of SP-A reduces intestinal levels of pro-inflammatory cytokines and TLR4 protein, and ameliorates adverse outcomes associated with gastrointestinal pathologies.

Keywords

SP-A; NEC; TLR4; immunomodulation; pro-inflammatory cytokines

INTRODUCTION

Necrotizing enterocolitis (NEC) continues to be one of the most feared complications of prematurity, affecting up to 10% of extremely low birth weight (ELBW) infants (1). Despite improvement in short-term survival with modern neonatal intensive care; long-term mortality for surgical NEC remains high (10–50%) with increased risk of neurodevelopmental delay for survivors (1, 2). Because the etiology of NEC remains incompletely understood, specific treatment strategies are lacking. Growing evidence points to stress or injury to the intestinal epithelium, combined with inadequate host defense and repair in the immature intestine, which results in intestinal injury mediated by multiple inflammatory mediators (3). It is thought that infectious agents are not directly responsible for NEC, but certain bacterial phenotypes in combination with bowel ischemia, immaturity of host mucosal defense and enteral cow milk-based feeding may initiate the development of NEC.

Bacterial pathogens interact with intestinal toll-like receptors (TLRs), which recognize pathogen-associated molecules such as lipopolysaccharides (LPS) (4). NEC in humans and animal models is associated with increased expression and activation of intestinal TLR4. Physiological stressors associated with the development of NEC (i.e. intestinal immaturity and hypoxia) appear to sensitize the intestinal epithelium to LPS via the up-regulation of TLR4 (5). In support of this concept, TLR4-null mice are protected from the development of NEC compared with wild-type mice in an experimental model of NEC (6). Increased intestinal expression of TLRs and production of inflammatory cytokines appear to precede histological injury in experimental animal models of NEC (7).

Pulmonary surfactant protein A (SP-A) is best known for its role in the function of pulmonary surfactant to reduce alveolar surface tension by maintaining surfactant homeostasis (8). However, SP-A is also a critical component of the innate immune system; SP-A assists in clearance of potential pathogens and has an important role in the immunomodulatory properties of surfactant (9, 10). SP-A is highly conserved among mammalian, avian, reptilian and amphibian species and lungfish, and polyclonal antibodies raised against rat, mouse or human SP-A typically cross-react with SP-A isolated from other species (11). While expression of SP-A is highest in respiratory tissues, expression does occur in a variety of other tissues that interact with the surrounding environment (i.e., stomach, intestine, eye, kidney, uterus, skin), albeit at much lower levels than found in lung

(12, 13). Mice deficient in SP-A have no obvious phenotype, other than greatly enhanced susceptibility to pulmonary infections (14, 15). Nevertheless, a connection between SP-A and gastrointestinal pathology was reported previously by George *et al.* (16), who found that SP-A null juvenile mouse pups raised in a nonhygienic environment had significant mortality. However, rather than the expected lung pathology, mortality was associated with significant gastrointestinal tract pathology. They also found that oral administration of exogenous purified SP-A to newborn SP-A null pups improved survival, suggesting that SP-A has a role in immunoprotection of neonatal mice.

The findings by George *et al.* illustrate interesting associations between the known functions of SP-A and the factors that contribute to the pathogenesis of NEC. SP-A has immunomodulatory properties, either increasing or decreasing pro-inflammatory or anti-inflammatory cytokines, depending on the context of the interaction (10). SP-A is a pattern recognition molecule and binds to invasive bacteria and viruses, facilitating clearance by macrophages (17). SP-A has the capacity to bind LPS and modulate TLR4 activity through LPS-binding or binding directly to the receptor (18, 19). As many of these functions involve factors implicated in the development of NEC, we hypothesized that orally-administered SP-A could play a role in the amelioration of pathologies associated with a rat pup model of experimental neonatal NEC.

METHODS

Preparation of purified SP-A

The SP-A used in these studies was isolated from human volunteers with pulmonary alveolar proteinosis which results in abnormal accumulation of surfactant in their lungs and must undergo periodic pulmonary bronchoalveolar lavage (BAL) to remove the material (20). Two independent preparations of human SP-A were used in the present study. SP-A was purified from the BAL as described previously (21, 22). Briefly, lavage material was pelleted and delipidated with isopropyl and 1-butanol. The aqueous phase underwent further precipitation and SP-A was purified by column affinity. The purity of the SP-A (~1 mg/ml) was assessed by SDS-PAGE and silver staining. Endotoxin content of the purified SP-A was determined by the Limulus Amoebocyte Lysate Assay (GenScript USA, Inc.; Piscataway, NJ): 260 fg endotoxin/μg SP-A (Floros) and 180 pg endotoxin/μg SP-A (McCormack). It has been reported that latent TGF-β has been detected in purified SP-A preparations as a result of non-covalent interactions, and this co-containment has profound unintended effects on the immunomodulatory properties of SP-A (23). We assessed the level of TGF-β in the SP-A preparations by immunoblot analysis. While immunoblot analysis was sensitive to detect 10 ng of purified, mature TGF-β, the SP-A used in these studies did not have detectable TGF-β. The consistency of our results using these independently-isolated SP-A preparations strongly suggests that differences between the preparations did not have a significant effect on the outcomes measured in this study.

