Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis

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McElroy SJ, Prince LS, Weitkamp J-H, Reese J, Slaughter JC, Polk DB. Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 301: G656–G666, 2011. First published July 7, 2011; doi:10.1152/ajpgi.00550.2010.— Necrotizing enterocolitis (NEC) is predominantly a disease of premature infants. The luminal surface of the small intestine is lined with a single layer of epithelial cells that separate it from the bacteria-rich luminal environment. One of the major components that protect the epithelial layer is intestinal mucus (35). Mucins are large glycoproteins that provide a physical barrier, facilitate removal of adherent bacteria, and concentrate enzymes near the epithelial surface to aid in host nutrient digestion (21). The ideal portion of the small intestine expresses multiple mucin genes. Included in these are Muc1, Muc3, Muc4, and Muc17, which are anchored to the cell membrane by a transmembrane domain (2, 18, 53), and Muc2, which is the most abundant mucin in the small intestine and is secreted into the lumen (2). These genes are expressed early in development and reach adult levels by 27 wk of gestation (6). However, the mucus produced by immature intestinal tracts have different viscosity (1), buoyancy, and carbohydrate composition (51) than mucus produced by adults. It is unknown whether these differences render the mucins of premature infants less effective at protective functions than that of mature adults, but, if they are less protective, this may help to explain why premature infants are more susceptible to NEC.

The role of intestinal mucus in NEC is unknown. Decreases in Muc2-stained goblet cells have been described in a rat model of NEC (11), but several important questions remain unanswered, including 1) whether mucin is affected in human samples of NEC, 2) whether this loss of mucin is due to changes of goblet cells or of their secretions, and 3) whether this loss of mucin is developmentally regulated. Identifying the role that mucins play in the mechanisms of gastrointestinal epithelial cell injury and repair during development is vital to our understanding of diseases such as NEC.

To answer our first question, we measured the number of mucin-containing goblet cells in human samples from infants who developed NEC. These infants had fewer mucus-containing goblet cells than age-matched and term controls. Because NEC is primarily an inflammatory disease of premature infants, we examined the role of inflammation on the quantity of mucus in immature small intestine of mice. Using this model, we were able to show that TNF-α has developmentally dependent effects on intestinal mucus expression, gene regulation, and secretion and that these effects are TNF-α receptor (TNFR) dependent. These findings have important implications...
tions for understanding injury and repair mechanisms in premature infants who are prone to develop NEC.

MATERIALS AND METHODS

**Mice and TNF-α injections.** C57Bl/6, TNFR1<sup>−/−</sup>, TNFR2<sup>−/−</sup>, and TNFR1<sup>−/−</sup>2/<sup>−/−</sup> mice were purchased from The Jackson Laboratory. All genetically altered mice were maintained on a C57Bl/6 background, and C57Bl/6 mice were used as wild-type controls. Mice were delivered vaginally, except for E19 mice that were delivered by Cesarean section on embryonic day 19. Mice at specific ages (E19, P0, P7, P14, P21, or P28 as indicated) were separated from their mothers, injected intrauterinely with 0.5 μg/g body wt TNF-α (Peprotech) and maintained without feeds in a temperature and humidity controlled chamber for 8 h before euthanasia. Small intestine was harvested and fixed by snap freezing or Carnoy solution. Tissues prepared for immunohistochemistry were paraffin embedded and sectioned and maintained without feeds in a temperature and humidity controlled chamber for 8 h before euthanasia. Small intestine was harvested and fixed by snap freezing or Carnoy solution. Tissues prepared for immunohistochemistry were paraffin embedded and sectioned at 5 μm. Tissues stained for anti-Muc2 were flushed with 0.5 mM dithiothreitol before fixation to decrease mucin polymers and improve staining sensitivity. Tissues prepared for quantitative PCR were homogenized and digested using the RNaseasy mini kit (Qiagen). All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

