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ORIGINAL RESEARCH—BASIC

Human Milk Supports Robust Intestinal Organoid Growth, Differentiation, and Homeostatic Cytokine Production

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BACKGROUND AND AIMS: Necrotizing enterocolitis is a severe gastrointestinal complication of prematurity. Using small intestinal organoids derived from fetal tissue of a gestational age similar to an extremely preterm infant, this study aims to assess the effect of diet on intestinal epithelial growth and differentiation to elucidate the role nutrition type plays in intestinal development and modifies the risk for necrotizing enterocolitis. **METHODS:** Organoids were cultured for 5 days in growth media and 5 days in differentiation media supplemented 1:40 with 4 different diets: parental milk, donor human milk, standard formula, or extensively hydrolyzed formula. Images were captured daily and organoids were quantified. Organoids were preserved for RNA sequencing and immunofluorescence staining with Ki67, cleaved caspase 3, and chromogranin-A. Media was saved for cytokine/chemokine and growth factor analysis. **RESULTS:** Human milk supplementation improved growth and differentiation of intestinal organoids generating larger organoids during the growth phase and organoids with longer and wider buds during differentiation compared to formula. Ki67 staining confirmed the proliferative nature of milk-supplemented organoids and chromogranin A staining proved that MM-supplemented organoids induced highest enteroendocrine differentiation. Human milk supplementation also upregulated genes involved in Wnt signaling and fatty acid metabolism pathways and promoted a homeostatic immune landscape, including via increased secretion of tumor necrosis factor-related apoptosis-inducing ligand among other cytokines. Conversely, organoids supplemented with formula had a downregulation of cell-cycle-promoting genes and a more inflammatory immune signature, including a reduced level of leukemia inhibitory factor. **CONCLUSION:** Our results demonstrate that parental milk, and to a lesser extent donor human milk, support robust intestinal epithelial proliferation, differentiation, and homeostatic cytokine production, suggesting a critical role for factors enriched in human milk in intestinal epithelial health.

Keywords: Intestinal Development; Breast Milk; Necrotizing Enterocolitis

Introduction

Necrotizing enterocolitis (NEC) is an inflammatory gastrointestinal complication of prematurity that causes intestinal necrosis and can lead to intestinal perforation, sepsis, shock, and death. With a prevalence of 5%–12% among very-low birth weight (VLBW; <1500 g) infants, NEC carries a mortality rate of up to 50% amongst neonates requiring surgical intervention and is associated with a numerous long-term complications, such as short bowel syndrome, intestinal strictures, and neurodevelopmental delay.[1] Yet despite decades of research, the exact cause of NEC remains elusive.

Compelling evidence has revealed that the largest risk factor for NEC apart from prematurity is formula feeding, while conversely, parental milk (PM) confers protection, with a 6–10 fold lower incidence of NEC among PM -fed infants compared to formula.[2,3] It is unknown whether this is due to the many known protective factors in PM or as a result of an injurious component present in formula or a combination of both. There is some evidence to show that intact bovine protein is injurious to the gut, making

Abbreviations used in this paper: **APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; CC3, cleaved caspase-3; CCL28, C-C motif chemokine ligand 28; CHGA, chromogranin A; CX3CL, C-X3-C motif chemokine ligand 1; CXCL10, C-X-C motif chemokine ligand 10; DGE, differential gene expression; DHM, donor human milk; EEC, enteroendocrine cell; EGF, epidermal growth factor; HF, hydrolyzed formula; IL-7, interleukin-7; IL-15, interleukin-15; IL-18, interleukin-18; LIF, leukemia inhibitory factor; M-CSF, macrophage colony-stimulating factor; MDC, macrophage-derived chemokine; MFI, mean fluorescence intensity; MIG/CXCL9, monokine induced by gamma interferon; MPIF-1, myeloid progenitor inhibitory factor 1; NEC, necrotizing enterocolitis; OGM, organoid growth medium; PCA, principal component analysis; PM, parental milk; SF, standard formula; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TSLP, thymic stromal lymphopoietin.**

Most current article

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hydrolyzed formulas (HFs) less damaging.[4] PM is known to contain a myriad of bioactives that have been demonstrated to have beneficial effects on immune development and other measures of neonatal health.[5,6] Unfortunately, PM is frequently unavailable as lactation insufficiency is common in parents of preterm infants; as such, it has become common at many centers to provide donor human milk (DHM) to preterm infants when PM is not available. Because DHM is typically expressed by donors at later stages of lactation and due to the pasteurization process it is required to undergo, it typically contains a lower protein and bioactives content; nevertheless, DHM has been shown to confer some degree of protection against development of NEC compared to formula.[5,7,8]