Experimental NEC model

Use of newborn Sprague-Dawley rat pups (weighing 5–6 g, Harlan Laboratories, Indianapolis, IN) was approved by the Animal Welfare Committee of the University of

Texas Health Science Center at Houston (#HSC-AWC-10-147). NEC was induced by oral gavage feeding of formula and exposure to hypoxia. Briefly, litters of newborn rats were separated from their dams after they suckled for 12 h. Rat pups were housed in an incubator and were then starved for 12 h before the initiation of formula feeding (day 1, 0.15 ml), 4 times daily (to deliver ~ 200 kcal/kg/day) for 3 days using sterile Solomon 22G 35 mm feeding needles (Instech Laboratories, Inc., Plymouth Meeting, PA). Rat pups were also subjected to 10 min of hypoxia (5% oxygen, 95% nitrogen), three times daily for 3 days, in a hypoxic chamber (Billups-Rothenberg, Del Mar, CA). The formula consisted of 15 g Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac canine milk replacement (Pet-Ag, Hampshire, IL) containing 1.86 kcal/ml. This technique was developed by Caplan (24) and Nadler *et al* (25). Modification of this well-established technique to induced intestinal injury in neonatal rats has been successfully used previously in our previous and current studies (7, 26, 27). Animals were monitored every three hours during the study. As in previously published studies, no analgesia was offered in this experimental NEC model (7, 28). Pups were euthanized on day 4 after NEC protocol to collect tissues. In some cases, pups were euthanized on day 3 if they were in pain, demonstrating labored respirations, severe abdominal distension or gastrointestinal bleeding.

To study the effect of SP-A in an experimental animal model of NEC, pups were randomly divided into 5 groups: dam-fed rats (DF, n = 6); formula-fed pups (FF, n = 7 or 8); formula-fed pups with formula supplemented with SP-A (FS, 5 µg/day; n = 7 or 8); formula-fed rat pups exposed to hypoxia to induce NEC (FH, n = 10), and formula-fed rat pups exposed to hypoxia with formula supplemented with SP-A (FHS, n = 10). To deliver SP-A to each pup, 5 µg of SP-A was diluted into 600 µl formula (the amount of formula required for feeding the pup daily). Both preparations were prepared at a concentration of 1 mg SP-A/ml.

Tissue harvest

Following incision of the abdomen, the gastrointestinal tract was carefully removed. The small intestine was evaluated visually for typical gross signs of NEC, such as intestinal distension, wall hemorrhage or necrosis. The terminal ileum was defined as the distal 20% of the length of the small bowel. The terminal 5 cm of small intestine (ileum) was excised. The distal 1 cm of each sample was formalin fixed and processed by the Cellular and Molecular Morphology Core Lab (the Texas Medical Center Digestive Diseases Center, Houston, TX) and stained with hematoxylin and eosin (H&E) for histological evaluation. The remaining 4 cm of small intestines was immediately frozen in liquid nitrogen then transferred to -80°C for further processing for protein and cytokine analysis.

Histopathological evaluation of NEC

Pathological changes in intestinal architecture were quantified using a modified NEC scoring system developed and used by the laboratories of M.S. Caplan and H.R. Ford (24, 25). Briefly, histological scores in the ileum were scored by 2 blinded evaluators on a scale of 0 (normal), 1 (mild NEC; separation of the villous core, without other abnormalities), 2 (moderate NEC; villous core separation, submucosal edema, and epithelial sloughing), and 3 (severe NEC; denudation of epithelium with loss of villi, full-thickness necrosis). The analysis was performed on 3–5 sections with 5 µm-thick each section for each sample

similar to other published studies (24). The final score was based on the worst-appearing microscopic field. Animals with histological scores ≥ 2 were defined as having NEC. Histological scores were only obtained from the tissues of live animals.

Tissue preparation for Western Blot and cytokine analysis

Ileal tissues were homogenized in 0.4 mL of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) containing protease inhibitors (2.5 mM Na_3VO_4 , 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ aprotinin and 1 mM PMSF). The homogenates were centrifuged at 14,000 g for 10 min at 4°C after incubation on ice for 30 min. The collected supernatants (tissue lysates) were used to measure the total protein using Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA). To detect the expression levels of SP-A and TLR4 by Western immunoblot analysis, proteins (50 μL /lane) were treated with antibodies specific for SP-A (#sc-13977; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), TLR4 (IMG-5031A, Imgenex Corp, San Diego, CA) and β -actin (#ab6276, Abcam, Cambridge, MA), respectively, for 12 hours at 4°C. After incubation with secondary IgG conjugated to horse-radish peroxidase (Bio-Rad; 1: 5000) for 1 hour at room temperature, immunoreactive bands were visualized by chemiluminescence (ECL+; GE Bioscience) and quantified on a Storm 840 Phosphorimager (GE Healthcare, Piscataway, NJ).

Cytokines were assessed by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (RTA00, RIF00 and RLB00 respectively, R&D Systems, Minneapolis, MN). Each rat intestinal tissue lysate sample was tested in duplicate on the 96-well ELISA plate, the samples with the percentages of confidence interval (CV) between the two readings <10 were included in the data analysis. Calibration curves were prepared with a range of 12.5 – 800 pg/ml for TNF- α , 31.2 – 2,000 pg/ml for IFN- γ , and 31.2 – 1,000 pg/ml for IL-1 β . Serum cytokine detection by ELISA is normally expressed as pg/ml, however, cytokine detection in tissue needs to be normalized based on either total protein concentrations in the tissue lysates or the weight of tissues homogenized, otherwise errors due to the variations in volumes of lysis buffer added for tissue homogenization or of the size of tissues collected (different tissue weight). Therefore, it was very important to normalize the values directly obtained from ELISA (pg/ml) to either pg/mg of total protein or pg/mg of tissue weight. Final cytokine levels were normalized by the total protein concentration (mg/mL) and reported as picograms of cytokine per milligram of total protein (pg/mg protein).

