Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation

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Ganguli K, Meng D, Rautava S, Lu L, Walker WA, Nanthakumar N. Probiotics prevent necrotizing enterocolitis by modulating enterocyte genes that regulate innate immune-mediated inflammation. Am J Physiol Gastrointest Liver Physiol 304: G132–G141, 2013. First published November 8, 2012; doi:10.1152/ajpgi.00142.2012.—Necrotizing enterocolitis (NEC), an extensive intestinal inflammatory disease of premature infants, is caused, in part, by an excessive inflammatory response to initial bacterial colonization due to the immature expression of innate immune response genes. In a randomized placebo-controlled clinical trial, supplementation of very low birth weight infants with probiotics significantly reduced the incidence of NEC. The primary goal of this study was to determine whether secreted products of these two clinically effective probiotic strains, Bifidobacterium infantis and Lactobacillus acidophilus, prevented NEC by accelerating the maturation of intestinal innate immune response genes and whether both strains are required for this effect. After exposure to probiotic conditioned media (PCM), immature human enterocytes, immature human intestinal xenografts, and primary enterocyte cultures of NEC tissue (NEC-IEC) were assayed for an IL-8 and IL-6 response to inflammatory stimuli. The latter two models were also assayed for innate immune response gene expression. In the immature xenograft, PCM exposure significantly attenuated LPS and IL-1β-induced IL-8 and IL-6 expression, decreased TLR2 mRNA and TLR4 mRNA, and increased mRNA levels of specific negative regulators of inflammation, SIGIRR and Tollip. In NEC-IEC, PCM decreased TLR2-dependent IL-8 and IL-6 induction and increased SIGIRR and Tollip expression. The attenuated inflammatory response with PCM was reversed with Tollip siRNA-mediated knockdown. The anti-inflammatory secreted factor is a 5- to 10-kDa molecule resistant to DNase, RNase, protease, heat stress, and acid exposure. B. infantis-conditioned media showed superior anti-inflammatory properties to that of L. acidophilus in immature human enterocytes, suggesting a strain specificity to this effect. We conclude that PCM promotes maturation of innate immune response gene expression, potentially explaining the protective effects of probiotics in clinical NEC.

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NECROTIZING ENTEROCOLITIS (NEC), the most common gastrointestinal emergency in neonatal intensive care units (NICU), is a life-threatening disease that occurs primarily in premature infants (13, 18, 24). NEC is characterized by intestinal inflammation leading to intestinal necrosis and perforation. It affects 8–13% of very low birth weight (VLBW) infants in the US (13, 18). Mortality associated with NEC is as high as 40% of affected infants and 50% of infants requiring surgery (11, 13). NEC in preterm infants is also associated with a significant risk for long-term morbidities such as short-gut syndrome and cholestatic liver disease due to chronic use of parental nutrition (5, 11, 13). Furthermore, the VLBW infants with NEC, and particularly those who require surgical intervention, are at increased risk for poor neurodevelopmental and growth outcomes (10). With these significant adverse outcomes and an increasing rate of premature births in the US (28), there is a need to develop novel interventional strategies that can reduce the risk for the development of NEC. Intestinal colonization with health-promoting microbiota may be crucial for the proper development of mucosal immunity and prevention against several immune-mediated diseases, such as allergy and autoimmunity (5, 27). Initial bacterial exposure in a sparsely colonized premature gut is likely associated with the inflammatory condition that precedes clinical NEC (5, 27).

The circumstances that allow initial microbial-induced inflammation to progress to fulminant, life-threatening NEC are incompletely understood, although an immature mucosal immune system, absence of human milk in the diet, and the acquisition of hospital acquired pathogenic organisms are thought to be major risk factors (5, 10, 11, 13, 18). Development of intestinal innate immunity is influenced by the process of intestinal colonization, which is disrupted in VLBW infants by mode of delivery, aseptic conditions of the NICU, and frequent use of antibiotics (5, 27). How aberrant gut colonization in VLBW infants leads to an excessive mucosal inflammatory response, compared with term infants, is incompletely understood (5, 27). We have attempted to define the mechanisms involved in excessive inflammation in premature infants by analyzing the expression of innate inflammatory genes using resected ileal mucosa from fetal intestine, noninflamed elective surgical specimens in older children, intestinal tissue from NEC patients, and established immature human enterocyte cell lines. These data suggest that the excessive intestinal inflammatory response occurring in the premature intestine (20, 21, 22), a hallmark of NEC, is due to a developmental immaturity of specific innate immune response genes (4, 23).

