Preterm human milk contains a large pool of latent TGF-β, which can be activated by exogenous neuraminidase

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Human milk contains substantial amounts of transforming growth factor (TGF)-β, particularly the isoform TGF-β2. We previously showed in preclinical models that enterally administered TGF-β2 can protect against necrotizing enterocolitis (NEC), an inflammatory bowel necrosis of premature infants. In this study we hypothesized that premature infants remain at higher risk of NEC than full-term infants, even when they receive their own mother’s milk, because preterm human milk contains less bioactive TGF-β than full-term milk. Our objective was to compare TGF-β bioactivity in preterm vs. full-term milk and identify factors that activate milk-borne TGF-β. Mothers who delivered between 23 0/7 and 31 6/7 wk or at ≥37 wk of gestation provided milk samples at serial time points. TGF-β bioactivity and NF-κB signaling were measured using specific reporter cells and in murine intestinal tissue explants. TGF-β1, TGF-β2, TGF-β3, and various TGF-β activators were measured by real-time PCR, enzyme immunoassays, or established enzymatic activity assays. Preterm human milk showed minimal TGF-β bioactivity in the native state but contained a large pool of latent TGF-β. TGF-β2 was the predominant isoform of TGF-β in preterm milk. Using a combination of several in vitro and ex vivo models, we show that neuraminidase is a key regulator of TGF-β bioactivity in human milk. Finally, we show that addition of bacterial neuraminidase to preterm human milk increased TGF-β bioactivity. Preterm milk contains large quantities of TGF-β, but most of it is in an inactive state. Addition of neuraminidase can increase TGF-β bioactivity in preterm milk and enhance its anti-inflammatory effects.

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Breast milk; necrotizing enterocolitis; transforming growth factor-β; inflammation; sialidase

HUMAN MILK CONTAINS biologically important amounts of transforming growth factor (TGF)-β, with a predominance of TGF-β2 over TGF-β1 (24). Although the precise function of milk-borne TGF-β is unclear, orally ingested TGF-β is presumed to promote gut barrier function, immune tolerance, and mucosal repair in the neonatal gastrointestinal tract (20, 30, 31, 57). We previously reported that TGF-β2 suppresses macrophage cytokine expression and mucosal inflammatory responses in the developing intestine, and in preclinical models, we showed that enterally administered TGF-β2 protects against intestinal injury similar to necrotizing enterocolitis (NEC), an inflammatory bowel necrosis of premature infants (34, 46).

Milk-borne TGF-β is composed of a smaller fraction of “bioactive” TGF-β, which is available to ligate its cognate receptors and induce downstream signaling, and a larger pool of latent TGF-β, in which TGF-β is bound noncovalently to a latency-associated peptide (LAP) (3). Unlike the situation in adult subjects, “preactivation” of milk-borne TGF-β may be particularly important in premature infants, because gastrointestinal mechanisms for in situ activation of orally ingested TGF-β, such as gastric acidity and intestinal gelatinases, are relatively deficient (2, 23, 27, 33, 45). In this context, we hypothesized that 1) premature infants remain at higher risk of NEC than full-term infants, even when they receive their own mother’s milk, because preterm human milk contains less bioactive TGF-β than full-term milk; 2) lower TGF-β bioactivity in preterm milk reflects the deficiency of one or more activators of TGF-β in preterm milk; and 3) TGF-β bioactivity in preterm milk can be increased to levels comparable to full-term milk by addition of TGF-β activator(s) that are expressed at lower levels in preterm than full-term milk. To investigate these hypotheses, we studied longitudinal changes in TGF-β bioactivity in milk produced by mothers who delivered prior to 32 wk gestation. We also measured TGF-β bioactivity in a few samples of stored donor human milk obtained from a milk bank and in a commercially available human milk-derived human milk fortifier. To elucidate the mechanistic basis for differences in TGF-β bioactivity in preterm vs. full-term milk, we compared preterm vs. full-term milk for the expression of known activators of TGF-β such as the glycoprotein thrombospondin-1 (THBS-1), proteases such as plasmin, matrix metalloproteinase (MMP)-2 and MMP-9, and glycoside hydrolases such as neuraminidase (23, 44). Finally, because preterm milk expressed significantly less neuraminidase activity than full-term milk, we tested whether the addition of a bacterial neuraminidase could activate the latent TGF-β fraction in preterm milk and enhance its anti-inflammatory effects.
**METHODS**