**Human intestinal samples.** Deidentified, archived human ileal sections from preterm infants with NEC (<n = 5; median gestational age: 27 wk; median age at tissue collection: 20 days), preterm infants with spontaneous intestinal perforation (SIP; <n = 5; median gestational age: 27 wk; median age at tissue collection: 47 days), and late preterm infants with noninflammatory intestinal diseases (NIID; <n = 6; hirschsprung's disease; ileal atresia: 1, jejunal atresia: 2, jejunal web: 1; median gestational age: 35 wk; median age at tissue collection: 69 days) were obtained under appropriate oversight and approval by the Institutional Review Board of Vanderbilt University, Nashville, TN. Sections were prepared and stained for immunohistochemistry as below. NEC samples were obtained at the time of surgery and were taken from the leading edge of damaged tissue. Infants in this cohort were deidentified, so their severity of NEC is not precisely known; however, all infants required surgical intervention and can be assumed to be Bell stage 3. All tissue samples were examined for live cells and villous architecture by immunohistochemistry, and half of the samples were additionally measured for tissue viability through use of flow cytometry for lymphocytes. Any samples not having viable cells were deemed to be necrotic and discarded. Traditionally, it is difficult to obtain suitable controls for NEC samples. To address this we used two separate controls. The first control contained age-matched infants who developed SIP and required surgery. The second control contained late-preterm infants who required surgery as a result of a noninflammatory, anatomical illness.

**Immunohistochemistry.** Ileal sections were deparaffinized, rehydrated, and antigen unmasked by boiling in a citrate-containing buffer (Vector Laboratories). Slides were incubated with 10% goat serum (Zymed) for 30 min and stained with antibodies to lysozyme (Dako), chromogranin A (Abcam), Muc2 (Santa Cruz Biotechnology), or active caspase 3 (BD Pharmingen) antibody at 4°C overnight. Anti-rabbit horseshardase peroxidase (Dako) was applied to slides for 30 min. Slides were developed using a diaminobenzidine substrate kit (Vector Laboratories), counterstained with methyl green or Meyer’s hematoxylin. Slides stained for presence of mucus were stained with Periodic Acid Schiff stain (PAS) (Sigma-Aldrich). The number of positive cells per 100 villous epithelial cells was counted by a single blinded investigator. Villous epithelial cells were defined as intestinal epithelial cells above position 5. Paneth cells, which are located below position 5, were counted with their associated villous epithelial cells. Goblet theca volume was determined by staining slides with Alcian Blue (Sigma-Aldrich). RGB images were obtained at 40 magnification and split into color channels using Image J (NIH). Pixel density of the blue channel was measured from 20 consecutive mucus-containing goblet cells from each sample, and mean density per volume was determined for both thecal areas and thecal area per goblet cell area.

**Quantitative PCR.** Ileal samples were homogenized, and RNA was purified from ileal samples using the RNeasy Mini Kit (Qiagen) according to manufacturer’s directions. First-strand cDNA was synthesized using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen). TaqMan real-time PCR primers for *Muc1*, *Muc2*, *Muc3*, β-actin, *Notch1*, *Notch2*, *HES-1*, *HES-3*, and *Sdf1* were used (Applied Biosystems). Two-step real-time PCR was performed with a Bio-Rad MyQ thermocycler and SYBR Green detection system (Bio-Rad). We normalized gene expression to β-actin in each sample. The 2<sup>−ΔΔCT</sup> method was used to compare gene expression levels between samples.