A major strategy for preventing NEC is to find a means to reduce the excessive immature inflammatory response and to accelerate the maturation of intestinal defenses. In previous clinical studies, several approaches have been used with varying success. These include oral administration of pooled human immunoglobulins (7), prenatal and postnatal steroids (12, 15), and expressed breast milk feedings from the infant’s mother (6). Although thought to be potentially beneficial in the prevention of NEC, use of steroids in the perinatal period, particularly postpartum, has been criticized recently because of other complications caused by their use (30). Accordingly,
other approaches are being considered. Probiotics are live microbial dietary supplements that promote health (8). In premature infants, they are thought to transiently improve the positive balance of colonizing bacteria that facilitate development of mucosal immunity and prevent the excessive inflammation associated with NEC (20). For example, several clinical studies have suggested that the introduction of probiotics with the first expressed breast milk feeding can signiﬁcantly reduce the incidence and severity of NEC (1, 16, 17). In 2005, Lin et al. (16) showed that Lactobacillus acidophilus and Bifidobacteria infantis, given in combination with expressed breast milk, signiﬁcantly reduced the incidence and severity of NEC compared with control infants given expressed breast milk alone. This study was repeated in 2008 in a multicenter trial that again demonstrated the effectiveness of these strains in NEC prevention (17). However, the mechanism by which these probiotics were able to reduce the incidence of NEC was not known and the use of these bacteria in premature infants is not sanctioned by the US Food and Drug Administration (FDA). We have thus chosen to investigate the use of secreted bioactive effectors of those probiotic bacteria that attenuate the intestinal inﬂammatory response.

Previously, we have identiﬁed an immature proﬁle of innate immune response gene expression in immature intestinal tissues (4, 20–23). On the basis of these observations, we have hypothesized that secreted factors of the clinically effective probiotic bacteria, L. acidophilus and B. infantis, attenuate the intestinal inﬂammatory response by accelerating the maturation of intestinal innate immune response gene expression, thereby reducing the risk of developing NEC in the immature intestine. Using both immature and mature human intestinal xenograft models and primary enterocytes isolated from freshly resected NEC mucosa, we report here that the PCM of L. acidophilus and B. infantis can attenuate the immature enterocyte response to inﬂammatory stimuli through modulating the expression of critical genes involved in the innate immune inﬂammatory response. Additionally, we investigated the role of secreted factors of L. acidophilus and B. infantis, when grown individually, to determine the extent by which each contributes to the anti-inﬂammatory effects shown in clinical trials.

METHODS

Chemicals

Ultra-pure D-glucose and BSA were obtained from Sigma (St. Louis, MO). Cell culture media, DMEM, or CMRL 1066, as well as nonessential amino acids, methionine, D-retinyl acetate, glutamine, penicillin, streptomycin, and gentamicin were obtained from GIBCO/Invitrogen (San Diego, CA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Tissue culture plastics were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were either reagent or molecular grade and obtained from Sigma. Ultra pure lipopolysaccharide (LPS, from Escherichia coli 055:B5) was obtained from List Biological Laboratories (Campbell, CA). Recombinant IL-1β and IL-8 ELISA kits were obtained from R&D Systems (Minneapolis, MN). Protein concentrations were measured using a BCA Protein assay kit (Pierce, Rockford, IL) against BSA standards in a colorimetric assay according to the manufacturer’s protocol.

Bacterial Cultures and Isolation of Probiotic-Conditioned Media

Lactobacillus acidophilus (ATCC no. 53103) and Bifidobacterium infantis (ATCC no. 15697) were obtained from American Type Culture Collection (ATCC, Manassas, VA), cultured as recommended by ATCC, and stored individually in Mann-Rogosa-Sharpe (MRS) medium containing 15% glycerol at −80°C. A 50-ml Falcon tube containing MRS broth (DIFCO, BD Bioscience, Franklin Lakes, NJ), supplemented with 0.5 g/l of cysteine, was inoculated with either a single colony of L. acidophilus or B. infantis. Additional Falcon tubes were inoculated with a single colony of each strain.

The inoculum was cultured at 37°C under anaerobic (BBL Campy Plus Microaerophilic System, Becton, Dickinson, Sparks, MD) conditions until they reached the midexponential phase of growth (OD600 0.5) or the stationary phase of growth (OD600 >1.0), as described previously (26). Probiotic-conditioned media (PCM) at the midexponential growth phase and stationary growth phase were prepared by centrifugation of probiotic cultures at 9,000 g for 10 min, repeated twice, and then by 0.22-μm ﬁltration to eliminate residual bacteria. The efﬁciency of bacterial depletion from the conditioned media was determined by plating serial dilutions.

Human Intestinal Models

Resected intestinal tissue. Selected segments of resected ileum from infants having elective surgery for necrotizing enterocolitis were obtained from Massachusetts General Hospital (MGH) [Partners 1999-P-003833 (Walker)]. In selected segments laser capture microdissection (LCM) was used to obtain epithelial RNA and then subjected to real-time RT-PCR, as described previously (23).