The Clevers' group developed intestinal epithelial cultures known as organoids that recapitulate intestinal tissue, maintaining the crypt-villus architecture and cellular heterogeneity and remaining phenotypically stable over long periods of time.[9,10] Intestinal organoids' morphological and functional characteristics depend on the subject's age.[11] As such, it is critical to use samples that are obtained from subjects close in age to the process being investigated. Using organoids derived from fetal intestinal samples of a similar gestational age to an extremely preterm infant, we investigated the effect of diets common in preterm infants (PM, DHM, standard formula (SF), and HF) on intestinal organoid growth and differentiation to elucidate the role diet plays in extra-uterine intestinal development and thereby modifying risk for NEC.

Methods

Patient Samples

Fetal small intestinal tissue samples were provided by the Konnikova Lab Biorepository that were collected at the University of Pittsburgh with IRB approval and informed consent. Sample information is provided in Table A1. Deidentified parental and DHM was acquired from Yale New Haven Children's Hospital under approved IRB 2000031633.

Human Milk Macronutrient Analysis

Parental and DHM were analyzed for their macronutrient content via mid-infrared spectroscopy (Miris Human Milk Analyzer, Sweden) provided in Table A2.

Organoid Culture

Crypts were isolated from cryopreserved fetal small intestinal tissue samples and cultured in 3D Matrigel following the manufacturer's protocol (Human-IntestiCult-Organoid-Growth-Medium, StemCell Technologies) with modifications as described in Supplementary Methods.

Once organoids were established, they were passaged according to manufacturer's protocol and plated in 30 wells, split between two 48-well plates. Each plate contained 3 technical replicates of 5 conditions: control, PM, DHM, SF, and extensively HF. Organoids were cultured for 5 days in growth media supplemented with diet conditions and 5 days in differentiation media supplemented with diet conditions. At the end of the culture period, organoids were preserved for RNA bulk sequencing and IF staining. Detailed culture methods are provided in Supplementary Methods.

Quantification of Organoids

Growth phase organoids were manually outlined in ImageJ using the paintbrush function, reducing full-color images to white (organoid present) or black (background) with hand watershedding on adjacent or overlapping organoids. An example image is provided in Figure A1. A python code adapted from Matthews et al quantified the number and area of organoids per image.[12]

Differentiating organoids were manually identified and counted. For each differentiating organoid, the number of buds, bud length, and bud diameter was recorded using ImageJ measure function.

Immunofluorescence Staining

Whole-mount immunofluorescence staining was performed as described[13] with modifications described in Supplementary Methods.

Quantification of Immunofluorescence Staining

ImageJ was used to subtract background and quantify mean fluorescence intensity from immunofluorescence images. Details are provided in Supplementary Methods.

Statistical Analysis

For individual case analysis of organoid number, area, percentage of differentiating organoids, bud length, bud diameter, and immunofluorescence staining, ordinary one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used in GraphPad Prism. For aggregate data combining all organoid cases, data was transformed by dividing each case by the average of its respective control group. Data was then analyzed with main effects-only two-way ANOVA with Tukey's correction for multiple comparisons in GraphPad Prism. For all experiments $P < .05$ was considered significant.

RNA Extraction and Sequencing

Organoids were frozen at -20 °C for storage prior to RNA extraction. RNA was extracted utilizing Qiagen RNeasy Plus Micro Kit per manufacturer instructions. RNA aliquots were treated with ezDNase Enzyme (Thermo-Fisher) and sent for QC assessment via electropherogram to the Yale Center for Genome Analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples that demonstrated lingering DNA peaks were cleaned up with Qiagen RNeasy MinElute Cleanup Kit and treated again with ezDNase Enzyme. cDNA libraries were prepared utilizing the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, USA) per manufacturer instructions. The RNA input was 100 ng for all samples except for those whose lower concentration required the utilization of all available RNA. Invitrogen UltraPure Distilled Water (Thermo-Fisher) was used for sample and reagent

dilution. SPRISelect beads (Beckman Coulter) were used for the sample cleanup steps. Samples were submitted for sequencing to the Yale Center for Genome Analysis. Following QC analysis with Agilent D1000 Screentape System, samples that required it underwent size selection. Libraries were sequenced on NovaSeq for 25 million reads per sample. Details of RNAseq analysis are provided in Supplementary Methods.