**Mucus secretion assay.** To determine the effects of TNF-α on mucus secretion, we modified a technique described by Garcia et al. (17). Fourteen-day-old C57Bl/6 mice were euthanized, and 4-cm ileal segments were harvested. Isolated tissues were transferred to a custom microvessel perfusion chamber (Living Systems International), which is a water-jacketed plastic chamber with proximal (inflow) and distal (outflow) fire-polished glass cannulae. The ileal segment was gently flushed with saline, threaded onto the proximal cannula, and secured with a braided nylone suture. The distal end of the sample was then secured onto the distal cannula so that the intestine was held without stretching. The luminal perfusion solution contained 150 mM Na<sup>+</sup>, 2.5 mM K<sup>+</sup>, 1 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, 150 mM Cl<sup>−</sup>, and 2.5 mM PO<sub>4</sub><sup>3−</sup>. The serosal perfusion solution was the same except that it also included 10 mM glucose and 25 mM HCO<sub>3</sub><sup>−</sup>. All solutions were adjusted and maintained at a pH of 7.4 and temperature of 37°C. Once the ileal segment was cannulated, the proximal cannula was connected to a variable-speed syringe pump (Harvard Apparatus) set to deliver 0.3 ml/min. The distal cannula was connected to a short piece of tubing that was directed into a perfusate-collecting tube. Serosal fluid was constantly circulated through a warmer and aerated with 95% CO<sub>2</sub>-5% CO<sub>2</sub> gas to maintain temperature and pH. After 15 min of equilibration, 200 μg/ml TNF-α (Peprotech), 10−4 M prostaglandin E2 (PGE<sub>2</sub>) (Caymen), or an equivalent volume of saline (control) were added to the serosal solution, and luminal perfusates were collected at 3- and 30-min intervals as indicated. After experimentation, the luminal perfusate mucus was measured using a PAS assay (17). In brief, 0.2 ml of periodic acid 0.1% was pipetted into each sample and incubated for 2 h at room temperature, after which 0.2 ml of Schiff reagent (Sigma-Aldrich) was added and incubated for 30 min at room temperature. The OD of the resulting solution at 550 nm wavelength was taken as a measure of the amount of PAS-positive product present (14, 17, 32) and compared with a known standard of porcine stomach mucin (Sigma-Aldrich).

**Replicates and statistical analysis.** All data are representative of at least three independent experiments. Comparisons of the percentage of epithelial cells in saline and TNF-injected mice at various ages were made using logistical regression. For quantitative PCR, gene expression was normalized to β-actin, and the 2<sup>−ΔΔCT</sup> method was used to compare gene expression levels between samples. PCR expression was analyzed using linear regression to estimate differences in intensity attributable to TNF injection status and age. Changes in secretion over time were estimated using a linear mixed-effects model. In this model we included a random intercept to account for the correlation arising from taking repeated observations on the same animal. In all models, first-order trends were estimated by including either age or time as continuous covariates. Minimum level of significance was set at <0.05, and error bars designate upper and lower confidence intervals.
RESULTS

Human infants with NEC have decreased numbers of mucin-laden goblet cells. To determine whether NEC affected small intestinal mucus, leading-edge nonnecrotic surgical samples were obtained from preterm human infants who had developed NEC. These were compared with gestational age-matched controls with SIP and to late-preterm human infants who had a NIID such as jejunal atresia. Samples were stained with PAS to quantify the number of mucin-containing goblet cells. Infants with NEC had significantly lower numbers of mucin-positive goblet cells and Paneth cells compared with controls ($P < 0.001$) (Fig. 1). This decrease was not seen in enteroendocrine cells.

TNF-α decreases Muc2-containing small intestinal goblet cells in immature small intestine. Because NEC is an inflammatory disease of premature infants, we wondered whether the loss of mucus-containing goblet cells seen in infants with NEC was unique to immature intestine. To test this, we induced intestinal inflammation by injecting mice with TNF-α (0.5 μg/g body wt) 8 h before tissue harvest. Mice were used for these studies because complete development of the mouse intestine is not achieved until 21 to 28 days after birth (23, 48). With the use of conserved developmental patterns such as crypt formation and enzyme expression, the small intestine of a newborn mouse is roughly equivalent to the intestine of a

![Graph showing the decrease of goblet cells in NEC, SIP, and NIID](image-url)
TNF-α DEPLETES MUCUS IN IMMATURE SMALL INTESTINAL TISSUE