**Human milk samples.** Milk samples were collected at Evanston Hospital (Evanston, IL) between October 2008 and September 2010 and at the University of Texas (San Antonio, TX) between January 2009 and January 2011, and deidentified samples were received at the University of Illinois at Chicago after approval by the local Institutional Review Board at each site. All biological samples were handled and conditions were monitored according to the Biospecimen Reporting for Improved Study Quality guidelines (42). Mothers who delivered between 23 and 31 6/7 wk or at ≥37 wk of gestation were enrolled after informed consent. Mothers who delivered prior to full term provided 2- to 5-ml milk samples at three time points after delivery: within 48 h (colostrum), on day 6–7 (1 wk), and on day 30–31 (1 mo). Mothers who delivered at full term provided samples within the 1st wk. Milk samples were refrigerated (4°C) immediately after they were pumped and collected within 1 h by the research personnel, who transferred 1-ml aliquots from the milk bags to sterile polyethylene microcentrifuge tubes (Sigma, St. Louis, MO) and processed the samples as described below. Donor human milk samples were purchased from the Mother’s Milk Bank (Austin, TX). Human milk-derived milk fortifier was donated by the manufacturer (Prolacta Bioscience, Monrovia, CA). All samples were centrifuged at 13,000 *g* for 10 min at 4°C. After gentle removal of the fat layer, the aqueous fractions and cell pellets were harvested and stored separately. Samples were stored at −80°C until testing and transported overnight to the University of Illinois at Chicago on dry ice in Styrofoam boxes. At the time of analysis, samples were thawed on ice, inspected visually for the presence of any signs of physical deterioration, and analyzed immediately. Samples were subjected to no more than two freeze-thaw cycles.

In some experiments, we added *Clostridium perfringens* neuraminidase (Invitrogen, San Diego, CA) to milk samples to activate latent TGF-β. The manufacturer defined 1 U of neuraminidase as the amount necessary to liberate 1 μmol of N-acetylgalactosamine acid per minute from bovine submaxillary mucin at pH 5.0 at 37°C.

**Animals.** Murine intestine was harvested after euthanasia from fetuses on embryonic day 18.5, pups on postnatal days 2 and 10, and adult mice (*n* = 6 animals per group). Studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

**ELISAs.** Commercially available ELISA kits (R & D Systems, Minneapolis, MN) were used to measure TGF-β1, TGF-β2, TGF-β3, and THBS-1 concentrations in the aqueous fraction of milk samples. Optical densities and standard concentrations were logarithmically transformed, and a linear equation was obtained (acceptable *r*^2^ ≥ 0.95). Analyte concentrations in test samples were calculated by regression. The linear standard range of measurement for all four assays was 3.2–1,000 pg/ml.

**TGF-β bioactivity.** One of two luciferase reporter cell lines was used to measure TGF-β bioactivity in milk samples: 1) mink lung epithelial cells (MLEC) stably transfected with a luciferase reporter construct containing the TGF-β-responsive plasminogen activator inhibitor-1 gene promoter (43) and 2) RAW 264.7 cells stably transduced with a luciferase lentiviral construct containing a Smad-response element (Cignal SRE luc reporter kit, Qiagen, Valencia, CA). Bioactive TGF-β and total TGF-β bioactivity in milk samples was measured by addition of milk samples (diluted 1:1 in serum-free medium) in the native state or after heat treatment at 80°C for 5 min (to activate latent TGF-β). After 16 h, cell lysates (M-PER reagent, Thermo Scientific, Rockford, IL) were used to measure luciferase activity with a commercially available kit (GloMax multidetection system, Promega, Madison, WI). In some experiments we added a neuraminidase inhibitor, N-acetyl-2,3-dehydro-2-deoxyuronic acid (50 μM, predetermined optimum; Sigma). All assays were performed in triplicate.