Cytokine Analysis

At each media change, cell culture supernatant was pooled between the technical replicates and frozen at −80C. Samples from day 5 of the growth phase and day 3 of the differentiation phase (day 8) for each condition were sent to Eve Technologies and analyzed using the Human Cytokine/Chemokine 96-Plex Discovery Assay® Array (HD96). Quantile normalization followed by log2 transformation was performed for each cytokine/chemokine, which was then analyzed in aggregate using principal component analysis. Pairwise differential expression was performed between each condition and each case replicate. Cytokines/chemokines were also individually analyzed using ordinary one-way ANOVA with Tukey's correction for multiple comparisons in GraphPad Prism. For all analyses, $P < .05$ was considered significant.

Results

Human Milk Supplementation Improves Organoid Growth

To investigate the effect of diet supplementation on fetal intestinal organoid generation and growth, we cultured organoids from 5 different subjects (Table A1) for 5 days in organoid growth media (organoid growth medium) supplemented with PM, DHM, SF, or HF. Macronutrient analysis of PM and DHM is provided in Table A2. Of note, parental and DHM came from different sources, making direct comparison difficult. However, macronutrients for both were similar to each other and to published reference ranges. There were no significant differences in day 5 organoid survival or the number of organoids generated between organoid growth medium (control) and organoids supplemented with any of the diet conditions in either a representative case (case 3) or across all 5 cases analyzed in aggregate (Figure 1A and B). Organoid counts from day 1 and 3 (case 3) and day 5 (all cases) are provided in Figure A2. Representative images from case 3 are provided in Figure 1C. In contrast, although on day 1 all diet

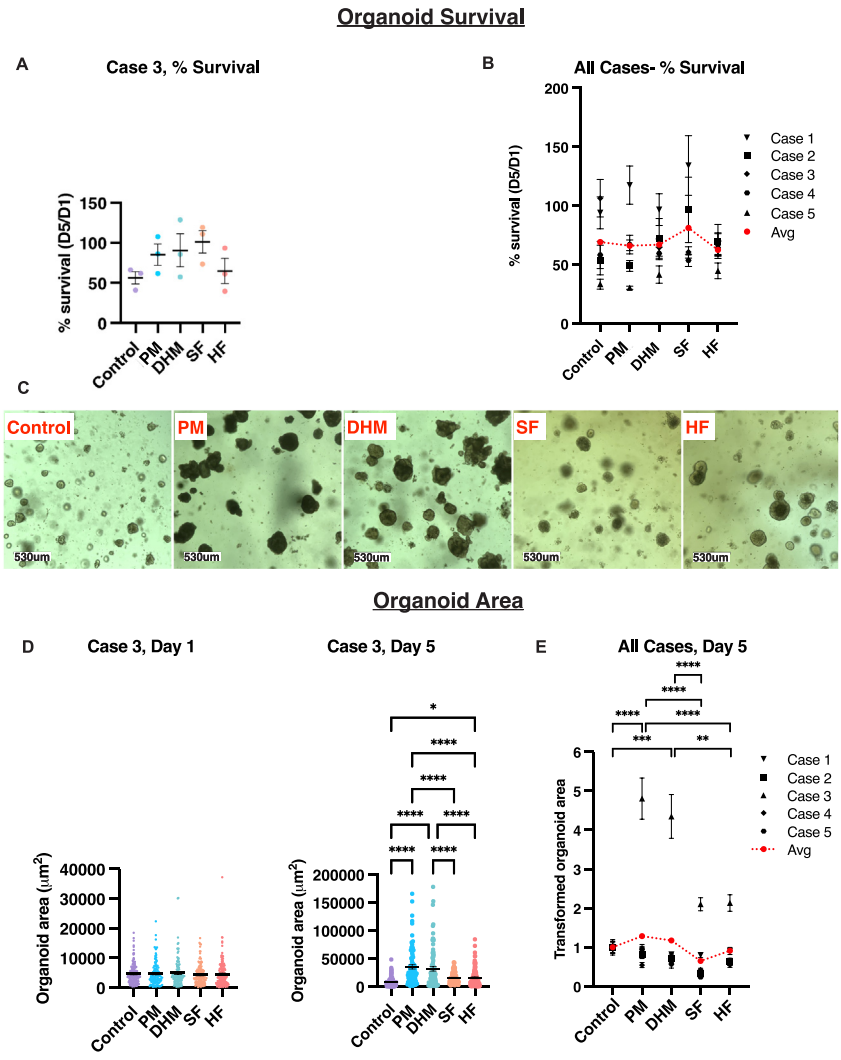

Figure 1. Human Milk Increases Organoid Size. (A) Day 5 organoid survival, case 3. 3. (B) Day 5 organoid survival, all cases. (C) Representative images from day 5, case 3. (D) Organoid area on day 1 and 5, case 3. (E) Transformed organoid area on day 5, all cases. Graphs display mean and standard error of the mean (SEM). One-way ANOVA with Tukey's correction for multiple comparisons in individual case analysis; for aggregate data analysis, data was transformed by dividing each case by the average of its respective control group and analyzed with main effects-only two-way ANOVA with Tukey's correction for multiple comparisons. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

conditions resulted in similar sized organoids, by day 5 of culture, organoids supplemented with human milk, either PM or DHM, were significantly larger compared to both formula conditions (SF and HF) and control organoids (Figure 1D and E). Notably, there was also a trend for HF to promote larger organoids than SF, which resulted in the smallest organoids (Figure 1E). There was no difference in organoid size between PM and DHM.