10-wk old human, a 14-day-old mouse is roughly equivalent to a 24-wk premature infant, and a 28-day-old mouse is roughly equivalent to a newborn term human (23, 48). Ileal tissue was harvested, and PAS-positive goblet cells were counted per 100 epithelial cells (Fig. 2A). TNF-α decreased the number of mucus-containing goblet cells in P0-, P7-, and P14-day-old mice (P < 0.001). This effect was not seen in older mice (P21 and P28), suggesting that the immature small intestine is particularly susceptible to inflammation (Fig. 2B).

Because PAS indiscriminately stains glycoproteins, we stained P14-day samples with antibodies to Muc2, the major macromolecular constituent of secreted intestinal mucus (20). TNF-α caused a significant decrease in Muc2-positive epithelial cells that was equivalent to what was measured by PAS staining (P < 0.001) (Fig. 2C).

Although TNF-α reduced goblet cell numbers in our immature intestinal samples, it was unknown whether this was attributable to TNF-α-induced apoptosis. To examine this, samples were stained with antibody to active caspase 3 and positive cells were counted per 100 epithelial cells. TNF-α caused a statistically significant increase in apoptosis in P7 mice (P = 0.01). However, TNF-α caused no significant change in P0, P14, or P21 mice and caused a significant decrease in apoptosis in P28 mice (P = 0.01) (Fig. 2D). Thus, although TNF-α causes a developmentally dependent increase in apoptosis, this does not appear to have a major role in TNF-α-induced changes in mucus.

To help determine whether TNF-α was causing a decrease of mucus itself, we measured the average volume of thecal mucus in the goblet cells of each sample. This was achieved by staining samples with Alcian Blue and utilizing densitometry to determine the mean volume of mucus contained per goblet cell theca and per goblet cell volume. Using this technique, we found that mucin concentrations decreased in volume as the intestine matured (P < 0.001). We also found that, although TNF-α caused a loss of mucin-containing goblet cells, it had no significant effects on the density of thecal mucus volumes (P = 0.17, Fig. 2E) or the density of thecal mucus volumes per goblet cell (P = 0.07, Fig. 2F) that were present compared with controls. In light of a lack of either necrosis or significantly increased apoptosis in P0–14 mice, our data suggest that TNF-α was causing a complete loss of intrathecal mucus.

**TNF-α increases Muc2 and Muc3 gene expression.** We next sought to determine whether loss of Muc2 in the intestinal epithelia was attributable to decreased mucin production. Ileal samples were analyzed with quantitative PCR for Muc1, Muc2, and Muc3. TNF-α caused significant increases in Muc2 mRNA in P14 and P28 mice (P ≤ 0.03). TNF-α also caused an overall increase in Muc3 expression (P < 0.001) and specifically caused a significant increase in P14 mice (P = 0.02). Levels of Muc1 were detected with low expression at all ages, but TNF-α caused no significant changes from baseline levels (Fig. 3).

**TNF-α has no effect on Notch and Hes1 expression.** Diferentiation of intestinal stem cells into goblet cells requires Notch pathway signaling (45). To determine whether TNF-α-induced decreases in Muc2 were due to loss of goblet cell differentiation, small intestinal samples from prior experiments were analyzed with quantitative PCR for key points of the Notch pathway: Notch1, Notch2, and Hes1. At all ages, Notch and Hes1 expression was either not detected (ND) or had no significant change with exposure to TNF-α (Fig. 4).

