Postnatal amiotic fluid intake reduces gut inflammatory responses and necrotizing enterocolitis in perterm neonates

Siggers, Jayda Lee Ann; Østergaard, Mette Viberg; Siggers, Richard Harvey; Kristine, skovgaard; Lars, Mølbak; Thymann, Thomas; Schmidt, Mette; Møller, Hanne Kristine; Stig, Purup; Fink, Lisbeth Nielsen; Frøkiær, Hanne; Mette, Boye; Sangild, Per Torp; Bering, Stine Brandt

Published in:
American Journal of Physiology: Gastrointestinal and Liver Physiology

Publication date:
2013

Document Version
Early version, also known as pre-print

Citation for published version (APA):
Postnatal amniotic fluid intake reduces gut inflammatory responses and necrotizing enterocolitis in preterm neonates

Jayda Siggers,1 Mette V. Østergaard,1 Richard H. Siggers,1 Kerstin Skovgaard,2 Lars Mølbak,2 Thomas Thymann,1 Mette Schmidt,3 Hanne K. Møller,4 Stig Purup,5 Lisbeth N. Fink,4 Hanne Frøkiær,4 Mette Boye,2 Per T. Sangild,1 and Stine B. Bering1

1Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark; 2National Veterinary Institute, Technical University of Denmark, Copenhagen, Denmark; 3Department of Veterinary Reproduction, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark; 4Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark; and 5Department of Animal Science, Aarhus University, Tjele, Denmark

Submitted 5 July 2012; accepted in final form 11 March 2013

Necrotizing enterocolitis (NEC) is a devastating disease of premature infants afflicting 1–5% of all newborns, and up to 7–14% of very low birth weight infants (500–1,500 g) admitted to neonatal intensive care (42). Risk factors for NEC include prematurity, enteral formula feeding, and bacterial colonization. Together these factors result in an exaggerated inflammatory response, leading to extensive hemorrhagic inflammatory necrosis, especially in the distal small intestine and proximal colon (31). Maternal milk has repeatedly been shown to reduce the risk of NEC, and the beneficial effects of maternal milk are believed to be due to antibacterial and immunomodulatory properties (6, 37, 39).

Maternal milk, especially colostrum, is capable of stimulating normal gastrointestinal development and maturation, together with regulating an immature gut immune system (14). These effects are largely dependent on the presence of colostrum-derived antimicrobial proteins (i.e., immunoglobulins, κ-casein, lysozyme, lactoferrin), and anti-inflammatory cytokines and growth factors (e.g., the anti-inflammatory cytokine IL-10, TGF-β, and EGF) (20, 22, 25). Many of the bioactive factors contained in maternal milk are also present in amniotic fluid (AF) (15). In utero, these bioactive AF peptides may play an important role in both fetal gastrointestinal development (3, 20, 30) and innate immunity development (1, 57). AF contains high concentrations of immunomodulatory peptides such as IL-10 (2), suggesting that AF helps to suppress inflammatory responses. Moreover, AF contains growth factors such as EGF, IGF-I, and TGF, indicating that AF plays a vital role in gastrointestinal development in utero (27, 55), as documented by reduced fetal intestinal growth after lacking exposure to amniotic fluid (38, 52). Hence, there are similarities between the prenatal biology of AF and the postnatal biology of maternal milk. For example, bovine, porcine, and human AF have all been shown in vitro to reduce the production of proinflammatory cytokines (IL-6, IL-12, and TNF-α) in bacteria-stimulated murine dendritic cells in a similar manner as bovine and porcine colostrum whey (28a). Furthermore, a simulated human amniotic fluid-like test solution, containing the enterocyte growth factors erythropoietin and granulocyte-colony stimulating factor, has been suggested to improve tolerance to milk feeding in preterm neonates (4).

Following preterm birth, maternal milk production is often limited and requires supplementation with infant formula or banked mature human milk during the difficult transition from parenteral to postnatal enteral feeding. Previously, we have documented in our preterm pig model of NEC, that enteral feeding induces histopathological changes and inflammatory responses within a few hours of enteral feeding initiation and that these are most pronounced with a formula diet devoid of the bioactive factors in mother’s milk (7, 43). In this model, only a very gradual transition to an enteral colostrum diet appears to prevent against NEC (13). However, AF has recently been shown to decrease NEC severity in the 10-day-old.

Address for reprint requests and other correspondence: S. B. Bering, Dept. of Nutrition, Exercise and Sports, 30 Rolighedsvej, DK-1958 Frederiksberg C, Denmark (e-mail: sbs@life.ku.dk).
hypoxic mouse model of NEC via Toll-like receptor (TLR) 4 and EGF receptor-dependent pathways (17). Given the aforementioned similarities between AF and colostrum, we wanted to test whether enteral AF could prevent feeding intolerance and overt intestinal inflammation in a model that incorporates preterm birth, parenteral to enteral feeding, and spontaneous NEC development. We hypothesized that minimal enteral feeding with porcine AF during parenteral nutrition, coupled with the presence of AF in formula, inhibits bacteria-dependent inflammatory responses and NEC development. To determine the effects of enteral AF, we investigated the impact of AF on the intestinal structure, function, microbial ecology, and immunological responses of the preterm pig during the parenteral to enteral transition phase. A preliminary account of the findings was published previously (44). In support of the in vivo pig model of NEC, we performed in vitro dendritic cell and intestinal epithelial cell culture studies to further verify the potential anti-inflammatory and epithelial repair properties of AF.

**MATERIALS AND METHODS**

**Preterm Caesarean Section and AF Collection**

Thirty preterm pigs were obtained from three litters delivered by caesarean section at 105–107 days of gestation (Danish Landrace × Yorkshire × Duroc, Askelygaard Farm, Roskilde, Denmark; term = 116 ± 2 days). Surgery, rearing, and catheterization of piglets were performed as previously described (39). Porcine AF was collected aseptically, centrifuged, and stored (−80°C) from the above sows as well as others during late gestation (experiment 1: 90–92 days gestation; experiment 2: 105–106 days of gestation, 500–1,000 ml per sow). The National Committee on Animal Experimentation in Denmark approved all procedures.