Fetal tissue. Human small intestine was obtained from elective prostaglandin/saline-induced therapeutically aborted 12- to 20-wk fetuses after informed, written consent from Partners for Human Research Committee [protocol 1999-P-003833 (Walker)]. All experiments involving the utilization of human tissues were approved by Partners for Human Research Committee (protocol 1999-P-003833). These tissues were then transported to the laboratory in ice-cold fresh organ culture medium as described previously (20–22). Fetal intestinal tissues were processed for organ cultures and for small intestinal xenografts as described below.

Small intestinal xenografts. Four-week-old homozygous SCID mice were housed in a speciﬁc pathogen-free facility and maintained on rodent laboratory chow 5001 and water ad libitum. The animals were raised in air-conditioned quarters at 21°C on a 12:12-h light-dark cycle with lights on at 0600. Sterilized food (rodent laboratory chow 5001, Ralston Purina, St. Louis, MO) and deionized water were provided ad libitum from the day of arrival until the completion of the experiments. Two-centimeter sections of fetal ileum, stripped of its mesentery, were implanted subcutaneously into 4- to 6-wk-old homozygous SCID mice as reported previously (21, 22). Surgery and postsurgical care were monitored according to an approved animal protocol from the Research Animal Care Committee (protocol 2005-N-000040) of the MGH. To avoid circadian inﬂuences, all animals were euthanized between 1100 and 1300. Mature xenografts (30 wk posttransplantation) display high sucrase and lactase whereas immature xenografts (20 wk posttransplantation) display high sucrase but low lactase. Both xenografts were challenged with inﬂammatory stimuli (LPS 50 ng/ml or IL-1β 1 ng/ml) for 16 h in organ culture, and the intestinal IL-8 and IL-6 response was measured by ELISA (20–22). In separate experiments, total RNA was isolated from intestinal xenografts.

For probiotic studies, the lumen of selected xenografts was cleaned with sterile PBS and then infused with up to 1 ml of PCM or MRS medium as a control. After 2 days, human intestinal xenografts were harvested and challenged with inﬂammatory stimuli (LPS 50 ng/ml or IL-1β 1 ng/ml) for 16 h in organ culture and the intestinal IL-8 response was then measured in the culture media by ELISA, as described previously (21–23). A lactate dehydrogenase (LDH) cyto-
toxic assay and sucrase activity assay were performed to monitor cell viability and mucosal integrity during the course of the experiments.

In separate experiments, immature human ileal xenographs were cleaned and infused with probiotic-conditioned or MRS medium alone. After 2 days, intestinal xenographs were harvested, rinsed with sterile saline, and frozen at −80°C until RNA extraction as described below for quantitative RT-PCR (qRT-PCR) analysis. In another study, treated and control xenographs were harvested and embedded in OCT and 8-µm cryosections were made at −20°C for Toll-like receptor (TLR) protein detection by immunofluorescence, as described previously (9, 20).

Intestinal Organ Cultures

Immature and mature human intestinal xenographs were exposed to LPS and IL-1β in organ culture (20–22). Organ culture was performed as described previously (20). Quadruple organ cultures from each xenograph were challenged with 50 µg/ml of ultra pure LPS, 1 ng/ml of IL-1β, or PBS (control), and medium was collected after 18 h and assayed for IL-8 and IL-6 by ELISA. Tissue was homogenized in 9 volumes of 0.154 M KCl and assayed for total protein with a BCA protein assay kit with a BSA standard. Sucrase activity was determined to assess morphological integrity of the mucosa and structural viability of the organ culture after each experiment (21). In some studies, additional organ cultures were subject to histological sections to assess structural integrity of the mucosa. After similar experiments were performed, tissues frozen at −80°C were used to isolate total RNA and then analyzed for changes in human IL-8 and IL-6 mRNA by real-time qRT-PCR using GAPDH mRNA as a standard, as described previously (21, 22).

Epithelial Cell Isolation and Culture

Primary enterocytes were isolated and cultured from the viable margins of resected ileal NEC tissues from a 25-wk gestation neonate (NEC-IEC), modified from procedures as described previously (20). Segments of discarded intestine were rinsed with ice-cold sterile PBS and incubated with a 1.25% trypsin-0.5 M EDTA solution at room temperature for 10 min. Mucus and most of the villi on the apical surface were scraped lightly and discarded. Harder scraping was applied to yield intact crypts, leaving behind only serosal layers. Crypt structures were collected in serum-free OptiMEM supplemented with antibiotic-antimycotic solution, washed, and incubated with serum-free OptiMEM containing 0.4 mg/ml collagenase type IV at 37°C for 90 min, with gentle and brief shaking every 10–15 min. The dissociated epithelial cells were spun down at 1,000 rpm in 4°C and washed with DMEM containing 10% FBS. The final pellet-containing crypts were suspended in “fortified OptiMEM” (OptiMEM supplemented with 10 mM HEPES, pH 6.5, 2.5 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM GlutaMAX-I, 20 ng/ml epidermal growth factor, 150 mM hydrocortisone 21-hemisuccinate sodium salt, 10 µg/ml insulin, and 4% FBS). This cell suspension was then plated in 100-mm-diameter dishes coated with extracellular matrix ECL (Upstate Biotechnology, Lake Placid, NY) and incubated at 32°C and 5% CO2.