**TNF-α has developmentally dependent effects on Spdef expression.** S-adenosyl-l-methionine-pointed domain-containing E-twenty-six family transcription factor (Spdef) was originally found in high concentrations in the prostate and functioned as an enhancer of prostate-specific antigen (44) but has since been found to be expressed in many epithelial cells including those in the small intestine (46). In the small intestine, Spdef plays a key role in regulating mucus synthesis, mucus glycosylation and packaging, and goblet and Paneth cell differentiation (10, 19, 43). To determine whether TNF-α induced changes in Spdef expression, levels, samples were analyzed with quantitative PCR for Spdef. Unlike Notch signaling, TNF-α induced developmentally dependent changes in Spdef expression. TNF-α caused a significant increase in Spdef expression in P0 mice and a significant decrease in Spdef expression in P21 mice (P = 0.04 and 0.02, respectively) (Fig. 4).

**TNF-α increases ileal mucus secretion.** We next examined the effects of TNF-α on mucus secretion. Adapting methods described by Garcia et al. (17), we harvested 4-cm segments of ileum from P14 mice and secured them in custom microvessel perfusion chambers (Fig. 5A). To determine the effects of TNF-α on mucus secretion, 100 ng/ml TNF-α was added to the serosal perfusate, and the luminal perfusate was sampled every 30 min for 3.5 h. Luminal perfusate mucin content was measured by staining the collected perfusate with PAS and comparing its optical density at 550 nm to that of a known standard of porcine stomach mucin (17). Measurements were compared with saline-treated controls. With the use of this technique, TNF-α caused a significant initial increase in secreted mucin that was sustained over 3.5 h compared with controls (P = 0.01) (Fig. 5B). This could be seen in the total amount of mucus secretion, where TNF-α caused a significant increase in total secreted mucus over the entire experiment (P = 0.01) (Fig. 5C).

**TNF-α sensitizes goblet cell secretion to PGE2.** Intestinal mucus is secreted by both constitutive and exocytotic pathways (12). Our data show that TNF induces an acute increase in mucus secretion compared with controls, but we were interested to know whether TNF had affects on subsequent exocytotic potential of the goblet cells. To determine this, we used the mucus secretion model and pretreated small intestinal segments with 100 ng/ml TNF-α for 20 min followed by 10^4 M of the secretagogue PGE2. There was no significant difference between TNF-α- and PGE2-treated samples, but samples treated with TNF-α before PGE2 experienced a significant increase in mucus secretion compared with controls (P < 0.001) (Fig. 5D). These data suggest that TNF-α causes increased secretion of small intestinal mucus as well as an increased sensitivity of the goblet cells to secretagogue such as PGE2.

**TNF-α-induced changes in small intestinal mucin expression are TNFR dependent.** TNF-α signals through two distinct receptors, TNFR1 and TNFR2 (22, 29, 36). These receptors mediate distinct effects in epithelial cells with TNFR1 promoting growth arrest and cytokine-mediated inflammation and TNFR2 promoting cellular migration and proliferation (13, 29, 30). To determine the roles of these receptors in TNF-α-induced changes in small intestinal mucus, P14-day-old TNFR1-/-, TNFR2-/-, and TNFR1-/- TNFR2-/- mice were examined for the presence of mucin-positive goblet cells using PAS and α-MUC2 staining and compared with wild-type mice.
Fig. 2. TNF-α decreases Muc2-positive small intestinal goblet cells in immature small intestine. A: C57Bl/6 mice at ages E19, P0, P7, P14, P21, and P28 were given 8-h injections with saline or 0.5 μg/g body wt TNF-α. Following euthanasia, ileal tissue was harvested and stained with PAS, and positive cells/100 epithelial cells were counted as above. N = 8 for each treatment at each age. Representative P14 slides are shown. B: TNF-α significantly decreased epithelial mucin on days P0, P7, and P14 (*p < 0.001). TNF-α had no effect on mucin presence at days E19, P21, or P28. C: P14 samples from were stained for Muc2, and positive cells/100 epithelial cells were counted. TNF-α treatment caused a significant decrease in Muc2 staining (*p < 0.001). D: samples from all ages were stained for active caspase 3, and positive cells/100 epithelial cells were counted. TNF-α significantly increased apoptosis at P7 and significantly decreased apoptosis at P28 (*p = 0.01). No statistical difference was found in apoptosis at E19, P0, P14, or P21. E: samples were next stained with Alcian Blue and analyzed with densitometry to determine the mean volume of mucus contained in the goblet cell theca of each sample. Mucin concentrations decrease in volume as the intestine matures (*p < 0.001), but TNF-α has no significant effects on mucus volumes (*p = 0.17). F: thecal densities per goblet cell were also determined and compared as in E. TNF-α again had no significant effects on mucus volumes (*p = 0.07).
controls. TNF-α had no effect on mucus positive goblet cell quantity in mice lacking TNFR1 (TNFR1−/− and TNFR1−/−-2−/−). However, TNFR2−/− mice showed goblet cell decreases comparable to wild-type controls (P < 0.001 for PAS and P = 0.004 for α-MUC2) (Fig. 6A). Representative samples of PAS-stained TNFR1−/−-2−/− mice are shown. These data suggest that TNFR1 signaling is required for TNF-α-induced secretion of mucus.