**RT-quantitative PCR.** Primers (Table 1) were designed using Beacon Design software (Bio-Rad, Hercules, CA). We used a standard RT reaction and SYBR green-based method to measure mRNA expression and normalized data against GAPDH or the 18S rRNA gene. Groups were compared by the cycle threshold (2^(-ΔΔCt)) method (5, 65).

**Gelatinase zymography.** Gelatinolytic activity of MMP-2 and MMP-9 was measured in milk samples by zymography. Milk-borne gelatinases were separated electrophoretically using 10% SDS-PAGE (gel copolymerized with 1 mg/ml gelatin) and then renatured, stained with Coomassie blue, and destained using established methods (1).

**Immunohistochemistry.** Deidentified human mammary tissues from biopsies and healthy margins of surgically resected neonatal intestinal tissues from preterm (24–27 wk gestation) and full-term neonates were immunostained as previously described (40). Briefly, tissue sections were deparaffinized, and antigen retrieval was achieved using EZ-AR solution (Biogenex, San Ramon, CA). All assays were performed in triplicate.

### Table 1. Primer sequences used for reverse transcriptase real-time PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>TGF-β1</td>
<td>GACACCAACTATTTGCTTCAAG</td>
</tr>
<tr>
<td></td>
<td>TGF-β2</td>
<td>GCTTCTCCTTGTGCTGCTTC</td>
</tr>
<tr>
<td></td>
<td>TGF-β3</td>
<td>GAGCCACTCTGTGCTGCTTC</td>
</tr>
<tr>
<td></td>
<td>THBS-1</td>
<td>ATGTACGTAGCTGCTGCTTCA</td>
</tr>
<tr>
<td></td>
<td>MMP-2</td>
<td>AGCTGAGCCACAGCAGTGAAG</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>TTCTCGAGCAAGATCTTCACTC</td>
</tr>
<tr>
<td></td>
<td>ITGAV</td>
<td>TTCTTATTGCTGAGATGCTCTG</td>
</tr>
<tr>
<td></td>
<td>ITGB6</td>
<td>AACAGATGTCTACATCTGTG</td>
</tr>
<tr>
<td></td>
<td>ITGB8</td>
<td>TCTTACGTCTTCAAGAAGTCTTC</td>
</tr>
<tr>
<td></td>
<td>CD90</td>
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<td></td>
<td>GAPDH</td>
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<tr>
<td>Murine</td>
<td>TGF-β2</td>
<td>GCTTCTGAGATGAGAAGAATGAG</td>
</tr>
<tr>
<td></td>
<td>18S rRNA</td>
<td>TCGGATAGAAGGAGGAG</td>
</tr>
</tbody>
</table>

TGF, transforming growth factor; THBS-1, thrombospondin-1; MMP, matrix metalloproteinase; ITGAV, integrin chain αv; ITGB6, integrin chain β6; TGF-β2, TGF-β receptor 2.
Secondary staining was performed with Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 30 min at room temperature. Nuclear staining was achieved with 4′,6-diamidino-2-phenylindole (Calbiochem, San Diego, CA). Fluorescence imaging was performed using a confocal microscope (model LSM 710, Zeiss).

Western blots. TGF-β2 was immunoprecipitated from milk samples using a standard protocol (35), separated in nondenaturing polyacrylamide gel, and immunoblotted as previously described (46). To measure TGF-β signaling, Smad2 and phosphorylated Smad2 expression was assessed in explanted intestinal tissue from 10-day-old C57BL/6 mouse pups after treatment with milk samples. The intestine was opened longitudinally, rinsed gently in PBS, and cut into 3- to 5-mm² explants, which were placed in serum-free RPMI 1640 medium in 5% CO₂ at 37°C. Some explants were treated with equal volumes of milk samples or PBS added 1:1 (vol/vol) for 25 min. After treatment, explants were homogenized in ice-cold lysis buffer (T-PER reagent containing protease and phosphatase inhibitors, Thermo Scientific).

Enzymatic activity assays. A commercially available fluorometric assay (Anaspec, Fremont, CA) was used to measure plasmin activity in human milk. The assay measures plasmin activity using a substrate that releases the yellow-green fluorophore 7-amido-4-trifluoromethylcoumarin (M). 4-Trifluoromethylcoumarin, which can be quantified at 380-nm excitation and 500-nm emission. The assay has a sensitivity of 0.2 mU/ml.