Fetal enterocytes (FHs74) (ATCC) were grown in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 200 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 1% HEPES buffer. Cells were incubated at 32°C with 5% CO2.

Laser Capture Microdissection

The epithelial cells were isolated from immature (20-wk) and mature (30-wk) intestinal xenographs, as described previously (23). The xenograph tissue was frozen in OCT, cut into 8-µm thin cryosections, and mounted on microscopy slides (Gold Seal Rite-On Micro Slides, Portsmouth, NH), and epithelial cells were isolated as described previously (3). After cryosections were fixed in 70% ethanol, they were rinsed with RNase-free water, stained with toluidine blue, and then dehydrated with increasing concentrations of ethanol and xylene prior to LCM of the epithelial cells. Toluidine blue-stained epithelial cells were captured onto polyethylene collecting caps (Macro Cap, Arcturus, MDS Analytical Technologies, Sunnyvale, CA), yielding ~600 stained cells from each mucosal section. Using the Qiagen kit and protocol (Qiagen, CA), total RNA was collected and the quality of RNA was evaluated by using an Agilent bioanalyzer (Waldbronn), utilizing an RNA 6000 Pico chip. The total RNA was used for qRT-PCR analyses of specific mRNA, using GAPDH as a standard housekeeping gene for normalization and the SYBR green Mastermix system (3).

Knockdown of TOLLIP Expression by siRNA Transfection

Specific siRNA oligonucleotide were designed and purchased from Invitrogen. Epithelial cells at 50% confluence were transfected with 10 nM of specific siRNA or negative control by using an RNAiMAX kit according to the manufacturer’s recommendations.

Transfection efficiency was assessed by transfecting the cell with BLOCK-iT Alexa Fluor Red fluorescent control and quantifying total number of cells by DAPI staining and transfected cells by red fluorescence to calculate the efficiency. Specific knockdown efficiency for each gene was measured by qRT-PCR and/or by Western blots.

Characterization of Secreted Probiotic Factors

After the secretions of the two probiotic strains were isolated as described above, they were then size fractionated by use of Amicon (Fisher Scientific) columns. The size-fraction showing the greatest anti-inflammatory effect was subsequently treated with DNase, RNase, protease, heat stress, and trichloroacetic acid, as described previously (2), to further characterize the physiological properties of the anti-inflammatory effector(s).

Real-Time qRT-PCR

Total RNA was prepared from human intestinal model systems as described above and quantified. Real-time qRT-PCR was performed as described previously (20, 22).

Immunofluorescence

To detect TLR-4 protein expression in treated and untreated human intestinal xenographs, 8-µm cryosections were evaluated by using specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody conjugated to FITC label. Frozen intestinal sections were photographed with 1,000 ASA film in a Zeiss fluorescence microscope. When needed, tissue sections were examined by confocal microscopy.

LDH Cytotoxicity Assay

For each experiment with PCM, LDH cytotoxic assay was performed to determine the mucosal integrity and viability during the course of the experiment. The LDH Assay Kit is based on the reduction of the tetrazolium salt (INT) in a NADH-coupled enzymatic reaction to formazan, which is water soluble and exhibits an absorption maximum at 492 nm. Since the intensity of the red color formed is proportional to the lactate concentration, the assay, using a set of lactate standards, can measure the concentration of lactate released to the culture media in a quantitative manner as recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI). The amount of the end-product formazan produced was read as optical density (OD) by a spectrophotometer at 492 nm. The results are expressed as a percentage of the maximum LDH release.
IL-8 and IL-6 ELISA

Secreted human IL-8 and IL-6 were measured by ELISA using 96-well high-bond microtiter plates (Nunc-Maxisorp, Fisher Scientific) and human IL-8 and IL-6 detection kits (R&D Systems), as described previously (20–22).

Statistical Analysis

Results are presented as means ± SE. The effect of gestational age and treatment conditions on chemokine secretion were analyzed by a two-way ANOVA. After overall significance was determined, post hoc analyses for individual variables were performed by a two-tailed unpaired t-test, and when necessary a Bonferroni correction was used with post hoc t-tests. Differences with a P value of <0.05 were considered significant.