To test whether TNF-α-induced Muc2 and Muc3 mRNA upregulation was also TNFR-dependent, ileal samples from our P14 TNFR mutant mice were analyzed with quantitative PCR for Muc2 and Muc3. Whereas TNF-α significantly increased gene expression of Muc2 in P14 wild-type mice and trended toward significance in TNFR1−/− mice (P = 0.05), it had no effect on Muc2 expression in mice lacking TNFR2. Similarly, TNF-α caused an increase in Muc3 in both wild-type and TNFR1−/− mice (P = 0.02 and 0.04) but had no effect on mice lacking TNFR2 (Fig. 6B). These data show that loss of mucus-containing goblet cells is dependent on TNFR1 signaling and suggest that TNF-α-induced upregulation of Muc2 and Muc3 requires TNFR2.

**DISCUSSION**

NEC is unique to premature infants, which suggests that intestinal development may regulate susceptibility. Intestinal mucus is a major innate immune system component that protects the intestine from the type of injury seen in NEC. In this study, we provide evidence that mucin expression is altered in infants with NEC. We show evidence that TNF-α can alter the expression and secretion patterns of small intestinal mucin and that these alterations are dependent on TNFR signaling. Most importantly, we show that these TNF-α-induced changes are developmentally dependent. TNF-α treatments significantly alter mucin production, expression, and secretion differently in immature intestinal tissue compared with mature intestinal tissue. These conditional effects may play an important role in development of NEC, and TNF-α-induced changes in mucus of the immature gut may provide new insights into the pathogenesis of NEC.

Using small intestinal samples from premature human infants, we determined that mucus-containing goblet cells are decreased in infants with NEC. It was interesting to note that our human NEC samples also showed a statistically significant decrease in Paneth cells. These cells, which are an important source of TNF-α, have been linked to the pathogenesis of NEC through antibacterial activity (50). However, the role of Paneth cells in NEC is poorly understood because present rodent models of NEC use neonatal mice whose intestinal developmental stage has not matured to the point where Paneth cells are present. Because NEC is an inflammatory disease of premature infants, we wondered whether the loss of mucus-containing goblet cells seen in infants with NEC was unique to immature intestine. We were able to experimentally induce a similarly significant loss by injecting preweaning mice with
TNF-α. Although our model does not exactly mimic NEC, our model is relevant to NEC because the intestines of preweaning mice are developmentally similar to the intestines of premature human infants (23, 48), and TNF-α/H9251 is important in the initiation and propagation of NEC (8). Additionally, our model may represent an early pathophysiology in NEC that occurs before the intestinal invasion of bacteria that leads to intestinal fermentation and tissue necrosis.