**Nutrition Protocols and Treatments**

**Experiment 1: AF supplementation during TPN and enteral nutrition.** Immediately after birth, pigs were stratified according to body weight and sex and randomly assigned to three treatment groups defined by the later enteral feeding regimen (15 ml/kg⁻¹·h⁻¹): porcine colostrum (COLOS, n = 7), control formula (FORM, n = 13), or formula supplemented with porcine AF (AF, n = 10). The colostrum pigs were used as a reference group to indicate the state of pigs fed an optimal diet under the given conditions. For the first 48 h total parenteral nutrition (TPN) was provided as previously described (45). During the TPN period, boluses of pure porcine AF or sterile water (placebo) were administered (10 ml/kg) every 3 h. Following the TPN period, enteral formula or colostrum was provided every 3 h as previously described (23). For the AF group, the water fraction (80%) of the formula was replaced with AF.

**Experiment 2: AF supplementation only during enteral nutrition.** Pigs were randomly assigned into two treatment groups defined by the later enteral feeding regimen: control formula (FORM, n = 16) or formula supplemented with porcine AF (AF, n = 14); TPN was provided exclusively for the first 48 h and then gradually reduced with initiation of gradual enteral formula feeding provided every 3 h to reach 15 ml/kg after 9 h.

**Tissue Collection and NEC Evaluation**

At tissue collection, the gastrointestinal tract was evaluated macroscopically for signs of NEC. The following scoring system was applied to characterize the extent of damage, as indicated by hemorrhage and/or necrosis that occurred in the stomach, small intestine (proximal, mid, and distal), and colon: 0, no damage; 1, occasional areas of violaceous mucosa (0–25% affected); 2, multiple areas of violaceous mucosa (25–50% affected); 3, severe hemorrhagic mucosa (50–75% affected); 4, extensive hemorrhagic mucosa (>75% affected), with or without areas of necrosis.

Samples from the gastrointestinal tract were collected and stored as previously described (45). For histological (7) and in situ hybridization (46) analyses, tissue samples were processed as previously described. For enzyme activity and gene expression analyses, tissue samples were snap frozen in liquid nitrogen. Villous height and crypt depth were evaluated (7) and disruption of normal mucosal architecture was noted. Tissue sections were evaluated for the presence of eight characteristics of NEC: submucosal edema, vacuolization of the enterocytes, congestion of vessels, regional villi sloughing, loss of villi, hemorrhage, pyknotic nuclei, and infiltration of inflammatory cells. One point was given for each characteristic present. The evaluators were blinded to treatment group. A cumulative NEC disease score was calculated by summing the gross pathological score for each region with the histopathological score for the distal intestinal tissue section. Pigs with a cumulative score of 12 or greater were considered to suffer from NEC (range: 6–38). The NEC scoring system and the associated histopathology have been described in detail earlier (43).

**Intestinal Enzyme Activities and Microbiology**

Activities of brush-border peptidases [aminopeptidase A (ApA), aminopeptidase N (ApN), dipeptidylpeptidase IV (DPPIV)], and disaccharidases (lactase, maltase, sucrase) were all measured as previously described (40).

The density and location of bacteria adhering to the mucosa were investigated by using specific bacterial oligonucleotide probes and fluorescent in situ hybridization on formaldehyde-fixed sections of distal small intestine, as previously described (46). Additionally, the bacterial assemblage of the colon contents was analyzed by terminal-restriction fragment (T-RF) length polymorphism (T-RFLP) using colon contents as previously described (45), with minor modifications. Bacterial DNA was extracted and purified using QIAamp DNA Mini Kit (Qiagen, Ballerup, Denmark), as described in the manufacturer’s protocol, and gels were analyzed on an automatic sequence analyzer (Applied Biosystems Genetic Analyzer 3130/3130xI, Nærum, Denmark).

**In Vivo Absorption and Gut Permeability**

Gastrointestinal function was assessed by measuring the concentration of blood galactose following oral administration of a bolus of galactose. The galactose test was performed after the transition to enteral formula feeding (5 h after birth). An oral bolus (15 ml/kg) of 5% galactose was given and an arterial blood sample drawn after 20 min followed by analysis of blood plasma galactose concentration as described in detail elsewhere (51). Gastrointestinal permeability was tested at the time of euthanasia. Pigs received an enteral bolus (15 ml/kg) of 5% lactulose and 5% manitol 3 h before euthanasia and urine was collected at euthanasia. Urine manitol and lactulose contents were determined as described previously (51).

**RNA Extraction**

Total RNA from snap frozen middle and distal small intestine was thawed in RNAiqe and then extracted by using RNaseasy Midi Kit with TRIZOL reagent (Qiagen) and total RNA from IPEC-J2 cells was extracted by use of AllPrep DNA/RNA Mini Kit (Qiagen). Concentration and OD260/280 ratio of extracted total RNA was measured using a Nanodrop ND-1000 spectrophotometer (Saveen and Werner AB, Limhamn, Sweden). Quality of extracted total RNA was estimated by Agilent 2100 bioanalyzer using the Nanochip 6000 (Agilent Technologies, Nærum, Denmark).
Microarray Analysis

Pigs from each treatment group were randomly selected for microarray analysis of frozen distal small intestine samples (COLOS, n = 6; FORM, n = 6; and AF, n = 6). The FORM group was further divided into formula-fed healthy pigs (F-HEA, n = 3) and formula-fed NEC pigs (F-NEC, n = 3), to compare sick vs. healthy formula-fed pigs. Samples and reference pools were labeled with Oyster 550 (FORM), or formula + amniotic fluid. Means in a row with superscripts without a common letter differ significantly (P < 0.05).

Quantitative Real-Time PCR

Validation of microarray results and supplementary expression analysis of a small group of other inflammation-related genes were performed on tissue from the middle and distal intestine [TLR-4, IFN-γ, IL-1α, IL-8, TNF-α, IL-6, Toll interacting protein (TOLLIP), myeloid differentiation primary response gene 88 (MYD88), lysozyme (LYZ), LPS binding protein (LBP), prepro β defensin 1 (PBD1), TNF-related apoptosis-induced ligand (TRAIL), aminopeptidase N (ANPEP)]. Extracted RNA was converted into cDNA by reverse transcription of 1,000 ng total RNA by using Quantitect Reverse Transcription Kit (Qiagen) as described previously (47) and was stored at -20°C until use. Quantitative real-time PCR (qPCR) was performed by using the Fast Real-Time PCR System (Applied Biosystems) to quantify changes in gene expression. Primers and hydrolysis probes were designed with use of PrimerExpress software v2.0 (Applied Biosystems) and synthesized at Applied Biosystems (Supplementary Data Table S1). Sequences used for primer and probe design were obtained from public databases (GenBank, NCBI and TIGR, Institute for Genome Research).