RESULTS

Effect of PCM on the Immature Intestinal IL-8 and IL-6 Inflammatory Response

The lumen of immature and mature human ileal xenografts were infused with 5% PCM from Lactobacillus acidophilus and Bifidobacteria infantis or a pH-adjusted MRS medium. After 48 h, xenografts were harvested and the intestinal mucosa challenged with LPS (50 ng/ml), IL-1β (1 ng/ml), or a vehicle control (PBS) by use of the organ culture system. IL-8 and IL-6 levels were measured after 16 h by ELISA (Fig. 1A). Both LPS and IL-1β were able to significantly induce high IL-8 and IL-6 levels in the immature intestinal xenografts (P < 0.001), whereas only a modest increase was measured in the mature xenografts (P < 0.05). Exposure of the immature xenograft to PCM significantly reduced the IL-8 and IL-6 response to both LPS and IL-1β (P < 0.001) in immature xenografts. As expected, given the modest inflammatory response in mature xenografts, exposure to PCM prior to LPS or IL-1β stimulation resulted in a highly significant attenuation (P < 0.001) of IL-8 and IL-6 mRNA (Fig. 1B), which was not demonstrated in the mature xenografts (P > 0.1). These data suggest that the L. acidophilus and B. infantis secretions present in conditioned media have a profound effect in reducing the excessive inflammatory response to LPS or IL-1β as observed in immature human intestinal xenografts. The differential of effect noted in the mature and immature xenografts suggests that probiotic secretions have a developmental influence on innate immunity.

Effect of PCM on Innate Immune Inflammatory Gene Expression

To determine the mechanism of the inhibitory effect of PCM on the inflammatory response, immature human intestinal xenografts were infused with PCM or MRS media alone. After 48 h, the intestinal mucosa was collected, epithelial cell RNA were isolated by LCM, and qRT-PCR analysis was performed. Figure 2A shows a highly significant reduction of TLR2 and TLR4 mRNA (P < 0.001) and a highly significant induction of the negative regulators (Fig. 2B), SIGIRR and Tollip (P < 0.001), in immature but not mature xenografts (P < 0.2). A similar induction of IRAK-M mRNA was not demonstrated.

Fig. 1. Probiotic secretions attenuate LPS/IL-1β-induced excessive IL-8 and IL-6 secretion in the immature human intestinal xenografts. Organ cultures of immature and mature intestinal xenografts were challenged by LPS (50 μg/ml) or IL-1β (1 ng/ml), with and without 48-h preexposure to probiotic-conditioned media (PCM). A: IL-8 and IL-6 secretion (pg/mg of protein) was measured after 16–18 h; B: IL-8 and IL-6 mRNA expression was measured after 12 h. A highly significant induction of IL-8 and IL-6 secretion as well as IL-8 and IL-6 mRNA were observed in immature xenografts. However, exposure to PCM before stimulation results in a highly significant decrease in IL-8 and IL-6 secretion (**P < 0.001) as well as IL-8 and IL-6 mRNA levels (**P < 0.001) were noted in immature xenografts. A modest increase in IL-8 and IL-6 secretion (pg/mg of protein) and IL-8 and IL-6 mRNA (*P < 0.05) in the mature xenografts was not altered with PCM pretreatment. n.s., Not significant.
Fig. 2. Probiotic secretions regulate innate immune response genes in immature but not mature human intestinal xenografts. Immature and mature human intestinal xenografts were incubated with or without PCM. After PCM exposure, quantitative RT-PCR (qRT-PCR) analysis of laser capture microdissected epithelial RNA showed a highly significant (**P < 0.001) decrease in TLR2 and TLR4 mRNA expression (A) and a highly significant (***P < 0.001) increase in negative regulators SIGIRR and Tollip, but not IRAK-M, mRNA in immature, but not in the mature intestinal xenografts (B). TLR, Toll-like receptor.

These observations suggest that PCM can induce the maturation of specific intestinal innate immune inflammatory genes in the immature but not the mature gut, again suggesting a developmental effect.

In selected experiments, frozen sections from immature xenografts exposed to PCM vs. control MRS media alone were analyzed by immunofluorescence with antibodies specific for TLR2 and TLR4 protein. Figure 3 is a representative photomicrograph showing a decrease in epithelial TLR4 in the immature human intestine exposed to PCM compared with the control MRS media exposure. Similar results were obtained for TLR2 (data not shown). These studies suggest that human TLR protein is expressed predominantly on the surface of the epithelium of the immature human intestinal xenograft and that PCM can downregulate its expression.

The inflammatory response in primary enterocytes isolated from NEC-IEC is attenuated by exposure to PCM. Since it was difficult to distinguish between viable and necrotic tissue, limiting the amount of tissue available for analysis, we isolated primary enterocytes from morphologically normal appearing tissue adjacent to NEC-IEC. After confirmation of viability, the NEC-IEC cells were pretreated with 5% PCM or control MRS media for 24 h and then stimulated with a TLR2 ligand, Pam3Cys (10 μg/ml), for 12 h. The IL-8 and IL-6 mRNA expression was determined by qRT-PCR. Figure 4A depicts a significant reduction in the IL-8 and IL-6 mRNA in response to Pam3Cys stimulation (P < 0.05) in PCM-treated NEC-IEC cells.