Statistical analysis

Initial analysis of mortality and histological scores between the groups was performed using a Fischer exact test. If significant results were identified in these overall contingency tests, subsequent *post-hoc* pairwise comparisons were performed to identify differences between two groups of rat pups. Protein and cytokine levels were compared across groups using a Kruskal-Wallis test or a Mann-Whitney test. If a Kruskal-Wallis yielded significant results, subsequent *post-hoc* pairwise comparisons were performed using a Mann-Whitney test. Statistical significance was assumed at $p < 0.05$. All analysis was performed using STATA (v. 10, College Station, TX).

RESULTS

Orally administered SP-A reduced mortality in a rat model of experimental NEC

To address the hypothesis, an established rat model of experimental neonatal NEC was utilized (7, 27, 29–31). Newborn rat pups were divided into 5 treatment groups described in the Methods sections. Pup survival was determined after 4 days of treatment (Table 1). There was a significant difference in mortality between the different groups ($p = 0.019$). The highest mortality was observed in the FH group, where nearly a third of the rat pups died. A *post-hoc* pairwise comparison between the FH group pups and the other pups, demonstrated that the mortality was significantly higher in the formula-fed pups exposed to hypoxia compared to the formula-fed pups exposed to hypoxia and SP-A (35% vs 5% mortality, respectively; $p = 0.043$). Furthermore, mortality rates were similar between the FHS and the FF groups (5% vs 7%, respectively; $p = 0.999$). Although there were more deaths in the FH group compared to the FF group, this difference failed to achieve statistical significance (35% vs 7%, respectively; $p = 0.101$).

Orally administered SP-A reduced intestinal pathology in a rat model of experimental NEC

We next assessed the effect of orally administered SP-A on the incidence and severity of NEC in a rat model of experimental NEC. The ileum was harvested from surviving animals in each group and processed for histological examination. Shown in Figure 1A are representative histological examples of the sections obtained from the animals. As expected, sections from dam-fed control pups showed normal, healthy villi and submucosal structure. The sections isolated from the formula-fed pups had more vacuolated villi indicating stress, but the overall structure of the ileum lacked the hallmarks of severe NEC. In pups fed formula supplemented with purified SP-A, ileum structure was similar to that seen in pups fed formula alone. On the other hand, severe damage is apparent in sections isolated from formula-fed pups exposed to hypoxic conditions; the villi were short and sparse, and the lamina propria was reduced in thickness. Oral administration of SP-A to formula-fed pups exposed to hypoxia resulted in ileum sections that appeared healthier, with taller villi and more normal lamina propria.

The incidence and severity of NEC in the sections of all surviving pups after 4 days of formula gavage were quantified using a modified NEC scoring system developed and employed by the laboratories of M.S. Caplan and H.R. Ford (24, 25). In this scoring system, the assessment of NEC was graded by three separate masked evaluators, and animals with histological scores of ≥ 2 were defined as having NEC, a score of 3 indicates severe NEC (32). A summary of the analysis of two independent experiments is shown Figure 1B. Not surprisingly, sections prepared from the DF group showed no evidence of NEC (0 or 1). On the other hand, the hallmarks of NEC were apparent in some of the sections prepared from all other groups. Analysis of the results as a whole indicated significant changes between some of the groups ($p = 0.019$). While the incidence and severity of NEC was more apparent in animals not exposed to hypoxia (FF and FS) as compared to the dam-fed group, these changes were not significantly different. As predicted, the group exposed to hypoxia had significantly higher numbers of pups with NEC (61%) compared to dam-fed pups (0%; $p = 0.002$). Importantly, oral administration of SP-A to formula-fed pups exposed to hypoxia

significantly reduced the numbers of pups with NEC (16%) as compared to the FH group ($p = 0.021$). Administration of SP-A to pups to exposed to hypoxia also reduced the numbers of pups assessed to have NEC (18%) as compared to the formula-fed group (28%), but these changes were not significant.

Orally administered SP-A reduced intestinal levels of pro-inflammatory cytokines in a rat model of experimental NEC

Increases in inflammatory cytokines are associated with both neonatal NEC in humans and experimental animal models of NEC (7, 33). We sought to determine if oral administration of SP-A reduces the pro-inflammatory tone of the intestine. We measured three Th1 cytokines, IL-1 β , IFN- γ and TNF- α , in the intestinal tissue lysates from rats in the five groups by ELISA. As seen in Figure 2A, intestinal levels of IL-1 β were significantly increased in the FH group compared to the DF, FF and FS groups ($p = 0.004$, *post hoc* Mann-Whitney test). Oral administration of purified SP-A to animals exposed to hypoxia (FHS) resulted in a 2.5-fold decrease in IL-1 β levels compared to the FH group ($P = 0.009$, *post hoc* Mann-Whitney test). The effect of SP-A on intestinal levels of TNF- α was then determined. This pro-inflammatory cytokine was only detectable in the ilea of formula-fed animals exposed to hypoxia (FH)(Figure 2B). The presence of exogenous SP-A significantly reduced intestinal levels of TNF- α by 90% ($p = 0.01$, *post hoc* Mann-Whitney test). However, not all Th1 cytokines were reduced by the presence of SP-A. As seen in Figure 2C levels of IFN- γ were significantly increased in animals exposed to hypoxia (FH) as compared to the DF, FF and FS groups ($p = 0.025$, *post hoc* Mann-Whitney test). In contrast to the results shown with IL-1 β and TNF- α , administration of SP-A did not significantly reduce these elevated levels.