Using our mouse model, we were able to determine that the developmental stage of the small intestine plays a significant role in the effect of TNF on intestinal mucus. Postnatal, preweaning mice whose small intestinal stage of development closely approximates the stage of human intestinal development that is most susceptible to develop NEC demonstrate a TNF-α-induced loss of mucus-positive goblet cells. This mucus loss may again represent the early stages leading up to the near-complete loss of mucus seen in NEC. However, TNF-α did not significantly change Spdef expression at P0 (*P = 0.01) and significantly decrease Spdef expression at P21 (*P = 0.02).

To better understand the mechanism of TNF-α-induced loss of intraepithelial mucin in premature intestine, we examined the potential contributions of TNF-α-induced apoptosis and alterations in goblet cell differentiation. The role of TNF-α-mediated apoptosis (4) was especially attractive because apoptosis is increased in animal models of NEC (28). Our results showed that TNF-α induced a significant increase in apoptosis in P7 mice, but not at other ages, and a significant decrease in P28 mice. Although apoptosis may be a contributing factor to TNF-α-induced changes in epithelial mucin, the percentage of cells affected by apoptosis is small compared with the total number of affected goblet cells, so it is unlikely that increased apoptosis is the primary determinant of TNF-α-induced mucin reduction. It is also unlikely that TNF-α alters differentiation of progenitor epithelial cells into goblet cells. Differentiation of intestinal stem cells into mucin-secreting goblet cells requires suppression of Notch signaling, leading to suppression of Hes1 (27). Our data showed that TNF-α had no significant effects on Notch or Hes1 expression. Although this does not directly rule out TNF-α-induced changes in goblet cell differentiation, our data suggest that, in the acute time period, it is not the major contributing factor. However, we recognize that TNF-α may indeed have an effect on goblet cell differentiation.

Fig. 4. TNF-α has minimal effect on genes controlling cell differentiation. C57Bl/6 mice at ages P0, P7, P14, P21, and P28 were given 8-h injections with saline or TNF-α (0.5 μg/g body wt). Ileal samples were harvested and examined by quantitative PCR analysis for Notch1, Notch2, Hes1, and Spdef (N = 3 for all time points, all samples were run in triplicate). Control levels of Notch1, Notch2, Hes1, and Spdef were either not detectible (ND) or did not significantly change over development. TNF-α had no effect on Notch1, Notch2, or Hes1 expression. However, TNF-α did significantly increase Spdef expression at P0 (*P = 0.01) and significantly decrease Spdef expression at P21 (*P = 0.02).
with longer exposures. One interesting caveat to this hypothesis is the effect of TNF-α on Spdef. Spdef is expressed in epithelial cells of the small intestine (46) and has been shown to play a key role in regulating mucin synthesis, mucin glycosylation, and packaging, and goblet and Paneth cell differentiation (10, 19, 43). Our data show that there are developmentally dependent effects of TNF-α on Spdef, but it is not clear from our data whether this is attributable to effects on cell differentiation or on mucin synthesis and packaging. Given our other supporting data, it is reasonable to hypothesize that this is indeed an effect on mucus synthesis, but further studies are necessary to elucidate this mechanism.

Many mucin secretagogues have been identified including PGE2 and inflammatory mediators (16). Specifically, TNF-α is known to stimulate PGE2 production in epithelial cells of the small intestine (46) and has been shown to act as a ligand for two distinct transmembrane receptors, TNFR1 and TNFR2, each with distinct downstream signaling pathways (22, 29, 36). Our data suggest that TNFR2 signaling may have significance for the immature intestine. Mice with immature intestines (younger than P14) exposed to TNF-α develop an organ bath model to quantify mucus secretion (34). To examine whether TNF-α-induced inflammation and potentially increased intestinal mucus without the benefit of TNF-α-induced increase in mRNA was also developmentally dependent. TNF-α increased Muc2 at P14 and P28 but not at other ages. TNF-α also increased Muc3 mRNA at P14 but not at other ages. These developmental differences in TNF-α-induced Muc gene regulation may have significance for the immature intestine. Mice with immature intestines (younger than P14) exposed to TNF-α have increased secretion of intestinal mucus without the benefit of TNF-α-induced increased mucus production seen in mature mice (P28). This leaves immature mice with a deficiency in intestinal mucin following TNF-α-induced inflammation and potentially increases their susceptibility to bacterial invasion. This may be a key reason why NEC is primarily a disease of premature infants.