To determine whether intestinal innate immune response genes are also regulated by the PCM in NEC-IEC, the levels of TLR2, SIGIRR, Tollip, and IRAK-M mRNA were measured by quantitative qRT-PCR (Fig. 4B). TLR4 was not expressed in the primary enterocyte cultures from the NEC-IEC. Five percent fractionated PCM was able to reduce TLR2 mRNA levels by 6.1-fold and increase SIGIRR mRNA levels by 5.9-fold and Tollip mRNA levels by 7.1-fold (P < 0.05). In contrast, there was no effect in regulating the level of IRAK-M mRNA (P > 0.39).

Effect of Negative Regulator Expression on the Inflammatory Response of Immature Fetal Enterocytes

To determine whether the increased expression of negative regulators was, in part, responsible for the reduction of IL-8 and IL-6 mRNA expression in NEC-IEC exposed to PCM, knockdown experiments using Tollip-specific siRNA were performed. Specific siRNA were transfected 48 h prior to treatment. After pre-treatment with PCM for 2 h, the cells were stimulated with Pam3Cys. Figure 5A shows that Pam3Cys-induced IL-8 and IL-6 mRNA expression (P < 0.001) was enhanced by knockdown of Tollip (P < 0.01), and the inhibition by pretreatment with PCM was partially abolished by Tollip-specific siRNA. The efficacy of Tollip knockdown was demonstrated by a significant reduction in Tollip mRNA and protein levels (P < 0.001), as shown in Fig. 5, B and C. These observations suggest that the absence of the negative regulator Tollip is partly responsible for the excessive inflammatory response of the NEC tissue epithelium, and PCM appears to regulate selective genes involved in the intestinal innate immune response.

Initial Characterization of the Probiotic Secreted Factor(s) That Regulates LPS-Induced IL-8 Secretion in Immature Fetal Human Intestine

To enrich the soluble probiotic factor(s), the PCM from L. acidophilus and B. infantis, grown together, was size fractionated by use of Amicon (Fisher Scientific) columns. Each fraction was then assayed for its ability to attenuate the LPS-
induced IL-8 response in 20-wk-old fetal human intestinal mucosa in organ culture. Figure 6A shows the most significant attenuation was observed with the 5- to 10-kDa fraction ($P < 0.01$). Neither control MRS media nor media conditioned with a commensal bacterium, *E. coli* (strain F18), inhibited the inflammatory response. Furthermore, the exposure of the 5- to 10-kDa fraction to DNase, RNase, protease, heat stress, and trichloroacetic acid had no effect on the anti-inflammatory properties of the probiotic secreted factor(s) (Fig. 6B). Therefore, from initial characterization of the secreted factor(s) of *L. acidophilus* and *B. infantis*, grown together, the anti-inflammatory effect(s) seems to be a heat-stable, nonprotein, non-nucleic acid, aqueous molecule between 5 and 10 kDa.

**Effect of Secreted Factors of B. infantis and L. acidophilus, Grown Separately, on the Inflammatory Response of Immature Fetal Enterocytes**

To determine whether the inflammatory attenuation of PCM is primarily due to a single probiotic strain, secreted products were isolated from *B. infantis* and *L. acidophilus*, grown together and separately. Fetal human enterocytes, FHs74 cells, were concomitantly treated with 5% *B. infantis* - or *L. acidophilus*-conditioned media and 10 ng/ml of TNF-$\alpha$ for 18 h. The induced IL-8 response was then measured in the cell culture media by ELISA. A statistically significant reduction in IL-8 levels was demonstrated in the cells pretreated with media conditioned with *B. infantis* and *L. acidophilus*, grown together ($P < 0.01$), as well as media conditioned with *B. infantis*, grown individually ($P < 0.01$). *L. acidophilus*-conditioned media showed only a minimal attenuation of the inflammatory response (Fig. 7). This observation suggests that the anti-inflammatory properties of PCM of *B. infantis* and *L. acidophilus* in immature intestinal epithelium may be due primarily to the secretions of *B. infantis*.

**DISCUSSION**

*L. acidophilus* and *B. infantis* when given together to premature infants have been clinically proven to prevent NEC (16, 17). Despite promising data, the FDA currently prohibits the
standard administration of whole bacteria to neonates for clinical purposes without IRB approval. Accordingly, we elected to isolate secreted products of these probiotic bacteria and investigate their potential role in attenuating the inflammatory response of immature intestinal epithelia. Initially, we tested PCM isolated from these organisms grown together. The PCM infused into xenograft loops not only reduced the excessive inflammatory (IL-8 and IL-6) response to stimuli (LPS/H9262) but also selectively stimulated a maturation of specific innate inflammatory response genes. When we subsequently infused live bacteria (10^3) washed free of their secretions in media, we were unable to demonstrate an anti-inflammatory response (data not shown). Thus in this study we provide evidence that the conditioned media of these two probiotics can reduce the excessive intestinal inflammatory response previously reported in fetal but not mature human enterocytes after exposure to exogenous (LPS) and endogenous (IL-1β) stimulation (20, 22).