A normalization factor for each pig was calculated based on the stability of the two best reference genes [phosphoglycerate kinase 1 (PGK1) and β-actin (ACTB)] of a panel of five putative endogeneous reference genes [β-2-microglobulin (B2M), ACTB, PGK1, 18S rRNA subunit (RPS18), and TATA box binding protein (TBP)] using geNorm (54). For IPEC-J2 cell samples, RPS18 was used for relative quantification calculations. To compare the fold change in gene expression between the treatment groups, normalized gene expression values for the treatment groups were compared relative to the negative control (set to 1, COLOS pigs).

Intestinal Cytokine Analysis

Samples from the distal small intestine were homogenized in RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris base) by using the gentleMACS Dissociator (MACS, Miltenyi Biotec), and homogenates were centrifuged for 10 min at 12,000 g and 4°C. Concentrations of IL-6 and IL-8 were quantified by use of porcine IL-6 and IL-8 DuoSet ELISA Development Kits (R&D Systems) according to the
manufacturer’s protocol and expressed as cytokine content per tissue weight.

**In Vitro Dendritic Cell and Enterocyte Studies**

Cell studies were aimed to characterize the general immunomodulating effects of porcine AF by using three well-established intestinal cell systems previously used to detect effects of dietary and bacterial components on isolated intestinal cells (10, 34, 41). As such, the results are limited to the immunomodulatory actions of AF in these isolated in vitro epithelial cells and may provide ideas for AF effects at the cellular level, although results cannot be used to predict the full in vivo effects of AF on the immature pig intestine.

For analysis of cytokine synthesis in dendritic cells, bone marrow-derived murine dendritic cells (DCs) were generated and stimulated as previously described (10). Briefly, to DCs (10^6 cells/500 μl) were added 10 μg/ml of UV-killed bacteria [Clostridium perfringens NECA20 (a pure type A isolate from a premature piglet with NEC) or Escherichia coli Nissle (strain 1917, serotype O6:K5:H1)] with or without porcine AF (0.9, 2.8, 8.3, or 25% vol/vol). Porcine AF from two different sows was tested. IL-10 and TNF-α content in supernatants harvested 18–20 h after stimulation were determined by DuoSet ELISA Kits (R&D Systems, Minneapolis, MN). The upregulation of CD40 and CD86 surface markers was measured 16 h after stimulation by flow cytometry. Phycoerythrin-conjugated anti-CD40 (eBioscience, San Diego, CA), allophycocyanin-conjugated anti-CD86 antibodies (Southern Biotech, Birmingham, AL), and appropriate isotype control antibodies were used. Cell viability was checked by incubating the stimulated DCs with the DNA stain 7-amino-actinomycin D (BD Biosciences, San Jose, CA) and was consistently >80%.

For analysis of cytokine expression in intestinal epithelial cells, IPEC-J2 cells were derived as previously described (41). Cells were seeded at 1 × 10^6 cells/cm² on 12-mm diameter Collagen-Coated Transwell membranes of pore size 0.4 μm (Corning Incorporated Life Sciences, Acton, MA). During culture 5 μg/ml insulin and 5 μg/ml EGF were added to the medium and transepithelial electrical resistance (TEER) was measured to follow cell polarization. Cells were stimulated 14 days after culture when TEER reached maximum. IPEC-J2 cells were washed in PBS (Sigma-Aldrich) and incubated with 10 ng/ml LPS (E. coli K-235, Sigma-Aldrich) in culture medium (without antibiotics) with or without 25% (vol/vol) porcine AF for 4 h at 37°C in a 5% CO2 humidified atmosphere. After incubation, cells

---

**Fig. 2.** A: graphical presentation of the dominating bacteria terminal restriction fragments (T-RFs) in colon contents of healthy pigs fed colostrum (COLOS) or formula-amniotic fluid (AF), and in formula-fed pigs. T-RFs with a mean intensity below 200 were excluded. Significant differences between treatment groups are indicated (*P < 0.05). B: principal component (PC) analysis plot of bacteria terminal restriction fragment length polymorphism (T-RFLP) data for the microbial communities of colon contents FORM (■), AF (□) and COLOS (○) pigs. The 2 components (x = 24% and y = 6%) separated the microbial communities on the basis of quantitative band patterns.
were harvested with trypsin-EDTA (Sigma-Aldrich), washed in PBS, snap frozen in liquid nitrogen, and stored at −80°C until further analyses of cytokine mRNA (IL-6, TNF-α).

For cell proliferation, IEC-6 cells (DSMZ, Braunschweig, Germany) were cultured in 96-well plates (3,000 cells/well) in Dulbecco’s Minimal Essential Medium (GIBCO, Life Technologies, Nærum, Denmark), supplemented with 10% fetal calf serum (FCS, Cambrex Bio Science, Copenhagen, Denmark), 0.4 mmol/l sodium pyruvate (Invitrogen, Life Technologies), 2 mmol/l Glutamax (Invitrogen), and penicillin-streptomycin solution (Sigma-Aldrich) for 24 h. At 60–80% confluence, cells were washed with PBS followed by incubation with 0–15% (vol/vol) porcine AF in culture medium without FCS for 72 h. Cell proliferation was determined by a resazurin metabolism assay according to the manufacturer’s instruction (AlamarBlue, BioSource, AH diagnostics, Aarhus, Denmark). Experiments were performed for AF collected from four different sows in quadruplicate wells in two replicate assays.

For the cell migration assay in IEC-6 cells, a scratch wound assay was performed as described earlier (34). Cells were seeded in 12-well plates (200,000 cells/well), and monolayers were scratched with a 200-μl pipette tip after 1 day to create a standardized cell-free area. Cells were washed and incubated with 0–15% (vol/vol) porcine AF in culture medium without FCS. Serum-free medium and medium containing 10% FCS served as negative and positive control, respectively. The migration distance was determined by photomicrographs after wounding and after 5 h. Experiments were performed for AF collected from four different sows in six measurements in each of two replicate wells in two replicate assays.