NF-κB activation. NF-κB activation was measured using RAW 264.7 cells stably transfected with the pNF-κB/SEAPporter assay kit (Invitrogen). This assay is based on the detection of H₂O₂ generated by galactose oxidase oxidation of desialylated galactose, the end result of neureaminidase action. In the presence of horseradish peroxidase, H₂O₂ reacts with the Ampex Red reagent to generate the red oxidation product resorufin, which can be read spectrophotometrically at 571-nm excitation and 585-nm emission. Data are expressed as the final concentrations of 7-amido-4-trifluoromethylcoumarin (μM).

Neureaminidase activity was measured in human milk and murine intestinal tissue using the Amplex Red neuraminidase assay kit (Invitrogen). This assay is based on the detection of H₂O₂ generated by galactose oxidase oxidation of desialylated galactose, the end result of neuraminidase action. In the presence of horseradish peroxidase, H₂O₂ reacts with the Ampex Red reagent to generate the red oxidation product resorufin, which can be read spectrophotometrically at 571-nm excitation and 585-nm emission. The assay has a sensitivity of 0.2 mU/ml.

NF-κB activation. NF-κB activation was measured using RAW 264.7 cells stably transfected with the pNF-κB/SEAP, which expresses the secreted alkaline phosphatase (SEAP) protein under the control of the NF-κB promoter (NF-κB SEAPorter assay kit, Imgenex, San Diego, CA). RAW 264.7 reporter cells were treated with milk samples mixed 1:1 with serum-free medium for 24 h, and culture supernatants were assayed for SEAP activity according to the manufacturer’s protocol. Briefly, 250 μl of culture supernatant were first diluted 1:10 in assay buffer, and equal volumes of the diluted sample and H₂O₂ were incubated at 65°C for 30 min to inactivate endogenous alkaline phosphatase. Then 100 μl of a colorigenic substrate (p-nitrophenyl phosphate, 1 mg/ml) were added to each well, and the samples were incubated for 1 h. Absorbance was read at 405 nm after 30 min and 1 h. The linear range of measurement of the assay was 3.1–200 ng/ml.

Statistical methods. Statistical analysis was performed using Sigma Stat 3.1.1 software (Systat, Point Richmond, CA). Parametric data were depicted using bar diagrams, whereas nonparametric data were shown using Tukey-Koopman box-whisker plots. For PCR data, crossing-threshold (ΔΔCt) values for genes with an at least twofold change were compared by the Mann-Whitney U-test. Comparison across multiple groups was performed using the Kruskal-Wallis H-test. In all tests, P < 0.05 was accepted as significant.

RESULTS

Preterm human milk contains minimal TGF-β bioactivity in the native state but contains a large pool of latent TGF-β, which can be readily activated. Using MLEC reporter cells, we first measured TGF-β bioactivity in preterm human milk from mothers who delivered between 23 0/7 and 31 6/7 wk and provided milk samples within 48 h (colostrum), 1 wk, and 1 mo after delivery (n = 50 mothers per group). Figure 1A shows TGF-β bioactivity in colostrum, 1-wk, and 1-mo samples of preterm milk in the native state and after heat treatment. Taken together, these data showed low levels of bioactive TGF-β in preterm milk in the native state. However, a substantial pool of latent TGF-β was detected in 1-wk and 1-mo samples that could be activated by heat treatment.

To investigate our hypothesis that preterm human milk contains less bioactive TGF-β than full-term milk, we next compared preterm and full-term milk samples received within the 1st wk after delivery (n = 20 mothers in each group). Preterm milk contained less bioactive TGF-β in the native state (Fig. 1B, left), but after heat treatment to activate latent TGF-β, preterm milk showed significantly more TGF-β bioactivity than full-term milk (Fig. 1B, middle). These data showed that preterm milk not only showed less TGF-β bioactivity than full-term milk in absolute terms but also contained less bioactive TGF-β when expressed as a proportion of the total pool (latent + active) of milk-borne TGF-β (Fig. 1B, right). Based on these findings, we hypothesized that preterm milk is relatively deficient in TGF-β-activating mechanisms present in full-term milk. In view of emerging clinical evidence indicating that donor human milk and a human milk-derived human milk fortifier may provide some protection against NEC (6, 54, 67), we also measured TGF-β bioactivity in donor milk (n = 10 donors) and the human milk-derived fortifier (n = 10 samples). Similar to fresh preterm milk, donor milk and human milk-derived fortifier showed minimal TGF-β bioactivity in the native state but revealed substantial TGF-β bioactivity after heat treatment (Fig. 1, C and D).