Additionally, although some protective effects of probiotic bacteria require the presence or adherence of intact bacteria, the anti-inflammatory properties of the two stains investigated in this study are a function of bacterial secretions. Using human models of the premature intestine and primary enterocyte cultures of NEC intestine, we have demonstrated a probable mechanism for reducing the inflammatory response by these probiotic bacteria. We have previously reported a devel-
opmental alteration of genes involved in the innate inflammatory immune response to microbial molecular pattern molecules in immature enterocytes as well as an enhanced alteration in NEC enterocytes (4, 23). An increased expression of TLR2 and TLR4, their signaling molecules MyD88 and TRAF6, the transcription factor NF-κB1, and its effector chemokine IL-8 has been previously demonstrated (4). Additionally, the expression of negative regulators of NF-κB activation, SIGIRR, Tollip, and IRAK-M was decreased in fetal enterocytes and particularly in NEC enterocytes compared with controls (23). Therefore, we tested the hypothesis that *L. acidophilus* and *B. infantis* attenuate the excessive inflammation of immature intestine by accelerating a maturation of the NF-κB signaling pathway. The data suggest that secretions from these two probiotics cause maturation of specific genes involved in the NF-κB signaling pathway (23). Since these observations were made by using the secretions from the two bacterial strains grown in coculture, we were left unable to draw conclusions about each strain individually.

Additionally, we demonstrated that PCM downregulates TLR2 and TLR4 expression in xenograft epithelium (Figs. 2A and 3) and upregulates the negative regulator genes, SIGIRR and Tollip, but not IRAK-M (Fig. 2B). These data suggest that maturation of the innate immune inflammatory signaling pathway in immature enterocytes may represent the mechanism by which these probiotics reduced the excessive inflammatory response to colonizing bacteria in premature infants. Interestingly, this is in contrast to the effects of corticosteroids on immature enterocytes, which result in a more global, nonspecific cellular maturation. Since the safety of corticosteroid use in preterm infants has been questioned, given various broad, off-target effects, a potential preventive agent with superior cellular specificity may be more clinically acceptable. It should be noted that the PCM-induced maturation of specific innate immune response genes occurred only in immature and not in mature xenografts, confirming that the PCM contained a specific factor(s) that can developmentally regulate the intestinal innate immune response. We further confirmed that the effect of PCM on intestinal innate immune inflammation was in part due to an upregulation of selective negative regulator genes by knocking down the expression of Tollip before exposing immature xenografts to probiotics and Pam3Cys, a TLR2 ligand (Fig. 5A). These studies showed that the reduced intestinal IL-8 response to inflammatory stimulation in the presence of PCM was lost with knockdown of the expression of a specific negative regulator. The decreased IL-8 inflammatory response, due to maturation of innate immune response genes, was further confirmed by immunofluorescence showing a decreased TLR4 protein expression in probiotic treated, but not control MRS media-treated, intestinal mucosa (Fig. 4). Importantly, the incomplete loss of the anti-inflammatory effect with Tollip knockdown suggests additional targets of the secreted anti-inflammatory effectors.

We have begun to characterize the secreted product(s) of *Lactobacillus acidophilus* and *Bifidobacteria infantis*, when grown together. Our initial observations suggest that this is a heat-stable, aqueous molecule fractionating predominantly between the sizes of 5 and 10 kDa. This factor(s) is neither a protein nor a nucleic acid (Fig. 6), and, given our observations, we suspect the anti-inflammatory factor may be a glycolipid or glycan. Investigations to further define the structure of this factor(s) are currently underway with the assistance of a collaborating glycobiology team.

When evaluating secretions of *B. infantis* and *L. acidophilus*, grown separately, we demonstrated an anti-inflammatory effect of *B. infantis*-conditioned media that is similar to the media conditioned with both strains simultaneously. Additionally, the *L. acidophilus*-conditioned media had minimal to no anti-inflammatory effect (Fig. 7). The clinical trials that showed a significant reduction in the incidence of NEC, prompting the choice of strains for our investigation, used only a combination of probiotics. The effective regimens included both *B. infantis* and *L. acidophilus*. However, neither strain was clinically investigated individually. It is possible that only one strain of the effective regimen was required for the clinical effect, and from our data we would suspect the more effective strain was

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Fig. 7. Effect of secreted factors of *B. infantis* (Bi) and *L. acidophilus* (La), grown separately, on the inflammatory response of immature fetal enterocytes (FHs74). Primary enterocytes (FHs74) were pretreated with secretions of either Bi or La, cultivated individually, or secretions of Bi and La, cultivated together, in cell culture. The IL-8 response was then evaluated after TNF-α stimulation (10 ng/ml). The TNF-α-induced IL-8 response was significantly reduced in fetal enterocytes treated with PCM, isolated from both strains grown together (†*P < 0.01*). A significant reduction in IL-8 response was also shown in cells treated with *B. infantis*-conditioned media (†*P < 0.01*); however, only a minimal reduction was shown in cells treated with *L. acidophilus*-conditioned media. Cont., control.
B. infantis. Although L. acidophilus secretions were minimally anti-inflammatory in our NEC models, it is possible that when administered orally in an in vivo system the L. acidophilus bacterium or bacterial secretions enhance the anti-inflammatory properties of B. infantis in a synergistic manner. Investigating the L. acidophilus secretions for properties to support this hypothesis should be pursued in future investigation.