Human Milk Supplemented Organoids are Highly Proliferative

To assess if the increase in organoid size was due to increased proliferation, we examined the effect of diet supplementation on organoid proliferation with Ki67 immunofluorescence staining. During the growth phase, organoids from representative case 3 had the highest proliferation rate in the human milk groups (PM and DHM) with the lowest rates of proliferation in SF (Figure 2A). Insufficient number of organoids was obtained for analysis in the control group in case 3. The aggregate data were even more significant, with PM again showing the highest Ki67 staining of all groups with significant difference between PM and both SF and HF groups (Figure 2B). DHM had similar levels of Ki67 expression as the control group, and both were significantly higher than SF, which had the lowest proliferation of all the groups (Figure 2B). No statistically significant difference was observed within the human milk groups. Representative images of staining from case 3 are provided in Figure 2C.

We also assessed the amount of apoptosis occurring via cleaved caspase-3 (CC3) staining. In representative case 3, there was a trend for HF to have the highest apoptosis level; in the aggregate data this was significant between HF and PM and DHM (Figure 2D and E). After HF, SF had the next highest level of apoptosis while PM and DM had the lowest (Figure 2E). Representative images of staining from case 3 are provided in Figure 2F.

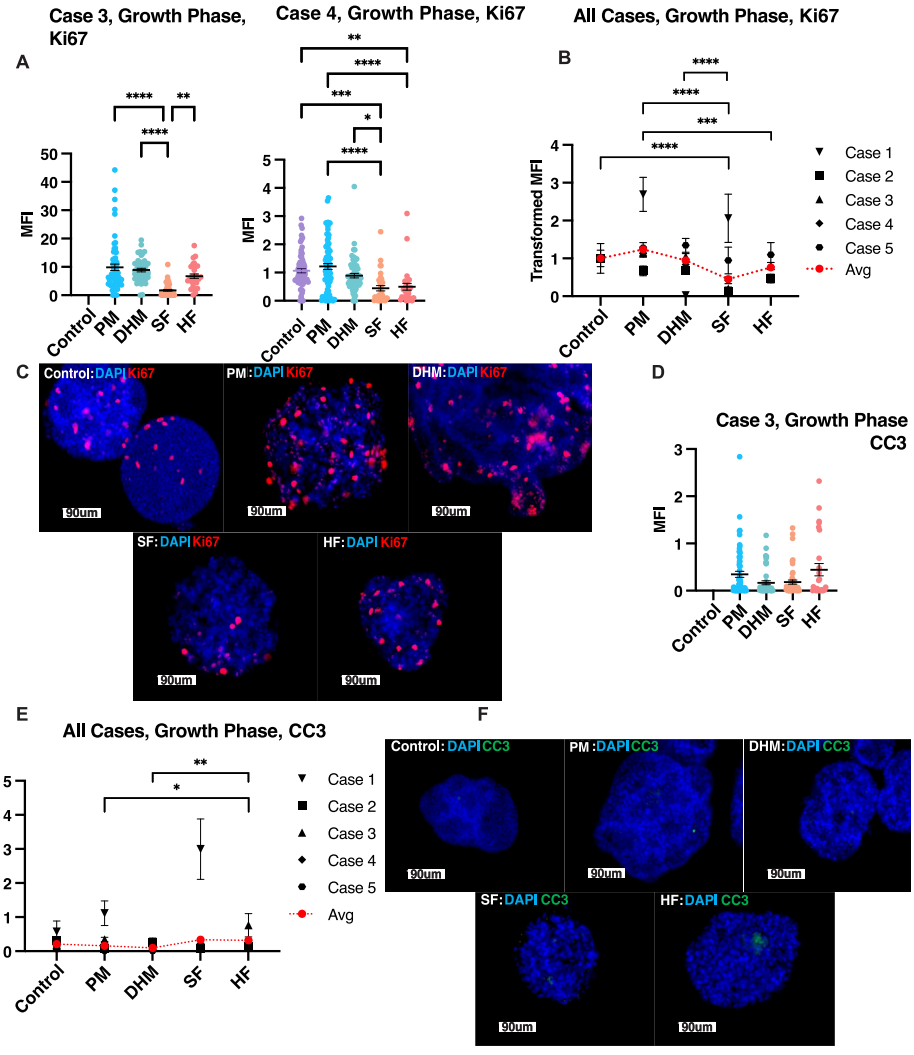

Figure 2. Human Milk Increases Organoid Proliferation. (A) Mean fluorescence intensity (MFI) of Ki67 staining in growth phase organoids, case 3 and case 4. (B) Transformed MFI of Ki67 staining in growth phase organoids, all cases. (C) Representative growth phase images from case 3 when available; control from case 4. (D) MFI of cleaved caspase-3 (CC3) staining in growth phase organoids, representative case 3. (E) Transformed MFI of CC3 staining in growth phase organoids, all cases. (F) Representative growth phase images from case 3 when available; control from case 4. Graphs display mean and standard error of the mean (SEM). One-way ANOVA with Tukey's correction for multiple comparisons in individual case analysis; for aggregate data analysis, data was transformed by dividing each case by the average of its respective control group and analyzed with main effects-only two-way ANOVA with Tukey's correction for multiple comparisons. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