Intestinal levels of TLR4 were decreased by upon oral administration of SP-A in a rat model of experimental NEC

Toll-like receptor 4 (TLR4) activity has a central role in the pathogenesis of NEC by altering the inflammatory milieu and resulting in a pro-inflammatory environment (34, 35). It has been reported that SP-A alters activation and expression of TLR4 in the lung (32, 36). We reported previously that levels of TLR4 protein were increased in formula fed pups compared to dam-fed pups in this rat pup model of neonatal NEC, however the change was not significant (7). As seen in Figure 3A, a representative blot of TLR4 protein in the intestine of DF and FF rat pups showed similar results in these investigations. To determine if exogenous SP-A has an effect on expression of TLR4 in the intestine, levels of TLR4 protein in the ileum were assessed by western analysis. In Figure 3B is shown a representative blot of the resulting analysis. As can be seen, TLR4 expression was evident in ileal tissue of FF, FS and FH animals, but expression was decreased in the FHS pups. Densitometry was used to quantify TLR4 levels relative to the loaded control, β -actin (Figure 3C). As can be seen, exposure of the pups to hypoxia significantly increased TLR4 expression compared to the FS animals. However, compared the FF group, increased levels of TLR4 protein in the intestine of FH pups did not reach significance. On the other hand, intestinal levels of TLR4 in animals exposed to hypoxia and administered SP-A (FHS) were significantly reduced compared to the FH group ($p = 0.014$, Kruskal-Wallis test and *post hoc* Mann-Whitney test).

Intestinal levels of SP-A are not significantly altered in a rat model of experimental NEC

SP-A is recognized as one of the major pulmonary surfactant-associated proteins and the majority of the SP-A produced in mammals is expressed in the lung; however, SP-A is expressed in non-pulmonary tissues, albeit at lower levels (12, 16). This includes the intestine, where differential allele-specific expression of SP-A has been observed (12, 13). We previously reported that SP-A expression is reduced in a human bronchiolar cell line (H441) upon transient exposure to infant formula (37). Based on those results and the fact that this model of neonatal NEC requires formula feeding, we postulated that a reduction in intestinal levels of SP-A may play a role in development of NEC in our experimental rat model. To investigate this possibility, we assessed levels of SP-A in the ileum of treated animals by western analysis. We first sought to determine if SP-A can be detected in intestinal tissue of rats. As seen in Figure 4A, western analysis of adult rat lung and intestinal tissue demonstrates that SP-A protein can be detected in intestinal tissue. Western analysis for SP-A in the treated pups is shown in Figure 4B. The results indicate that while SP-A was detected in the intestine of the newly-born rat pups, levels of SP-A did not appear to significantly change between the various groups.

DISCUSSION

In 1823, Charles Billard published what may be the first case report of necrotizing enterocolitis in a “small, weak infant with infection, inflammation and necrosis of the gastrointestinal tract”. NEC continues to be a major problem in pediatrics and despite numerous advances in medical treatment of premature infants, the pathogenesis and pathophysiology of NEC remain incompletely understood, and morbidity and mortality remain unacceptably high. One leading theory holds that NEC is initiated by factors that induce intestinal epithelial damage, which precipitate a dysregulated inflammatory process driven by activation and overexpression of TLR4 (7, 38). Pulmonary surfactant protein-A (SP-A) is a key component of the innate immune system in the lung that participates in the clearance of pathogenic microorganisms and necrotic cells, down-regulation of allergic reactions, and resolution of inflammation (39). We sought to determine if enteral administration of SP-A could reduce inflammation in the intestine using a well-established rat model of experimental neonatal NEC. Our results demonstrate that prophylactic administration of purified human SP-A can reduce morbidity and mortality in experimental NEC. Gross examination of the ileum sections from neonatal rat pups suggests that SP-A can improve damage of the ileum resulting from formula feeding and hypoxic stress, indicating that oral administration of SP-A reduces the incidence and histological severity of NEC in a rat model of experimental neonatal NEC. SP-A also reduces intestinal levels of two pro-inflammatory cytokines, IL-1 β and TNF- α , consistent with its known immunomodulatory activity in the lungs. These results suggest that oral administration of SP-A to neonatal rats exposed to hypoxia can reduce intestinal levels of pro-inflammatory cytokines associated with experimental NEC. SP-A is known to modulate expression and activation of TLR4, a key mediator of inflammation during NEC, and these investigations demonstrate that SP-A reduces intestinal levels of TLR4 in new-born rats treated to induce experimental neonatal NEC. This finding suggests that the ability of SP-A to reduce assessment of NEC and the levels of pro-inflammatory cytokines in an experimental model

of neonatal NEC may involve expression of TLR4. Our investigations suggest that the well-described functions of SP-A to modulate inflammation potentially can be employed as a means to ameliorate processes and adverse outcomes associated with NEC.