Preterm human milk contains a larger pool of TGF-β2 than full-term milk. We next used specific ELISA to compare TGF-β1, TGF-β2, and TGF-β3 concentrations in preterm vs. full-term milk. Each of these ELISAs includes an acid-activation step to activate latent TGF-β and, therefore, measures the sum of the active and latent forms of the specific TGF-β isoform. We detected a significantly larger pool of active and latent TGF-β2 in preterm than full-term milk [median 8,837 (range 1,771–15,849) pg/ml in preterm milk vs. median 4,125 (range 982–10,927) pg/ml in full-term milk, P = 0.04]. These findings are consistent with higher levels of total TGF-β2 bioactivity in heat-treated preterm (vs. full-term) milk (Fig. 1B, middle). The concentrations of TGF-β1 and TGF-β3 in preterm and full-term milk samples were comparable [TGF-β1: median 110 (range undetectable to 227) pg/ml in preterm milk vs. median 152 (range undetectable to 318) pg/ml in full-term milk (P = 0.88); TGF-β3: median 37 (range undetectable to 48) pg/ml in preterm milk vs. median 83 (range undetectable to 95) pg/ml in full-term milk (P = 0.89)]. These data are depicted in Fig. 2A; a consistent y-scale was used in the three box plots to highlight the predominance of TGF-β2 among the three TGF-β isoforms in milk.

To confirm that preterm milk contains less bioactive TGF-β2 than full-term milk, we immunoprecipitated TGF-β2 from milk samples and resolved active and latent TGF-β2 by nondenaturing PAGE (n = 10 samples per group). Consistent with bioactivity measurements in MLEC reporter cells, native preterm milk expressed less active TGF-β2 (14 kDa) than full-term milk (Fig. 2B, left). Immunoprecipitation of TGF-β2 depleted most of the TGF-β bioactivity from milk samples,

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confirming that TGF-β2 is the predominant isoform of TGF-β in milk (Fig. 2B, right).

We next asked whether the differences in the concentrations of TGF-β isoforms in preterm vs. full-term milk resulted from differential expression of these isoforms in milk cells. In RT-quantitative PCR assays, we did not detect a significant difference in mRNA expression of TGF-β1, TGF-β2, or TGF-β3 in the cellular fractions of preterm and full-term milk (Fig. 2C). We next performed immunohistochemistry on human mammary tissue to identify the cellular source of milk-borne TGF-β2. TGF-β2 was immunolocalized to epithelial cells (Fig. 2D, inset), indicating a vectorial discharge pattern directed into the mammary glands (17).

Low neuraminidase activity in preterm milk can explain lower TGF-β bioactivity in preterm than full-term milk. We next investigated our second hypothesis: that lower TGF-β bioactivity in preterm milk reflects the deficiency of one or more activators of TGF-β in preterm milk. Pro-TGF-β2 lacks the tripeptide motif arginyl-glycyl-aspartic acid and is, therefore, not sensitive to integrin-dependent activation (43). Current evidence indicates a role for the glycoprotein THBS-1, proteases such as plasmin, MMP-2, and MMP-9, and glycoside hydrolases such as neuraminidase (23, 44).
Plasmin activity was comparable in preterm and full-term milk (Fig. 3A). THBS-1 mRNA was expressed at significantly lower levels in the cellular fractions of preterm than full-term milk (0.02 ± 0.01 fold change, \( P < 0.05 \); data not depicted). However, there was no difference in the THBS-1 protein concentrations in preterm vs. full-term milk (Fig. 3B). Preterm and full-term milk showed considerable variability in MMP-2 and MMP-9 mRNA/ enzyme activity; the two groups did not show a significant difference (Fig. 3, C and D).