Human Milk Supplementation Induces Increased Organoid Differentiation

To investigate if there was an effect of diet supplementation on organoid differentiation, we cultured organoids for an additional 5 days in supplemented organoid differentiation media. Upon provision of differentiation media, spherical organoids give rise to more complex structures with buds that resemble the crypt-villus-axis. By day 3 of differentiation (day 8 of culture), in representative case 3, DHM had produced the highest percentage of differentiating organoids (defined as an organoid with a nonspherical shape with one or more buds) per high power field, with significance to both SF and HF (Figure 3A). There was also a trend towards lower differentiation in both of the formula conditions but especially in HF (Figure 3A). This was consistent in the aggregate data, with added significance in the percentage of differentiating organoids between DHM and control; additionally, PM had a significantly higher percentage of differentiating organoids as compared to SF (Figure 3A). DHM also produced organoids with significantly higher number of buds per organoid compared to PM and SF in representative case 3 and compared to all other diet conditions in the aggregate data (Figure 3B).

We also assessed the length and diameter of the buds of the differentiating organoids. In representative case 3, PM supplementation resulted in the longest buds, with significance to DHM and control (Figure 3C). This held true in the aggregate data, with both PM and DHM supplementation leading to significantly longer buds compared to SF (Figure 3C). This effect was even stronger in bud diameter, with PM again having significantly wider buds than DHM or control in representative case 3 (Figure 3D). DHM also induced significantly wider buds than control media alone (Figure 3D). Interestingly, in the aggregate data PM produced a significantly wider bud diameter compared to all conditions with a similar strong trend compared to SF. Finally, non-supplemented control differentiation media yielded the thinnest organoid buds compared to all other conditions (Figure 3D). Representative images from case 3 and an example of bud measurement is provided in Figure 3E.

Finally, we assessed proliferation via Ki67 staining during the differentiation phase. As expected, organoids of