The impetus for these investigations was provided by a report from George *et al.* (16). In the relatively sterile environment of modern-day laboratories and animal care facilities, mice deficient in the gene encoding SP-A (SP-A^{-/-}) displayed few detrimental phenotypes other than enhanced susceptibility to pulmonary infections, (14, 15). George *et al.* wished to determine if the effect of a nonhygienic environment in juvenile mice would predispose the SP-A^{-/-} mice to lung pathologies as a result of recurrent infections. Indeed, they found a significant increase in mortality of the mice. However, upon examination, the mice lacked the expected pathologies associated with pulmonary infections. Rather, an intestinal pathology resembling NEC was observed in the afflicted mice. They also found that while SP-A^{-/-} pups born from crosses of SP-A^{-/-} dams with SP-A^{+/-} males displayed enhanced mortality, mortality of SP-A^{-/-} pups arising from SP-A^{+/-} dams with SP-A^{-/-} males were not significantly different from SP-A^{+/-} pups, suggesting transmission of a protective agent, presumably SP-A, from the dams. Since SP-A protein has been detected in human mammary epithelium (40), they surmised that SP-A may be transmitted to the pups via breast milk to provide protection to the gut. They then demonstrated that oral administration of SP-A (5 µg/day) to SP-A^{-/-} pups significantly decreased mortality compared to pups not treated with SP-A. However, neither SP-A protein nor RNA was found in lactating mammary tissues in this murine model (George CLS, K Goss, P McCray, J Snyder, abstract 3756.10, Surfactant Protein-D in milk, Society of Pediatric Research meeting, 2008). In addition, it has been reported that milk from SP-A null murine dams has significant cytokine alterations compared to normal milk with an associated increase in pup mortality (41). Thus, while it seems that orally administered SP-A can transit through the stomach to provide protection to immature mature intestine, the precise physiological mechanism responsible for this function of SP-A remains unclear.

This study demonstrates an interesting and unexpected intersection of the mechanisms that are believed to initiate and propagate NEC with the known functions of pulmonary SP-A. Factors that are associated with development of NEC, including hypoxia and the exposure to high concentrations of LPS, are known to increase TLR4 expression within the intestinal epithelium (42). Once activated, TLR4 initiates a cascade of events, including synthesis of pro-inflammatory cytokines, which in turn produces chemotaxis, transmigration and activation of leukocytes. Targeted treatments (such as probiotics, pentoxifylline, alkaline phosphatase and anti-TNF-α antibodies) can reduce expression or activity of pro-inflammatory cytokines and can reduce the incidence and severity of experimental NEC (43–46). These reports suggest that pathogenesis of NEC involves the intersection of LPS, TLR4 and pro-inflammatory cytokines. In the lung, SP-A is known to alter the cytokine environment (9, 10, 39). SP-A has also been shown to regulate TLR4 expression and activity in human macrophages and dendritic cells in the lung (32, 36). SP-A can bind LPS and antagonizes LPS-enhanced TLR4 activity in alveolar macrophages (18, 19, 47). In the absence of SP-A, alveolar macrophages exhibit increased activation of NF-κB, due to reduced levels of the inhibitory protein I-κB (48). SP-A also interacts with TLR4-MD2 protein and inhibits the LPS-stimulated release of TNF-α in JAWS II dendritic cells (32). It

is possible that SP-A may interact directly with TLR4 and other receptors to prevent activation; alternatively SP-A may interact directly with LPS, preventing its binding to TLR4 (49). The fact that SP-A participates in the clearance of pathogenic microorganisms and necrotic cells may indicate that SP-A reduces colonization by pathogenic microorganisms (17). As each of these factors have been implicated in the pathogenesis of NEC, our findings that a pulmonary protein can have a role in gastrointestinal pathology are not that surprising. Shown in Figure 5 is a schematic of a proposed mechanism by which the known functions of SP-A can affect the factors believed to result in NEC.

For years clinicians and researchers have attempted to develop safe and effective strategies to prevent the development of NEC by a variety of agents, such as by IgA supplementation, intravenous dexamethasone, enteral antibiotics, polyunsaturated fatty acid supplementation, or lactoferrin, and arginine supplementation. However, due to the inability to reproduce results and because potential toxicities, these approaches are not widely used. The potential beneficial effects of pulmonary surfactant on NEC have been tested previously (50). An artificial surfactant consisting of the hydrophobic SP-B and SP-C proteins and surfactant lipids had no protective or therapeutic effect in an animal model of NEC; however, SP-A was not included in that preparation. The theoretical advantages of the therapeutic use of SP-A in patients with NEC or at risk for NEC may be its favorable safety profile, its endogenous expression in the gastrointestinal tract, its lack of systemic effects and its efficacy with an oral route of administration (enteral feeding). While more research is necessary to define the mechanism by which SP-A acts to alter the balance of factors that contribute to the development of NEC in the premature host, our results suggest that SP-A can potentially be exploited in the prophylaxis of NEC of the newborn.