Statistical Analysis

Data were analyzed by using SAS (SAS/STAT version 8.1, SAS Institute, Cary, NC) and R (version 2.15.0) for experiments 1 and 2, respectively. Treatment (FORM, AF, and COLOS) and intestinal region (proximal, middle, and distal) were considered as fixed effects, and pig and litter were included as random effects. When no significant effect of region was detected, data were pooled and analyzed across regions. The results in tables and figures are given as the least square means ± SE, and differences between two means were tested by Fisher’s exact test, with subsequent pairwise comparisons. Data from the in vitro experiments were evaluated by one-way ANOVA with Dunnett’s multiple comparison post hoc test using GraphPad Prism (version 5.0, GraphPad Software, Chicago, IL) for DC experiments and Holm-Sidak multiple comparison test using SigmaPlot (version 11.0, Systat software, Chicago, IL) for intestinal epithelial cell assays. For T-RFLP data comparisons between individual T-RFs, a two-tailed Monte Carlo Estimates Mann-Whitney U-test and Kruskal-Wallis tests were used (SAS Institute, Cary, NC). T-RFs smaller than 60 bp and larger than 820 bp were excluded because these were outside the range of the standards. BioNumerics version 4.0 (Applied Math, Sint-Martens-Latem, Belgium) generated principal component analysis (PCA) on the quantitative band values. Division by the variances over the entries and subtraction of the averages over the characters was included in the PCA. Identification of specific bacteria characterized by T-RFs was done in silico by inserting primer sequences and restriction enzymes in the MiCA home page (http://mica.ibest.uidaho.edu.digest.php) by using the RDPII database (Release 9, Update 37, Bacterial SSU 16S rRNA). IL-8 data were log-transformed before analyses. P < 0.05 was used as the critical level of significance for all statistical evaluations.

RESULTS

Clinical Observations, Histology, and NEC Incidence

In experiment 1, the highest NEC incidence was found in the FORM group (54%, 7/13). The lowest incidence was observed in COLOS pigs (0%, 0/7), with intermediate incidence in AF pigs (20%, 2/10). NEC severity in FORM pigs (17.3 ± 2.0) was higher than in AF pigs (9.9 ± 2.2, P < 0.05) and COLOS pigs (7.7 ± 2.7, P < 0.01). AF pigs had lower cumulative NEC scores compared with FORM pigs (P < 0.05). The two pigs defined as suffering from NEC in the AF group had NEC scores just above the defined cutoff (scores equal to 13 and 16; NEC defined as ≥12), indicating only minor intestinal lesions whereas all FORM pigs with NEC had NEC scores >20. Weight gain over the experiment was lower for COLOS (79 ± 20 g) and FORM (34 ± 15 g) pigs than for AF pigs (143 ± 17 g, P < 0.05).

Compared with COLOS pigs, mucosal dry weight was lower in FORM and AF pigs (−8%, P < 0.05; Table 1) but villous heights and crypt depths did not differ across treatments. For AF pigs, spleen weights (1.88 ± 0.39 g/kg) and kidney weights (7.25 ± 0.67 g/kg) were reduced, relative to values in FORM pigs (2.37 ± 0.44 and 8.67 ± 0.68 g/kg, respectively, P < 0.05), whereas no effects were seen for weights of other internal organs (intestine, colon, heart, lung, pancreas, and spleen). There were increases in the intestinal activities of lactase (+42 and 44%), maltase (+59 and 63%), DPPIV (+13 and 25%), and Apn activity (+25 and 28%, only distal small intestine) in COLOS pigs, compared with FORM and AF pigs (all P < 0.05; Table 1), which did not differ.

In experiment 2, the incidence of NEC was 50% in both the FORM (8/16) and the AF (7/14) pigs, with no differences in regional, mean, or total NEC scores. A relative loss in body weight over the experiment was observed in both AF pigs (−59 ± 15 g/kg) and FORM pigs (−24 ± 14 g/kg), with no difference between the groups (P = 0.09).

Mucosal dry weight was the same in AF and FORM pigs. Crypt depth in the distal small intestine was higher in the AF compared with FORM pigs (106 ± 2 vs. 96 ± 2 μm, P < 0.005), whereas villous heights did not differ across treatments. Neither was any effect seen for weights of the internal organs (intestine, heart, lungs, liver, kidneys, and spleen). There were no effects for weights of other internal organs (intestine, colon, heart, lung, pancreas, and spleen).

Table 2. Significance values for the bacteria terminal restriction fragments in distal intestinal tissues based on pairwise comparisons between groups of pigs fed colostrum, formula+amniotic fluid, and formula

<table>
<thead>
<tr>
<th>T-RF</th>
<th>FORM vs. COLOS</th>
<th>FORM vs. AF</th>
<th>COLOS vs. AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>0.0155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>0.0140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>102</td>
<td></td>
<td>0.0137</td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>0.0270</td>
<td>0.0326</td>
<td></td>
</tr>
<tr>
<td>189</td>
<td></td>
<td></td>
<td>0.0105</td>
</tr>
<tr>
<td>191</td>
<td></td>
<td></td>
<td>0.0478</td>
</tr>
<tr>
<td>193</td>
<td>0.0030</td>
<td></td>
<td>0.0197</td>
</tr>
<tr>
<td>199</td>
<td></td>
<td></td>
<td>0.0419</td>
</tr>
<tr>
<td>205</td>
<td></td>
<td></td>
<td>0.0451</td>
</tr>
<tr>
<td>215</td>
<td>0.0424</td>
<td></td>
<td></td>
</tr>
<tr>
<td>219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>231</td>
<td>0.0311</td>
<td></td>
<td>0.0187</td>
</tr>
<tr>
<td>233</td>
<td>0.0586</td>
<td></td>
<td></td>
</tr>
<tr>
<td>237</td>
<td>0.0002</td>
<td>0.0040</td>
<td></td>
</tr>
<tr>
<td>351</td>
<td></td>
<td>0.0322</td>
<td>0.0103</td>
</tr>
<tr>
<td>363</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>576</td>
<td>0.0257</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T-RF, terminal restriction fragment; Arrows show the direction of changes. Proposed identity for T-RF 233: Clostridium perfringens.
Table 3. Significance values for differentially regulated genes in distal intestinal tissues based on pairwise comparisons between groups of healthy pigs fed colostrum or formula + amniotic fluid, healthy formula-fed pigs, and formula-fed pigs with necrotizing enterocolitis by microarray analysis