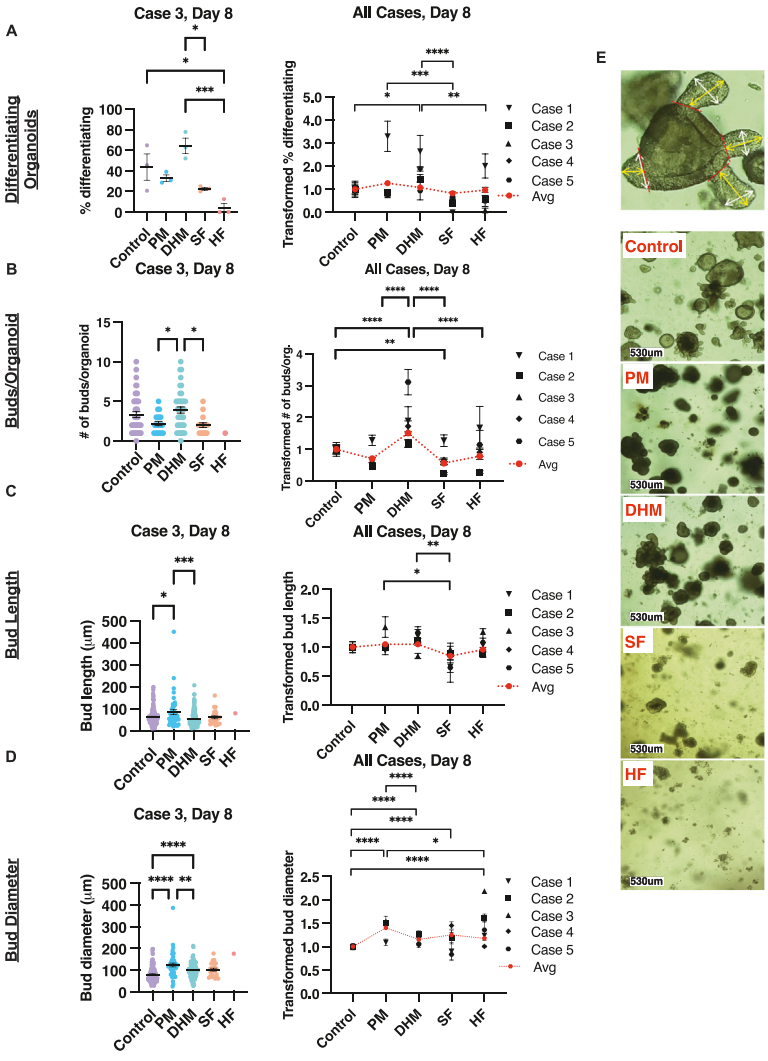

Figure 3. Human Milk Improves Organoid Differentiation. (A) Percentage of differentiating organoids per hpf, day 8, case 3 and transformed percentage of differentiating organoids per hpf, day 8, all cases. (B) Number of buds/organoid, day 8, case 3 and transformed number of buds/organoid, day 8, all cases. (C) Bud length, day 8, case 3 and transformed bud length, day 8, all cases. (D) Bud diameter, day 8, case 3, and transformed bud diameter, day 8, all cases. (E) Example bud measurement and representative day 8 images from case 3. Graphs display mean and standard error of the mean (SEM). One-way ANOVA with Tukey's correction for multiple comparisons in individual case analysis; for aggregate data analysis, data was transformed by dividing each case by the average of its respective control group and analyzed with main effects-only two-way ANOVA with Tukey's correction for multiple comparisons. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

all conditions, including control, demonstrated a log decrease in Ki67 mean fluorescence intensity from the growth phase (Figure A3A and B). No HF supplemented organoids from representative case 3 were available for analysis. Notably, although the trends between control, DHM, SF, and HF remained the same with both control and DHM showing significantly higher Ki67 staining compared to both formula types in the aggregate data, PM decreased to similar levels of Ki67 expression as SF (Figure A3B). Representative images of staining from case 3 are provided in Figure A3C.

Parental Milk Supplementation Supports Early and Robust Enteroendocrine Cell Differentiation

We next aimed to determine if the differentiation of any of the epithelial subtypes was affected by diet supplementation. We chose to focus on the enteroendocrine cells (EECs) as these cells secrete factors needed for proper digestion and peristalsis. Immunofluorescence staining using chromogranin A (CHGA), a marker of EEC, was measured separately in both the growth and differentiation phase of the organoids. Interestingly, even during the growth phase, data from both the representative case 3 and the aggregate data demonstrated that organoids supplemented with PM had a significant increase in CHGA staining compared to DHM and SF with a similar strong trend compared to all other conditions (Figure 4A and B). Representative images from case 3 are provided in Figure 4C. During the differentiation phase, case 3 had similar levels of CHGA expression between the control, PM, and DHM organoids which all had a trend towards higher CHGA staining compared to the formula supplemented organoids (Figure 4D). Insufficient numbers of case 3 HF organoids were available for analysis. In the aggregate data, control and PM groups had similar numbers of EECs that were statistically higher than in both of the formula groups (Figure 4E). A similar effect was observed in the DHM group, but this was not statistically significant. Representative images from case 3 are provided in Figure 4F.