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REFERENCES

1. Jesse N, Neu J. Necrotizing enterocolitis: relationship to innate immunity, clinical features, and strategies for prevention. *Neoreviews*. 2006; 7:e143–e49.
2. Vohr BR, Wright LL, Dusick AM, et al. Neurodevelopmental and functional outcomes of extremely low birth weight infants in the National Institute of Child Health and Human Development Neonatal Research Network, 1993–1994. *Pediatrics*. 2000; 105(6):1216–26. [PubMed: 10835060]
3. Matin, RJ.; Fanaroff, AA.; Walsh, MC. Fanaroff and Martin's Neonatal-Perinatal Medicine: Diseases of the Fetus and Infant. Mosby/Elsevier; St. Louis, MO: 2011.
4. Caplan MS, Simon D, Jilling T. The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis. *Semin Pediatr Surg*. 2005; 14(3):145–51. [PubMed: 16084401]
5. Hackam DJ, Good M, Sodhi CP. Mechanisms of gut barrier failure in the pathogenesis of necrotizing enterocolitis: Toll-like receptors throw the switch. *Semin Pediatr Surg*. 2013; 22(2):76–82. [PubMed: 23611610]
6. Sodhi CP, Neal MD, Siggers R, et al. Intestinal epithelial Toll-like receptor 4 regulates goblet cell development and is required for necrotizing enterocolitis in mice. *Gastroenterology*. 2012; 143(3): 708–18. e1–5. [PubMed: 22796522]

7. Liu Y, Zhu L, Fatheree NY, et al. Changes in intestinal Toll-like receptors and cytokines precede histological injury in a rat model of necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol*. 2009; 297(3):G442–50. [PubMed: 19608731]
8. Kishore U, Greenhough TJ, Waters P, et al. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol*. 2006; 43(9):1293–315. [PubMed: 16213021]
9. Pastva AM, Wright JR, Williams KL. Immunomodulatory roles of surfactant proteins A and D: implications in lung disease. *Proc Am Thorac Soc*. 2007; 4(3):252–7. [PubMed: 17607008]
10. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol*. 2005; 5(1):58–68. [PubMed: 15630429]
11. Sullivan LC, Daniels CB, Phillips ID, et al. Conservation of surfactant protein A: evidence for a single origin for vertebrate pulmonary surfactant. *J Mol Evol*. 1998; 46(2):131–8. [PubMed: 9452514]
12. Akiyama J, Hoffman A, Brown C, et al. Tissue distribution of surfactant proteins A and D in the mouse. *J Histochem Cytochem*. 2002; 50(7):993–6. [PubMed: 12070278]
13. Lin Z, Wang Y, Zhu K, et al. Differential allele expression of host defense genes, pulmonary surfactant protein-A and osteopontin, in rat. *Mol Immunol*. 2004; 41(12):1155–65. [PubMed: 15482851]
14. Korfhagen TR, Bruno MD, Ross GF, et al. Altered surfactant function and structure in SP-A gene targeted mice. *Proc Natl Acad Sci U S A*. 1996; 93(18):9594–9. [PubMed: 8790375]
15. LeVine AM, Whitsett JA. Pulmonary collectins and innate host defense of the lung. *Microbes Infect*. 2001; 3(2):161–6. [PubMed: 11251302]
16. George CL, Goss KL, Meyerholz DK, et al. Surfactant-associated protein A provides critical immunoprotection in neonatal mice. *Infect Immun*. 2008; 76(1):380–90. [PubMed: 17967856]
17. Haagsman HP. Interactions of surfactant protein A with pathogens. *Biochim Biophys Acta*. 1998; 1408(2–3):264–77. [PubMed: 9813361]
18. Nguyen HA, Rajaram MV, Meyer DA, et al. Pulmonary surfactant protein A and surfactant lipids upregulate IRAK-M, a negative regulator of TLR-mediated inflammation in human macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2012; 303(7):L608–16. [PubMed: 22886503]
19. Stamme C, Muller M, Hamann L, et al. Surfactant protein a inhibits lipopolysaccharide-induced immune cell activation by preventing the interaction of lipopolysaccharide with lipopolysaccharide-binding protein. *Am J Respir Cell Mol Biol*. 2002; 27(3):353–60. [PubMed: 12204898]
20. Greenhill SR, Kotton DN. Pulmonary alveolar proteinosis: a bench-to-bedside story of granulocyte-macrophage colony-stimulating factor dysfunction. *Chest*. 2009; 136(2):571–7. [PubMed: 19666756]
21. Beharka AA, Crowther JE, McCormack FX, et al. Pulmonary surfactant protein A activates a phosphatidylinositol 3-kinase/calcium signal transduction pathway in human macrophages: participation in the up-regulation of mannose receptor activity. *J Immunol*. 2005; 175(4):2227–36. [PubMed: 16081790]
22. Garcia-Verdugo I, Wang G, Floros J, et al. Structural analysis and lipid-binding properties of recombinant human surfactant protein a derived from one or both genes. *Biochemistry*. 2002; 41(47):14041–53. [PubMed: 12437362]
23. Kunzmann S, Wright JR, Steinhilber W, et al. TGF-beta1 in SP-A preparations influence immune suppressive properties of SP-A on human CD4+ T lymphocytes. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291(4):L747–56. [PubMed: 16648238]
24. Caplan MS, Jilling T. The role of polyunsaturated fatty acid supplementation in intestinal inflammation and neonatal necrotizing enterocolitis. *Lipids*. 2001; 36(9):1053–7. [PubMed: 11724457]
25. Nadler EP, Dickinson E, Knisely A, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *J Surg Res*. 2000; 92(1):71–7. [PubMed: 10864485]
26. Liu Y, Fatheree NY, Dingle BM, et al. *Lactobacillus reuteri* DSM 17938 changes the frequency of Foxp3+ regulatory T cells in the intestine and mesenteric lymph node in experimental necrotizing enterocolitis. *PLoS One*. 2013; 8(2):e56547. [PubMed: 23437165]