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>AF vs. F-HEA</th>
<th>AF vs. F-NEC</th>
<th>F-HEA vs. COLOS</th>
<th>F-NEC vs. COLOS</th>
<th>AF vs. COLOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM17</td>
<td>ADAM metallopeptidase domain 17 (tumor necrosis factor, alpha, converting enzyme)</td>
<td>0.015 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQP1</td>
<td>aquaporin 1</td>
<td>0.036 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>angiopoietin-like 4</td>
<td>0.013 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANPEP</td>
<td>aminopeptidase N</td>
<td>0.007 ▼</td>
<td>0.021 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANXA11</td>
<td>annexin A11</td>
<td>0.001 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOA1</td>
<td>apolipoprotein A-I</td>
<td>0.035</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVEN</td>
<td>apoptosis, caspase activation inhibitor</td>
<td>0.030 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIRC5</td>
<td>baculoviral IAP repeat-containing 5 (survivin)</td>
<td>0.040 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
<td>0.019 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4A</td>
<td>complement component 4A</td>
<td>0.014 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASP8</td>
<td>caspase 8, apoptosis-related cysteine peptidase</td>
<td>0.018 ▼</td>
<td>0.043 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD1A</td>
<td>CD1a molecule</td>
<td>0.033 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>CD2 molecule</td>
<td>0.003 ▼</td>
<td>0.005 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD247</td>
<td>CD247 molecule</td>
<td>0.033 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD55</td>
<td>decay-accelerating factor CD55 (DAF)</td>
<td>0.020 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFLAR</td>
<td>CASP8 and FADD-like apoptosis regulator</td>
<td>0.051 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein, pentraxin-related</td>
<td>0.008 ▼</td>
<td>0.002 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL3</td>
<td>chemokine (C-X-C motif) ligand 3</td>
<td>0.037 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEFB4</td>
<td>defensin, beta 4</td>
<td>0.026 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
<td>0.050 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENPEP</td>
<td>glutamyl aminopeptidase (aminopeptidase A)</td>
<td>0.010 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCGR3A</td>
<td>Fc fragment of IgG, low affinity IIIa, receptor</td>
<td>0.003 ▼</td>
<td>0.003 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGA</td>
<td>fibrinogen alpha fragment</td>
<td>0.016 ▼</td>
<td>0.050 ▼</td>
<td>0.050 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTH1</td>
<td>ferritin, heavy polypeptide 1</td>
<td>0.049 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.021 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGTA1</td>
<td>glycprotein, alpha-galactosyltransferase 1</td>
<td>0.005 ▼</td>
<td>0.030 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPX</td>
<td>hemopexin</td>
<td>0.035 ▼</td>
<td>0.038 ▼</td>
<td>0.030 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNA1</td>
<td>interferon, alpha 1</td>
<td>0.005 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNG</td>
<td>interferon, gamma</td>
<td>0.034 ▼</td>
<td>0.044 ▼</td>
<td>0.036 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL10RB</td>
<td>interleukin 10 receptor, beta</td>
<td>0.009 ▼</td>
<td>0.030 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1A</td>
<td>interleukin 1, alpha</td>
<td>0.026 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL2RG</td>
<td>interleukin 2 receptor, gamma</td>
<td>0.023 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL4R</td>
<td>interleukin 4 receptor</td>
<td>0.015 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF3</td>
<td>interferon regulatory factor 3</td>
<td>0.004 ▼</td>
<td>0.020 ▼</td>
<td>0.003 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITGB3</td>
<td>integrin beta 3</td>
<td>0.008 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMB2</td>
<td>laminin, beta 2</td>
<td>0.044 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
<td>0.050 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHA</td>
<td>lactate dehydrogenase A</td>
<td>0.028 ▼</td>
<td>0.035 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHC</td>
<td>lactate dehydrogenase C</td>
<td>0.023 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYZ</td>
<td>lysozyme</td>
<td>0.011 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK8</td>
<td>mitogen-activated protein kinase 8</td>
<td>0.014 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDH2</td>
<td>malate dehydrogenase 2, NAD (mitochondrial)</td>
<td>0.021 ▼</td>
<td>0.002 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP14</td>
<td>matrix metallopeptidase 14</td>
<td>0.027 ▼</td>
<td>0.054 ▼</td>
<td>0.049 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC1</td>
<td>mucin 1, cell surface associated</td>
<td>0.048 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUC5AC</td>
<td>mucin 5AC, oligomeric mucus/gel-forming</td>
<td>0.022 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYD88</td>
<td>myeloid differentiation primary respose gene 88</td>
<td>0.028 ▼</td>
<td>0.051 ▼</td>
<td>0.018 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOL3</td>
<td>nucleolar protein 3 (apoptosis repressor with CARD domain)</td>
<td>0.027 ▼</td>
<td>0.054 ▼</td>
<td>0.049 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS1</td>
<td>nitric oxide synthase 1 (neuronal)</td>
<td>0.001 ▼</td>
<td>0.02 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS2A</td>
<td>nitric oxide synthase 2A (inducible)</td>
<td>0.004 ▼</td>
<td>0.02 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOS3</td>
<td>nitric oxide synthase 3 (endothelial cell)</td>
<td>0.008 ▼</td>
<td>0.039 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCLN</td>
<td>occludin</td>
<td>0.031 ▼</td>
<td>0.029 ▼</td>
<td>0.009 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLG</td>
<td>plasminogen</td>
<td>0.009 ▼</td>
<td>0.015 ▼</td>
<td>0.044 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSMB9</td>
<td>proteasome subunit, beta type, 9</td>
<td>0.004 ▼</td>
<td>0.02 ▼</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL13</td>
<td>ribosomal protein L13</td>
<td>0.012 ▼</td>
<td>0.032 ▼</td>
<td>0.005 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL32</td>
<td>ribosomal protein L32</td>
<td>0.012 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SABP</td>
<td>soluble angiotein-binding protein</td>
<td>0.005 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFTPD</td>
<td>surfactant associated protein D</td>
<td>0.037 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC5A1</td>
<td>solute carrier family 5</td>
<td>0.037 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPP1</td>
<td>secreted phosphoprotein 1 (osteopontin)</td>
<td>0.003 ▼</td>
<td>0.034 ▼</td>
<td>0.020 ▼</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRY</td>
<td>sex determining region Y</td>
<td>0.016 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST3GAL4</td>
<td>ST3 beta-galactoside alpha-2,3-sialytransferase 4</td>
<td>0.016 ▼</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
increases in the distal small intestinal activities of lactase (44%) and ApN (28%) in FORM pigs compared with AF pigs. On the contrary, maltase activity was increased in the proximal (42%) and middle (39%) small intestine in AF pigs compared with FORM pigs (Table 1). In vivo intestinal barrier function did not differ between AF and FORM pigs. Furthermore, no difference was observed in galactose absorption during TPN on day 3 (A). A t-test was performed to compare differences in TPN-galactose absorption between AF and FORM pigs. Furthermore, no difference was observed in galactose absorption during TPN on day 5. A t-test was performed to compare differences in TPN-galactose absorption between AF and FORM pigs. In vivo intestinal barrier function did not differ between AF and FORM pigs. Furthermore, no difference was observed in galactose absorption during TPN on day 3. A t-test was performed to compare differences in TPN-galactose absorption between AF and FORM pigs.