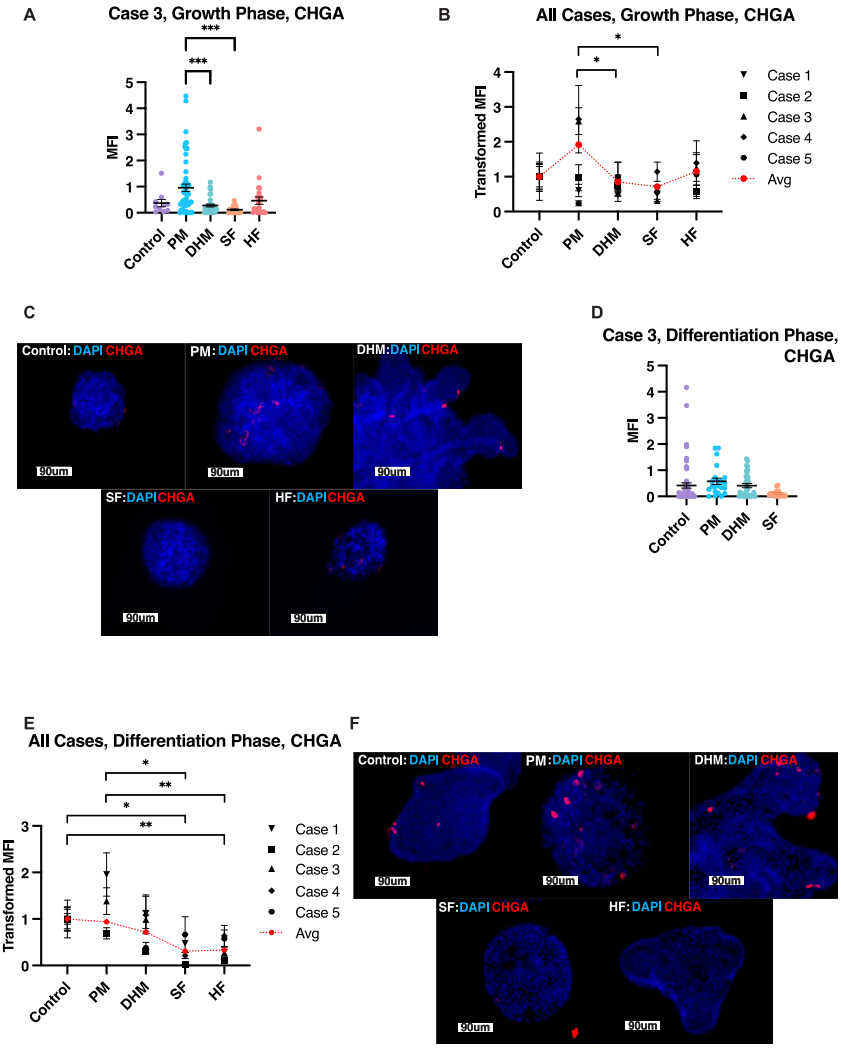

Figure 4. Parental Milk Induces Enteroendocrine Cell Differentiation. (A) MFI of chromogranin-A (CHGA) staining in growth phase organoids, case 3. (B) Transformed MFI of CHGA staining in growth phase organoids, all cases. (C) Representative growth phase images from case 3. (D) MFI of CHGA staining in differentiation phase organoids, case 3. (E) Transformed MFI of CHGA staining in differentiation phase organoids, all cases. (F) Representative differentiation phase images from case 3 when available; HF from case 4. Graphs display mean and standard error of the mean (SEM). One-way ANOVA with Tukey's correction for multiple comparisons in individual case analysis; for aggregate data analysis, data was transformed by dividing each case by the average of its respective control group and analyzed with main effects-only two-way ANOVA with Tukey's correction for multiple comparisons. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

Human Milk Supplemented Organoids Have Increased Expression of Genes Involved in Fatty Acid Metabolism and Induction of Wnt Signaling While Those Supplemented with Formula Have Downregulation of Cell-Cycle Genes

To determine how the various diets affect epithelial cells on a transcriptional level, we performed bulk RNA sequencing (RNAseq) of the organoids after the initial growth phase. Given that human milk supplementation performed similarly for both PM and DHM, RNAseq data was grouped into human milk (HM) group containing PM and DHM samples and formula group containing SF and HF samples. We performed differential gene expression (DGE) analysis between the integrated groups which demonstrated few differentially expressed genes between the organoids supplemented with PM and DHM (Figure A4A) and between the organoids supplemented with SF and HF (Figure A4B). We then compared the HM and formula groups. Compared to formula, HM samples had an upregulation of metabolic genes such as PLIN2,[14] HMGCS2,[15] PGK1,[16] and CPT1A[17] which have been linked to having important roles in cell proliferation as drivers of energy generation and upregulation of AGPAT2,[18] an essential enzyme in phospholipid synthesis (Figure 5A). Moreover, there was an upregulation of other genes involving diverse cellular processes, including iron transport (SLC11A2,[19] TFRC,[20] and LCN2,[21] which additionally acts as an antimicrobial peptide), glucose metabolism (LCN15,[22] a lipocalin also known to be upregulated in EEC), proliferation (ERO1a,[23] EIF4EBP1,[24] ALKBH2,[25] RPF2,[26] RPL17P6,[27] and LINC01315[28]), and genes involved in epithelial repair (TFF3,[29] secreted by goblet cells associated with protection from NEC).