TNF-α acts as a ligand for two distinct transmembrane receptors, TNFR1 and TNFR2, each with distinct downstream signaling pathways (22, 29, 36). Our data suggest that TNFR2 is required for TNF-α-induced upregulation of Muc2 and Muc3 gene expression, whereas TNFR1 is required for actual loss of intraepithelial mucus. One limitation of these studies was the

**Fig. 5.** TNF-α increases ileal mucus secretion. A: ileal samples were surgically removed from P14-day-old C57Bl/6 mice and mounted on a glass cannula in an organ bath chamber. Ileal samples were allowed to equilibrate for at least 15 min before experiments were started. Modified Krebs solution was circulated in the bath on the serosal side of the harvested ileum. TNF-α (100 ng/ml), prostaglandin E2 (PGE2) (10–4 M), or an equivalent volume of saline was added to the serosal perfusate as indicated. A separate modified Krebs solution was perfused through the ileal lumen and collected on the distal side. Perfusate was collected, stained with PAS stain, measured for optical density, and compared with a known standard of gastric porcine mucin. B: TNF-α causes a significant initial increase in secreted mucus that is sustained over 3.5 h compared with controls (*P = 0.01) and overall causes a significant increase of total mucus secretion during the entire experiment (*P = 0.01). C: Additionally, intestinal samples pretreated with TNF-α for 20 min had a significantly increased response to the secretagogue PGE2 (*P < 0.001, N = 4).
large variability we found in MUC2 and MUC3 mRNA expression levels in our TNFR2−/− mice. These data suggest that, whereas TNFR1 is required for TNF-α-mediated increased mucus secretion, TNFR2 activation is required for mucus replenishment. This hypothesis is congruent with our present understanding of TNFR signaling because TNFR1 promotes cytokine-mediated inflammation through activation of NF-κB and TNFR2 promotes intestinal restitution through cellular migration and proliferation (13, 29, 30). TNF-α and LPS activation of NF-κB has been shown to upregulate the intestine-specific transcription factor CDX2, which in turn upregulates Muc2 gene expression (26, 54). Our laboratory has also shown that TNFR1 signaling causes transient internalization of EGFR in intestinal epithelial cells (39). This may be important because EGFR signaling has been shown to increase airway mucus production (47, 52).

On the basis of these data, we propose a mechanism whereby TNF-α signaling through TNFR1 causes increased exocytotic secretion of intestinal goblet cell mucus in immature small intestine. TNF-α also causes a compensatory increase in Muc2 and Muc3 expression through TNFR2 but only in intestinal samples from P14 or older mice. This increased secretion without a compensatory increase in mucin gene upregulation in more immature intestine could predispose the intestinal epithelial layer to bacterial invasion, which is a hallmark of NEC.

In summary, we have shown that intraepithelial mucin levels are decreased in infants who have developed NEC. A similar decrease can be mimicked in mice where TNF-α induces excess Muc2 secretion in the small intestine. Although TNF-α causes an increase in mucus secretion and an increased sensitivity to secretagogues, it also causes a compensatory upregulation of mucin genes but only in more mature intestine. These
effects are mediated in specific TNFR-dependent manners with mucus gene secretion requiring TNFR1 and mucus gene up-regulation requiring TNFR2. Lastly, and perhaps most importantly, we found that TNF-α has different effects on intestinal Muc2 at different points during intestinal development. This developmentally regulated, TNF-α-induced effect on intestinal mucus may be a key reason why NEC is predominantly found in premature infants and may add to our understanding of the pathogenesis of NEC.

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