27. Liu Y, Fatheree NY, Mangalat N, et al. Lactobacillus reuteri strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-kappaB signaling in the intestine. *Am J Physiol Gastrointest Liver Physiol*. 2012; 302(6):G608–17. [PubMed: 22207578]
28. Dvorak B, Khailova L, Clark JA, et al. Comparison of epidermal growth factor and heparin-binding epidermal growth factor-like growth factor for prevention of experimental necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr*. 2008; 47(1):11–8. [PubMed: 18607263]
29. Caplan MS, Kelly A, Hsueh W. Endotoxin and hypoxia-induced intestinal necrosis in rats: the role of platelet activating factor. *Pediatr Res*. 1992; 31(5):428–34. [PubMed: 1603619]
30. Dvorak B, Halpern MD, Holubec H, et al. Maternal milk reduces severity of necrotizing enterocolitis and increases intestinal IL-10 in a neonatal rat model. *Pediatr Res*. 2003; 53(3):426–33. [PubMed: 12595590]
31. Kelly N, Friend K, Boyle P, et al. The role of the glutathione antioxidant system in gut barrier failure in a rodent model of experimental necrotizing enterocolitis. *Surgery*. 2004; 136(3):557–66. [PubMed: 15349102]
32. Awasthi S, Madhusoodhanan R, Wolf R. Surfactant protein-A and toll-like receptor-4 modulate immune functions of preterm baboon lung dendritic cell precursor cells. *Cell Immunol*. 2011; 268(2):87–96. [PubMed: 21439559]
33. Caplan MS, Sun XM, Hsueh W, et al. Role of platelet activating factor and tumor necrosis factor-alpha in neonatal necrotizing enterocolitis. *J Pediatr*. 1990; 116(6):960–4. [PubMed: 2348301]
34. Gripar SC, Sodhi CP, Richardson WM, et al. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis. *J Immunol*. 2009; 182(1):636–46. [PubMed: 19109197]
35. Sodhi CP, Shi XH, Richardson WM, et al. Toll-like receptor-4 inhibits enterocyte proliferation via impaired beta-catenin signaling in necrotizing enterocolitis. *Gastroenterology*. 2010; 138(1):185–96. [PubMed: 19786028]
36. Henning LN, Azad AK, Parsa KV, et al. Pulmonary surfactant protein A regulates TLR expression and activity in human macrophages. *J Immunol*. 2008; 180(12):7847–58. [PubMed: 18523248]
37. Chen MG, Atkins CL, Bruce SR, et al. Infant formula alters surfactant protein A (SP-A) and SP-B expression in pulmonary epithelial cells. *Pediatr Pulmonol*. 2011; 46(9):903–12. [PubMed: 21520433]
38. Frost BL, Jilling T, Caplan MS. The importance of pro-inflammatory signaling in neonatal necrotizing enterocolitis. *Semin Perinatol*. 2008; 32(2):100–6. [PubMed: 18346533]
39. Nayak A, Dodagatta-Marri E, Tsolaki AG, et al. An Insight into the Diverse Roles of Surfactant Proteins, SP-A and SP-D in Innate and Adaptive Immunity. *Front Immunol*. 2012; 3:131. [PubMed: 22701116]
40. Braidotti P, Cigala C, Graziani D, et al. Surfactant protein A expression in human normal and neoplastic breast epithelium. *Am J Clin Pathol*. 2001; 116(5):721–8. [PubMed: 11710690]
41. Gidvani MP, Theisen E, Leduc R, et al. Maternal surfactant protein A influences the immunoprotective properties of milk in a murine model. *Pediatr Res*. 2014; 76(2):135–41. [PubMed: 24858142]
42. Chan KL, Wong KF, Luk JM. Role of LPS/CD14/TLR4-mediated inflammation in necrotizing enterocolitis: pathogenesis and therapeutic implications. *World J Gastroenterol*. 2009; 15(38):4745–52. [PubMed: 19824106]
43. Wu SF, Chiu HY, Chen AC, et al. Efficacy of Different Probiotic Combinations on Death and Necrotizing Enterocolitis in a Premature Rat Model. *J Pediatr Gastroenterol Nutr*. 2013
44. Travadi J, Patole S, Charles A, et al. Pentoxifylline reduces the incidence and severity of necrotizing enterocolitis in a neonatal rat model. *Pediatr Res*. 2006; 60(2):185–9. [PubMed: 16864701]
45. Rentea RM, Liedel JL, Fredrich K, et al. Intestinal alkaline phosphatase administration in newborns decreases systemic inflammatory cytokine expression in a neonatal necrotizing enterocolitis rat model. *J Surg Res*. 2012; 177(2):228–34. [PubMed: 22687880]
46. Halpern MD, Clark JA, Saunders TA, et al. Reduction of experimental necrotizing enterocolitis with anti-TNF-alpha. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290(4):G757–64. [PubMed: 16269520]