Preterm milk showed significantly less neuraminidase activity than full-term milk (Fig. 3E). In human mammary tissue, neuraminidase-1, a membrane-associated protein in this enzyme family (41), was immunolocalized to epithelial cells (Fig. 3F). Neuraminidase-1 immunoreactivity was more prominent along the apical aspects of epithelial cells (Fig. 3F, inset), indicating that the protein is likely discharged into the mammary glands.

In addition to the activators of TGF-\( \beta \), we also compared the cellular fractions of preterm and full-term milk for mRNA expression of ITGAV (integrin chain \( \alpha_V \)), ITGB6 (integrin chain \( \beta_6 \)), ITGB8 (integrin chain \( \beta_8 \)), and Thy-1/CD90, genes involved in integrin-mediated activation of TGF-\( \beta \) and TGF-\( \beta \). Cells in preterm milk expressed less ITGB8, but the other analytes were expressed at comparable levels (Fig. 3G).

**Fetal/neonatal intestine is less efficient than the mature intestine at in situ activation of milk-borne TGF-\( \beta \).** We argued that milk-borne neuraminidase activity and the levels of pre-activated TGF-\( \beta \) may not be biologically relevant if the neonatal intestine had adequate capacity for in situ activation of...
orally ingested TGF-β. To investigate this question, we took advantage of the highly conserved nature of TGF-β across mammalian species: each of the TGF-β isoforms in humans and mice shows >98% amino acid sequence homology and cross-species reactivity (37, 45). To estimate the capacity of the neonatal intestine for in situ activation of milk-borne TGF-β, we treated preterm human milk with tissue lysates from murine fetal/neonatal and adult intestine and measured TGF-β bioactivity after 16 h. As depicted in Fig. 4A, neonatal intestinal tissue was less efficient than the adult intestine at TGF-β activation.

We next sought to determine the contribution of intestinal neuraminidase expression to the ability of intestinal tissue to activate milk-borne TGF-β. Similar to the previous experiment, we treated preterm human milk with tissue lysates from neonatal (postnatal day 10) mice and, in some experiments, added a neuraminidase inhibitor (N-acetyl-2,3-dehydro-2-deoxyneuraminic acid). The neuraminidase inhibitor blocked the effect of intestinal tissue lysates on TGF-β activation (Fig. 4B), indicating that neuraminidase was a major contributor to the in situ activation of milk-borne TGF-β in the intestine.

To explain the maturational increase in the ability of intestinal tissue to activate milk-borne TGF-β, we hypothesized that neuraminidase expression in the intestine is developmentally regulated and increases with maturation. We measured neuraminidase activity in the murine intestine on embryonic day 18.5, postnatal days 2 and 10, and 6–8 wk after birth. Consistent with our hypothesis, we detected low neuraminidase activity in the fetal intestine, which increased after birth through adulthood (Fig. 4C).

Finally, to validate our findings of the maturational increase in intestinal neuraminidase expression in the human developing intestine, we performed immunohistochemistry for neur-
aminidase-1 on histologically intact preterm (25–28 wk gestation) and full-term intestinal tissue that was resected during surgery for bowel obstruction (n/H11005 3 per group). Neuraminidase-1 was immunolocalized in epithelial cells in a discrete, punctate pattern along the apical membrane (Fig. 4D). Consistent with our findings in mice, preterm human intestine showed patchy neuraminidase-1 immunoreactivity, which was weaker than in the full-term intestine.

**Addition of bacterial neuraminidase to preterm human milk increased TGF-β bioactivity.** We next investigated our third hypothesis: that TGF-β bioactivity in preterm milk can be increased to levels comparable to full-term milk by addition of TGF-β activator(s) that are expressed at lower levels in preterm than full-term milk. To test this hypothesis, we added neuraminidase prepared from a bacterial source, *C. perfringens*, to preterm milk and measured its effect on TGF-β bioactivity in preterm milk. As depicted in Fig. 5A, supplemental neuraminidase increased TGF-β bioactivity in preterm milk in a dose-dependent fashion in the MLEC reporter cells. Similar results were obtained in RAW 264.7 SRE reporter cells (data not shown).