Microarray Analysis

ANOVA was performed to identify genes differentially expressed among COLOS (n = 6), AF (n = 6), formula-fed NEC pigs (F-NEC, n = 3), and formula-fed healthy pigs (F-HEA, n = 3) in experiment 1 (Table 3). Thirty-five differentially expressed genes (18 up, 17 down) were detected when AF pigs were compared relative to F-NEC pigs (indicating the combined effect of diet and NEC pathology). Several genes known to be involved in the innate immune response including IL-1α, inducible nitric oxide synthase (NOS2), and TNF-α was found to be significantly downregulated in AF pigs compared with F-NEC pigs (P < 0.01). Comparison of AF with F-HEA (indicating mainly the effect of diet), showed 24 genes that were differentially regulated (11 up, 13 down), including increased lysozyme and occludin, and decreased vascular endothelial growth factor, interferon-α1, and nitric oxide synthase 1 and 3 (neuronal and endothelial nitric oxide, respectively). Nineteen of the 24 differentially regulated genes (79%) in AF compared with F-HEA pigs were different from those differentially regulated in AF compared with F-NEC pigs, suggesting a separate effect of AF under healthy conditions, independent from the NEC-preventive effect. Seventeen differentially expressed genes (9 up, 8 down) were detected when AF pigs were compared relative to COLOS pigs.

Comparison of COLOS pigs relative to F-NEC pigs (indicating the combined effect of diet and NEC pathology), showed 28 genes that were differentially regulated (15 up, 13 down), including increased aminopeptidase N, survivin, occludin, osteopontin, TLR-1, TLR-10, and Tollip expressions, and decreased IL-α, mitogen-activated protein kinase 8 (MAPK8), mucin-5AC (MUC5AC), MYD88, TRAIL, and TNF-α. Nine of the 14 differentially regulated genes (64%) in F-HEA compared with COLOS pigs were different from those differentially regulated in F-NEC compared with COLOS pigs, again suggesting that diet and NEC pathology induce partly independent effects on the immature intestine. Sixteen of the 28 genes (58%) were found to be similar to and regulated in the same direction as differentially expressed genes detected in AF pigs compared with F-NEC pigs, indicating that some of the immune mechanism might be similar in response to dietary amniotic fluid and colostrum administration. Genes identified as differentially expressed in groups of healthy pigs (COLOS) and NEC pigs (F-NEC) were imported into Ingenuity Pathway Analysis.
Analysis (IPA) software, and the most significant functional network was associated with inflammatory disease and cell death. This IPA confirmed TNF-α to be in a central position, acting on several other cytokines and transmembrane receptors.

Quantitative Real-Time PCR

When tissues from AF pigs were compared with all the animals from the FORM group (both sick and healthy) in experiment 1, there were no changes in gene expression patterns. However, qPCR analyses on tissues from the middle intestine confirmed the proinflammatory cytokines IL-1α (−54%), TNF-α (−41%), and IL-6 (−59%) to be significantly downregulated in AF pigs, compared with F-NEC pigs (all \( P < 0.05 \)). Also the neutrophil chemoattractant IL-8 was found to be significantly lowered in AF pigs (−46%, \( P < 0.05 \)). Similarly, in AF and COLOS pigs, relative to F-NEC pigs, TNF-α (−56 and −58%, respectively) and IL-1α (−63 and −64%, respectively) in the distal small intestine were also downregulated (all \( P < 0.05 \)). Furthermore, MYD88 (−36%) was downregulated in AF pigs and ANPEP was upregulated (+68%) in COLOS pigs (all \( P < 0.05 \)).

In Vitro Dendritic Cell and Enterocyte Studies

Following both gram-positive (\textit{C. perfringens NECA20}) and gram-negative (\textit{E. coli Nissle}) bacterial stimulation of DCs, AF differentially modulated the DC cytokine responses. In response to stimulation by both bacteria, TNF-α production showed a dose-dependent decrease as AF concentrations increased (Fig. 3), highlighting the specific anti-inflammatory properties of AF, whereas the production of IL-10 was unaffected by AF at all concentrations used (data not shown). Furthermore, DCs stimulated with both bacteria showed an increased expression of the costimulatory molecules CD86 and CD40 (demonstrated as an increase in fluorescence intensity), both of which are important for T cell activation. When coincubated with 25% AF, DC CD86 and CD40 expression was downregulated (Fig. 4), indicating reduced DC maturation.

IPEC-J2 cells stimulated with LPS showed a significant upregulation in expression of TNF-α and IL-6 mRNA compared with AF-stimulated cells (+26 and 45%, respectively, \( P < 0.05 \)). The differential IL-6 gene expression was significantly downregulated when coincubating the cells with both LPS and AF, relative to LPS alone (−50%, \( P < 0.05 \)). The same trend was seen for TNF-α expression (−15%, \( P = 0.12 \)).