Using primary enterocytes from freshly resected NEC ileum, we showed that the probiotic secretions decreased the cellular IL-8 IL-6 response and modulated specific innate immune response genes relevant to this particular inflammatory condition. We have not examined other specific inflammatory conditions in the human small intestine that are age-related (e.g., bacteria/viral gastroenteritis), so we can only speculate that these probiotic strains may also be effective in preventing inflammation under these clinical conditions. However, on the basis of the observation of an anti-inflammatory effect in immature xenografts and primary fetal enterocytes, we would predict that probiotic secretions may be effective in other inflammatory conditions of the immature intestine. This will require further clinical investigation.

Again, the pathophysiology of NEC is multifactorial, including elements of an exaggerated inflammatory response, poor intestinal perfusion, hypoxia, bacterial translocation, and barrier compromise. The data presented here only address a single component of this complex cascade, and we are simultaneously investigating the effects of probiotic secretions on the other components of the pathophysiological pathways, which is beyond the scope of this study. Collectively, our present observations suggest that probiotic secretions reduce the IL-8 and IL-6 response to inflammatory stimuli by inducing a selective maturation of innate immune response gene expression, e.g., downregulation of TLRs, signaling molecules and transcription factor genes and upregulation of selective negative regulatory genes as suggested by previous observations (25, 26, 31, 32). Another recent study in an animal model for NEC caused by a combination of formula feeding and hypoxia shows an upregulation of TLR4 expression, compared with unaffected pups fed with mother’s milk (14). This supports our observation made in human NEC tissues. However, these data only show the effect on total RNA of enterocytes in fetal xenografts exposed to PCM and does not distinguish epithelial surface TLR2 and TLR4 expression from cytosolic expression, shown previously to occur in mature enterocytes (9, 26, 29). Nonetheless, from our prior studies using immature fetal enterocytes (H4) cells (9), we have hypothesized that TLR expression is likely to be on the apical surface of fetal enterocytes and internalized with maturation. This distinction will require additional experiments and is beyond the scope of the present study.

Another important consideration with regard to the use of probiotics to prevent NEC is that probiotics, as live organisms, are not currently approved by the FDA for routine use in premature human infants. The previous studies reported have been conducted outside of the United States (1, 16, 17). Accordingly, if we can show that a purified secreted probiotic factor(s), free of intact bacteria, is effective in vivo in an animal NEC model and then in a large multicenter clinical trial in the United States, this approach could be recommended for the standard care of premature infants in the US.

In conclusion, we believe that the reported observations made in developing human intestinal xenografts and in primary enterocytes isolated from resected NEC intestine suggest a mechanism for the protective effect of the probiotics L. acidophilus and B. infantis in the prevention of NEC. B. infantis-conditioned medium is significantly more effective in modulating the intestinal inflammatory response in immature enterocytes than L. acidophilus-conditioned medium. Furthermore, this study provides a possible pathophysiological basis for the prevention of NEC by soluble probiotic secretions, which have not been previously described or characterized. Additional investigations to demonstrate the mechanisms by which secretions of B. infantis attenuate inflammation in the immature intestine are required, as well as studies to define the physicochemical properties and structure of the effective secreted factor(s), which are currently ongoing. On the basis of our preliminary observations, we suspect that the secreted anti-inflammatory factor may be a glycan or glycolipid.

Additional in vivo animal model and clinical studies are needed to determine the efficacy of these secreted probiotic factors in altering the excessive inflammatory (IL-8) responses in the immature intestine. This observation, in conjunction with the previous clinical studies using the same probiotic strains (16, 17), should provide the basis for designing a multicenter trial with a fixed protocol to confirm usage of probiotic secretions by neonatologists in preventing necrotizing enterocolitis.

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AUTHOR CONTRIBUTIONS

K.G. designed and completed cell culture experiments, isolated probiotic secreted factors, performed ELISA and PCR analyses, and finalized the manuscript. D.M. had a critical role in preparing xenografts and analyzing data related to xenograft experiments. S.R. isolated RNA and completed analyses related to several experiments in this manuscript. L.L. contributed to study design and concept, isolated the NEC-IEC cells from the NEC resected tissue, and interpreted data. N.N. and W.A.W. provided the funding, supervision, and interpretation for all experiments included in this manuscript.

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