47. Sender V, Lang L, Stamme C. Surfactant protein-A modulates LPS-induced TLR4 localization and signaling via beta-arrestin 2. *PLoS One*. 2013; 8(3):e59896. [PubMed: 23536892]
48. Wu Y, Adam S, Hamann L, et al. Accumulation of inhibitory kappaB-alpha as a mechanism contributing to the anti-inflammatory effects of surfactant protein-A. *Am J Respir Cell Mol Biol*. 2004; 31(6):587–94. [PubMed: 15308505]
49. Kawai T, Akira S. Pathogen recognition with Toll-like receptors. *Curr Opin Immunol*. 2005; 17(4): 338–44. [PubMed: 15950447]
50. Canpolat FE, Yurdakok M, Ersin SC. Effects of enterally administered surfactant in a rat model of necrotizing enterocolitis. *Neonatology*. 2012; 102(1):53–8. [PubMed: 22508026]

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Figure 1. Effect of orally administered SP-A on ileal histology and assessment of NEC in an experimental rat model of neonatal NEC

A. Representative H & E sections of ileum isolated from the various groups. DF = dam-fed, FF = formula-fed, FS = formula-fed + purified SP-A (5 µg/day), FH = formula-fed with hypoxia, FHS= formula-fed with hypoxia + SP-A (original magnification ×100). **B.**

Assessment of NEC in sections of ileum of treated animals. A score of 2 was defined as NEC. Each point represents assessment of a single animal that survived 4 days of treatment; the graph represents animals from two independent experiments with animals treated with independent preparations of purified SP-A. $P < 0.05$, determined by Fisher exact test was considered significant.

Figure 2. Effect of orally-administered SP-A on ileal IL-1 β , TNF- α and IFN- γ cytokine levels in an experimental rat model of neonatal NEC

A. Levels of IL-1 β in the ileum of rats treated for 4 days determined by ELISA; DF = dam-fed, FF = formula-fed, FS = formula-fed + purified SP-A, FH = formula-fed with hypoxia, FHS = formula-fed with hypoxia + SP-A. **B.** Levels of TNF- α in the ileum of rats treated for 4 days determined by ELISA. ND indicates not detectable. **C.** Levels of IFN- γ in the ileum of rats treated for 4 days determined by ELISA. Data represent box plots of cytokines measures in animals shown in Figure 1B. $P < 0.05$, determined by the Kruskal-Wallis test and *post hoc* Mann-Whitney test, was considered significant.

Figure 3. Effect of orally-administered SP-A on ileal levels of TLR4 in an experimental rat model of neonatal NEC

A. Levels of ileal TLR4 protein in Dam-fed compared to formula-fed rat pups. Levels of ileal TLR4 protein were determined by western analysis of 2 pups. Shown is a representative immunoblot; DF = dam-fed, FF = formula-fed. **B.** Levels of ileal TLR4 protein determined by western analysis. Shown is a representative immunoblot. The TLR4 positive control (+ control) was purchased (Santa Cruz Biotechnology, Inc.; human erythroleukemia lysate, sc-2270). **C.** Densitometric quantification of TLR4 in the ileum of rats treated for 4 days; FF = formula-fed, FS = formula-fed + purified SP-A, FH = formula-fed with hypoxia, FHS = formula-fed with hypoxia + SP-A. Shown are the levels of TLR4 relative to β -actin levels (arbitrary units, means $\pm$ SEM). Data represent $n = 4 - 6$ animals from two independent experiments. $P = 0.05$, determined by the Kruskal-Wallis test and *post hoc* Mann-Whitney test, was considered significant.

Figure 4. Expression of SP-A in the lung and ileum of adult and newly-born rats

A. Levels of SP-A in adult rat lung and ileum determined by western analysis. Shown are western blots for SP-A and β -actin. Mouse BAL represents bronchoalveolar lavage material, used as a positive control for SP-A. Arrows indicate monomer and dimerized forms of SP-A.

B. Western analysis of ileal SP-A in rats treated for 4 days. Shown are western blots for SP-A and β -actin; FF = formula-fed, FS = formula-fed + purified SP-A, FH = formula-fed with hypoxia, FHS = formula-fed with hypoxia + SP-A.

Figure 5. Proposed model for the role of SP-A in ameliorating experimental NEC
Red indicates some of the factors believed to initiate NEC in the intestine. SP-A is known to dampen the activities of these factors. LPS = lipopolysaccharide, TLR4 = toll-like receptor 4.

Table 1

Mortality of rat pups exposed to various conditions in an experimental model of NEC.

Group*	Number of rat pups		p-value**	
	Total (n)	Died; n (%)	FF referent	FH referent
DF	12	0 (0)	ns	0.029
FF	15	1 (7)	referent	ns
FS	15	4 (26)	ns	ns
FH	20	7 (35)	ns	referent
FHS	20	1 (5)	ns	0.043
Total	82	13 (16)		

* DF = dam-fed; FF = formula fed; FS = formula-fed + SP-A; FH = formula-fed + hypoxia; FSH = formula-fed + hypoxia + SP-A.

** post-hoc pairwise comparison to specified referent group; ns = not significant