Existing studies indicate that TGF-β receptors are expressed at much lower levels in porcine fetal/neonatal than adult intestine (63). Because data depicted in Fig. 5A were obtained in reporter cell lines, we next sought confirmation that the neonatal intestine is not already saturated for TGF-β signaling and is capable of responding to increased TGF-β bioactivity in neuraminidase-treated preterm milk. We treated explanted murine intestinal tissue with preterm human milk pretreated with *C. perfringens* neuraminidase. Similar to the porcine intestine...
Fig. 5. Addition of bacterial neuraminidase to preterm human milk increased TGF-β bioactivity. A: TGF-β bioactivity in MLEC reporter cells. TGF-β bioactivity in preterm milk can be increased in a dose-dependent fashion by addition of Clostridium perfringens neuraminidase (1 and 10 mU). Values are means ± SE. B: addition of C. perfringens neuraminidase (10 mU) to preterm human milk samples before treatment of explanted murine intestinal tissue with these milk samples increased Smad2 phosphorylation in the explants. Top: densitometric data. Values are means ± SE. Bottom: immunoblots showing phosphorylated (phospho-Smad2) and total Smad2 expression in murine intestinal tissue. Inset: fold changes in TGF-β receptor 2 (TGFβR2) mRNA (normalized against 18S rRNA gene) on E18.5, P2, and P10 and in 6- to 8-wk-old (Adult) mice. TGF-β receptor 2 mRNA expression is significantly lower in fetal/neonatal than adult intestine. Values are means ± SE. C: secreted alkaline phosphatase (SEAP) concentrations in culture supernatants. NF-κB activation was measured in RAW 264.7 NF-κB/SEAP reporter cells. Addition of C. perfringens neuraminidase (10 mU) to preterm human milk increased its ability to suppress LPS-induced NF-κB activation to levels comparable to full-term milk. Values are means ± SE; n = 3 separate experiments, with each sample tested in triplicate. 

*P < 0.05, **P < 0.01, ***P < 0.001.

DISCUSSION

We present a detailed investigation into TGF-β expression and its bioactivity in preterm human milk. To our knowledge, this is the first study to specifically investigate the effects of preterm delivery on the expression of TGF-β in human milk. We show that even though preterm human milk shows minimal TGF-β bioactivity in the native state, there is a large pool of latent TGF-β in preterm milk, which can be readily activated.

Finally, we asked whether neuraminidase effects on milk-borne TGF-β were biologically relevant. Because TGF-β is a potent inhibitor of macrophage inflammatory responses (34), we asked whether neuraminidase treatment can improve the ability of preterm human milk to suppress NF-κB signaling in macrophages. We treated RAW 264.7 NF-κB/SEAP reporter cells with preterm and full-term human milk for 24 h and then with LPS. As depicted in Fig. 5C, addition of C. perfringens neuraminidase (10 mU) to preterm human milk increased its ability to suppress LPS-induced NF-κB activation to levels comparable to full-term milk.
TGF-β may protect against NEC and allergic disorders, these findings merit further investigation in preclinical and clinical settings.

We detected considerable TGF-β bioactivity in preterm milk, particularly in the 1-wk and 1-mo samples. At these time points, most TGF-β was in a latent form, which is consistent with earlier observations in human and animal milk by Srivastava et al. (66), Nakamura et al. (45), Rogers et al. (60), Pakkanen (50), and Xu et al. (71). In our study, we detected increased latent TGF-β at later time points. Existing studies show conflicting information on temporal changes in milk TGF-β concentrations after delivery. In some studies, colostrum showed the highest TGF-β concentrations, which then decreased as a function of time after delivery (8, 62, 68). In other cohorts, no clear trends were identified (19). Although the presence of TGF-β in human milk is well documented, most of the earlier studies on milk-borne TGF-β were conducted on mothers who gave birth at full term (4, 9, 11, 12, 14–16, 19–22, 24, 26, 28, 29, 38, 47–49, 51, 52, 57, 61, 69). Further study is needed to confirm whether the temporal kinetics of milk-borne TGF-β we observed represent a pattern characteristic of preterm milk. The data also need cautious interpretation in view of methodological differences; earlier studies relied on measurement of immunoreactive TGF-β1 and/or TGF-β2 by ELISA and did not measure bioactivity. Unlike ELISA, which records the presence of an immunogenic epitope, bioactivity measurements reflect the concentrations of all three isoforms of TGF-β in a given sample combined with differences, if any, in the potency of the individual isoforms (43).