Addition of AF to IEC-6 cells induced a dose-dependent increase in intestinal cell proliferation and migration (Fig. 5). Proliferation reached maximum at 10% AF (+21% relative to control, \( P < 0.01 \)), whereas migration continued increasing at 15% AF (+180% relative to control, \( P < 0.001 \)).

**Discussion**

During late gestation the human fetus swallows ~800 ml AF per day (33). This provides enteral nutrition (15–20% of body metabolic needs) as well as immunoregulatory, antimicrobial, and growth-promoting factors for immature enterocytes (53). In this manner, AF intake in utero functions to prepare the gut for the dramatic shift from a highly controlled in utero environment to the heavily burdened environment present immediately after birth (38, 52). Many of the beneficial factors present in AF are also present in maternal milk, and maternal milk (colostrum) has repeatedly been shown to prevent NEC in preterm infants, which is a leading cause of morbidity and mortality in neonatal intensive care units (26). Using our preterm pig model of NEC, we have shown that gradually increasing amounts of colostrum after a few days of total parenteral nutrition protects against NEC (13), whereas a more abrupt transition to enteral feeding is associated with rapid inflammatory responses, especially when using a formula diet (7, 29, 43). We now show that, similar to colostrum, postnatal administration of porcine AF as minimal enteral nutrition to preterm neonates is able to increase body weight gain, alter bacterial colonization and NEC severity, and induce differential expression of mRNA coding for genes involved in gut inflammatory responses. The beneficial effects of AF were seen only when provided both as minimal enteral nutrition during the TPN period and the following enteral nutrition period, whereas providing AF therapeutically only during the enteral period did not show consistent effects. The immunemodulating and growth-stimulating properties of porcine AF were further confirmed in vitro by using murine dendritic cells and neonatal porcine and adult rat intestinal epithelial cells. These results indicate that AF may be a useful supplement to the enteral diet during the parenteral-to-enteral transition phase in preterm neonates, particularly when maternal milk is not available. Further investigations are clearly required before this can be tested in infants (e.g., collection and preparation of human AF, time of administration, and the optimal dose), but these results open an intriguing new possibility for improved

![Fig. 3. Murine dendritic cell production (mean ± SD) of TNF-α following costimulation with porcine amniotic fluid (0–16.7% vol/vol) and 10 µg/ml of \textit{Clostridium perfringens} NECA20 or \textit{Escherichia coli} Nissle. Significant differences between amniotic fluid concentrations are indicated (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).](attachment:fig3.png)

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00278.2012 • www.ajpgi.org
nutritional care of preterm neonates just after birth. Although earlier studies in infants using a simulated AF-like solution (4, 11) indicate that such fluids are tolerated by preterm infants, our study is the first to provide both clinical and detailed histopathological evidence for feeding natural amniotic fluid to a preterm animal model that is spontaneously NEC-sensitive. Our results are supported by a study that show TLR-4 and EGF receptor mediated effects of AF in a 10-day-old mouse model of NEC (17). It is noteworthy, however, that the elevated sensitivity to NEC appears less dependent on TLR-4 overexpression in preterm pigs compared with postnatal mice (5, 18). Notwithstanding these differences, the effect of AF on preterm intestinal development and prevention of overt intestinal inflammation in differing models of NEC highlights the potential of AF as a possible prophylactic therapy for preterm infants.

In comparing F-NEC pigs to COLOS pigs, pathway analysis confirmed TNF-α to be a pivotal regulator of several genes involved in both the inflammatory response and apoptosis, which is consistent with results in other NEC model studies (9, 19, 49, 50). Genes involved in inflammation (CD55, IFN-γ, IL-1α, IL-2 receptor, IL-4 receptor, NOS2, TLR-3, TNF-α, and TNF receptor associated factor) were downregulated and genes associated with immune regulation (TOLLIP and IFN regulatory factor) were upregulated in AF pigs when compared with F-NEC pigs in the microarray analysis, suggesting that AF might provide protection against intestinal lesions through suppression of inflammatory pathways. Of the genes differentially regulated in the AF pigs compared with the F-HEA pigs 79% were different from the genes differentially regulated in the AF vs. F-NEC pigs, indicating a dietary effect of AF (including decreased VEGF and NOS 1 and 3 transcriptional activities, and increased occluding and lysozyme), beyond the effect related more specifically to NEC pathogenesis. The effect of AF on genes involved in inflammation was supported both by the qPCR analyses of tissue samples (IL-1α, TNF-α, and IL-6) as well as by data from the in vitro IPEC-J2 cells (TNF-α and IL-6). We were, however, not able to confirm effects on all genes by microarray by qPCR analyses. This may be due to variation in immune gene expressions resulting from differences in disease severity within the FORM group. Furthermore, expression from individual cell types may have been masked or diluted by analyzing intact tissue containing many cell types (56) rather than single cell-type populations by laser capture microdissection. We conclude that the progression of NEC itself in formula-fed pigs is the most important factor that consistently affects whole tissue intestinal immune markers, whereas dietary provision of AF beneficially moderates this immune response. Intestinal content of the proinflammatory cytokine IL-8 was unchanged with therapeutic AF administration during the enteral nutrition period in experiment 2, where NEC incidence was unchanged. This correlates well with a recent study showing correlation between IL-8 content and NEC severity in general (48). We have in another recent study shown that both IL-6 and IL-8 are reduced in pigs receiving MEN with AF despite unaffected NEC severity (32). Provision of therapeutic AF, concomitant with an acute proinflammatory effect of formula feeding, is therefore ineffective in protecting against a harmful intestinal immune response. Immature DCs express low densities of MHC class II and costimulatory molecules like CD40 and CD86, which increase dramatically in the presence of bacteria. We showed that coincubation of DCs with AF during bacterial stimulation decreased CD40 and...
CD86 expression, indicating that AF impedes DC maturation, possibly by blocking receptor-bacteria interaction. This pheno-
type of DCs, also referred to as tolerogenic, may prime but not
activate naïve T cells (16). Our additional DC studies have
shown that both porcine, human, and bovine AF dose depend-
ently decrease IL-12 production from C. perfringens- and E.
coli-stimulated murine DCs (28). Further studies are required
to verify both the dose-dependency and species-specificity of
AF administration to preterm neonates. Regardless, our results
suggest that within each species the anti-inflammatory activity
of AF may protect the immature mucosa against inflammation
at a time of high dietary and bacterial antigen challenge, when
provided from birth. Several T-RFs were lower in AF than in
FORM pigs. As such the reduced NEC severity with AF
administration may be related to the general capacity of AF to
decrease the bacterial load in the intestine. Accordingly, we
have recently shown improved NEC prevention and beneficial
effects on the intestinal proteome when administering enteral
antibiotics to preterm pigs (24). The marked increase in bac-
terial density in COLOS pigs may be more related to the
conserved colostrum matrix in the lumen, which is absent in
the pigs fed formula, whether or not supplemented with AF.
The colonization pattern in preterm pigs fed various diets has
been extensively reviewed earlier (12).