In this study we detected more TGF-β2 in preterm than full-term milk. The reasons for these differences are unclear. Milk TGF-β2 levels do not correlate with plasma concentrations, indicating that TGF-β2 is either endogenously produced and secreted in mammary tissue or preferentially transported via an active mechanism (19). In clinical studies, in mothers with psychosocial stress or depression, higher TGF-β via an active mechanism (19). In clinical studies, in mothers receiving supplements, Bifidobacterium reuteri, Lactobacillus rhamnosus, or Lactobacillus paracasei showed lower milk TGF-β concentrations in a randomized controlled trial, Böttcher et al. (8) showed lower milk TGF-β2 concentrations in mothers receiving Lactobacillus reuteri supplements than in mothers those receiving placebo. However, in other randomized controlled trials, supplementation with Bifidobacterium lactis or Lactobacillus rhamnosus in atopic mothers increased milk TGF-β2 and/or TGF-β1 concentrations (53, 55).

We have identified neuraminidase as a key activator of milk-borne TGF-β. To our knowledge, this is the first study to do so. Neuraminidases encoded by several pathogens, including the influenza virus H5N1 and C. perfringens, have been previously shown to activate latent TGF-β1, although the pathophysiological importance of this phenomenon is unclear (10, 39). Zou and Sun (72) showed that neuraminidases could be used to activate recombinant latent TGF-β2 in vitro. To explain the differences in TGF-β bioactivity in preterm vs. full-term milk, we evaluated neuraminidase and several other known activators of TGF-β, such as acid/alkaline pH, heat, and reactive oxygen species, which denature the LAP; proteases such as plasmin, MMP-2, and MMP-9, which release TGF-β through cleavage of latent TGF-β; and THBS-1, which induces a conformational change in the LAP (10, 23, 58). Integrins-αβ6 and -αβ3 activate TGF-β1 and TGF-β3 by binding to the RGD motif expressed on the LAP region of these two isoforms. However, in these studies, neuraminidase was the only analyte that was significantly different between preterm and full-term milk (Fig. 3). Neuraminidases are well documented in human milk and are presumed to play a role in the digestion of milk-based oligosaccharides (70). Neuraminidases can desialylate LAP amino acids 50–85, which are critical for the noncovalent association of LAP with the mature domain (64). The reasons for the low expression of neuraminidase we observed in preterm milk are unclear.

We show that bacterial neuraminidase can activate latent TGF-β and increase anti-inflammatory effects of preterm milk to levels similar to full-term milk. We previously demonstrated anti-inflammatory effects of enterally administered TGF-β2 in a neonatal mouse model of NEC-like injury (34). Using human immature human epithelial cells in vitro, Rautava et al. (56, 57) showed that milk-borne TGF-β2 inhibits IL-1β-induced and NF-κB-mediated inflammatory signaling. Because of neuro- logical immaturity, premature infants born prior to 32 wk gestation frequently receive expressed breast milk by gavage, and fortification of mother’s milk to increase caloric density and protein and mineral content in milk is a common practice. In this context, the possibility of using a bacterial/recombinant neuraminidase as a prefeed fortification agent to activate milk-borne TGF-β in mother’s own or donor milk to enhance the anti-inflammatory effects raises exciting therapeutic possibilities. However, further work is needed to ascertain the safety and safety profile of enterally administered neuraminidases; bacterial neuraminidases have been implicated in the pathogenesis of hemolytic anemia in infants with advanced NEC, hemolytic-uremic syndrome, and thrombotic microangiopathy, possibly through unmasking of the Thomsen-Friedenreich cryptantigen (T-antigen) on erythrocytes, platelets, and glomerular endothelium (7, 13).

In conclusion, we have shown that preterm milk contains substantial amounts of latent TGF-β and identified neuraminidase as a key regulator of milk-borne TGF-β bioactivity. Although the study has important limitations of a small sample size and paucity of information on clinical/demographic characteristics of mothers who donated milk samples, there is strong potential for knowledge translation. Further study is needed in preclinical and clinical settings to investigate these findings in vivo.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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