As in other studies, formula feeding was detrimental to the
digestive capacity of the preterm intestine, as indicated by
reduced digestive enzymes, compared with colostrum feeding
(35, 39, 45, 46). The observation that postnatal administration
of AF failed to improve digestive enzyme activity and mucosal
architecture may be explained by a relatively short enteral
feeding period in this study (~24 h). Keeping the enteral
feeding period short was necessary to ensure high RNA integ-
Rity for gene expression analysis, since severely hemorrhagic
and necrotic tissue yields low-quality RNA (46). Our results
suggest that villous atrophy and breakdown of the mucosal
barrier is a relatively late response to formula-induced gut
inflammation, albeit the presence of mucosal abnormalities
(submucosal edema, vacuolization of the enterocytes, congus-
tion of vessels, regional villous sloughing) may occur very
soon after the onset of enteral feeding, as shown in our previous
work (43). We showed that AF dose dependently increases
proliferation and migration of intestinal epithelial
cells in vitro, suggesting that AF acts as a wound-healing agent
after the formula-induced epithelial damage.

AF is recognized mainly for its growth-promoting properties
on the fetal intestine, just as colostrum and milk are important
for gut maturation postnatally. This study is the first to show
that AF administration may affect NEC in preterm neonates by
reducing the inflammatory response and bacterial load. Posi-
tive effects of AF supplemented as minimal enteral nutrition on
various growth and immune parameters have also been docu-
mented in another recent study, although NEC in this case was
not affected (32). The growth-promoting activity of AF on fetal
intestinal cells is severalfold less than for milk (20), and the
concentrations of immunomodulatory factors, like TGF-β, are
only 10–15% of that in colostrum (36). Further studies are
required to show whether the provision of concentrated human
AF to preterm infants as a supplement during parenteral nutri-
tion, or together with enteral milk feeding, will provide the
desired synergistic maturational, antimicrobial, and immuno-
logical effects on the immature gut.

ACKNOWLEDGMENTS
We thank Elin Skytte and Kristina Möller from Department of Nutrition,
Exercise and Sports, University of Copenhagen; Joanna Z. Amenuvor from
the National Veterinary Institute, Technical University of Denmark; and Annette
K. Nielsen from Department of Animal Science, Aarhus University, for skillful
technical assistance.

GRANTS
The work was supported by the Danish Research Councils and the Veteri-
inary Institute, Technical University of Denmark.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
J.L.S., M.V.Ø., R.H.S., T.T., M.S., and P.T.S. conception and design of
research; J.L.S., M.V.Ø., R.H.S., L.M., T.T., M.S., H.K.M., S.P., P.T.S., and
S.B.B. performed experiments; J.L.S., M.V.Ø., R.H.S., K.S., L.M., T.T., H.K.M.,
S.P., L.N.F., H.F., M.B., P.T.S., and S.B.B. analyzed data; J.L.S., M.V.Ø.,
interpreted results of experiments; J.L.S., R.H.S., H.K.M., and S.B.B. prepared
figures; J.L.S., R.H.S., P.T.S., and S.B.B. drafted manuscript; J.L.S., M.V.Ø.,
manuscript; J.L.S., M.V.Ø., R.H.S., K.S., L.M., T.T., M.S., H.K.M., S.P.,

REFERENCES
1. Akinbi HT, Narendran V, Pass AK, Markert P, Hoath SB. Host
defense proteins in vernix caseosa and amniotic fluid. Am J Obstet
Second-trimester amniotic fluid interleukin-10 concentration predicts pre-
epidermal growth factor in human amniotic fluid. Mt Sinai J Med 45:
4. Barney CK, Lambert DK, Alder SC, Scofield SH, Schmutz N, Chris-
tensen RD. Treating feeding intolerance with an enteral solution patterned
after human amniotic fluid: a randomized, controlled, masked trial. J
5. Bering SB, Bai S, Zhang K, Sangild PT. Prematurity does not markedly
affect intestinal sensitivity to endotoxins and feeding in pigs. Br J Nutr
6. Bjornvad CR, Schmidt M, Petersen YM, Jensen SK, Offenberg H,
Emlf J, Sangild PT. Preterm birth makes the immature intestine sensitive
to feeding-induced intestinal atrophy. Am J Physiol Regul Integr Comp
7. Bjornvad CR, Thymann T, Deutz NE, Burdin DG, Jensen SK, Jensen
BB, Molbak L, Boye M, Larsson LI, Schmidt M, Michaelsen KF,
Sangild PT. Enteral feeding induces diet-dependent mucosal dysfunction,
bacterial proliferation, and necrotizing enterocolitis in preterm pigs on
parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 295: G1092–
G1103, 2008.
8. Calvano SE, Xiao W, Richards DR, Feliciano RM, Baker HV, Cho RJ,
Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C,
Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry
SF. A network-based analysis of systemic inflammation in humans.
factor and tumor necrosis factor-alpha in neonatal necrotizing enterocoli-
10. Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially
modulate expression of cytokines and maturation surface markers in
11. Christensen RD, Havranek T, Gerstmann DR, Calhoun DA. Enteral
administration of a simulated amniotic fluid to very low birth weight
12. Cilleborg MS, Boye M, Sangild PT. Bacterial colonization and gut
development in preterm neonates. Early Hum Dev 88, Suppl 1: S41–S49,
2012.