We performed DGE analysis between the HM and control and formula samples to identify beneficial or detrimental factors associated with each condition. Genes involved in epithelial barrier protections such as PIGR,[30] an IgA transporter, IRF,[31] an IFNγ stimulated gene that induces PIGR expression, DUOX2,[32,33] an NADPH oxidase associated with inflammatory susceptibility, and CD177[34] were upregulated in HM treated organoid compared to controls (Figure 5B). Additionally, compared to control, organoids supplemented with HM had downregulation of negative regulators of the cell cycle genes including NOTUM,[35] NKD1,[36] and ZNRF3[37]

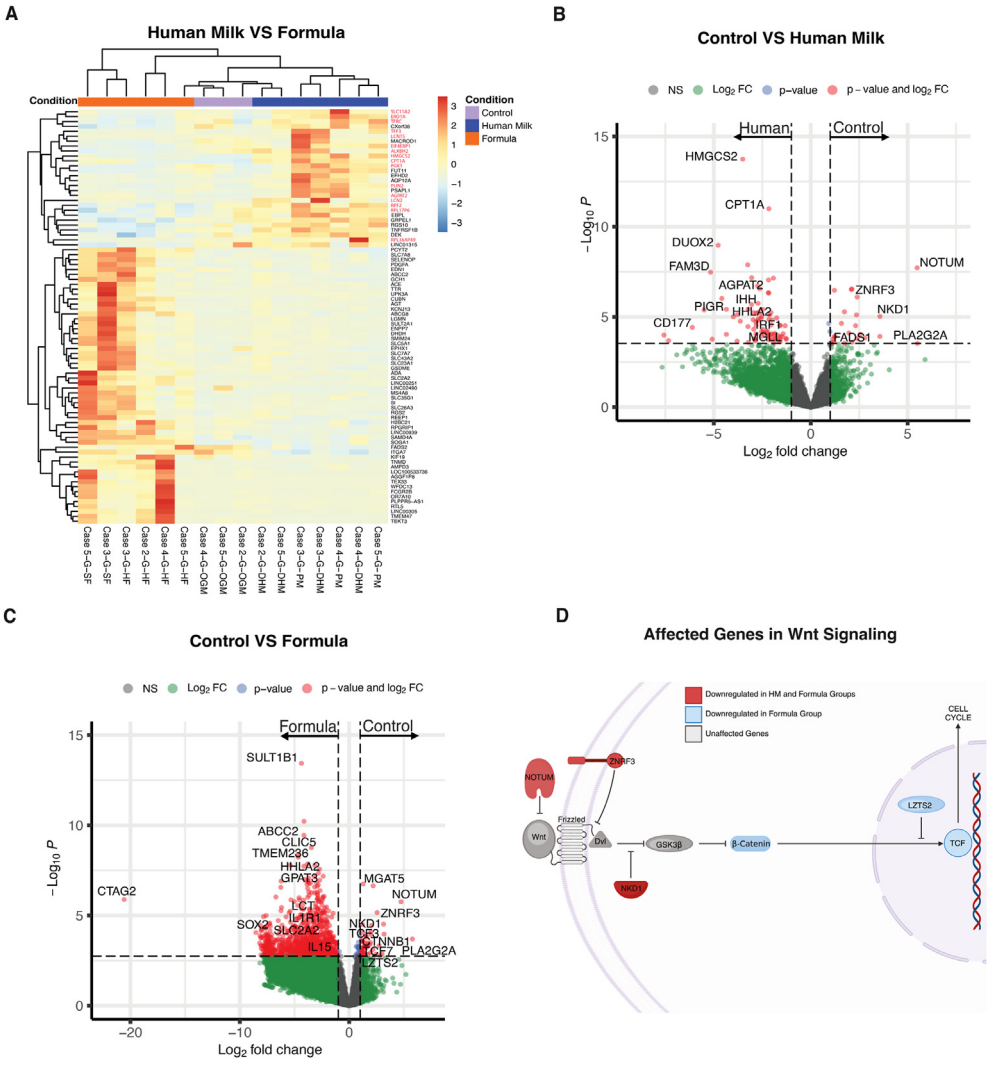

Figure 5. Bulk Sequencing Demonstrates Differential Wnt Signaling. (A) Heatmap showing differential gene expression (DGE) between HM and formula supplemented organoids. (B) Volcano plot showing DGE of HM supplemented organoids. (C) Volcano plot showing DGE of formula supplemented organoids. (D) Wnt signaling pathway schematic derived from DAVID Functional Annotation Analysis and created with BioRender.com with genes downregulated in both organoids supplemented with human milk and formula (red) and those supplemented with formula (blue) highlighted.

that would lead to increased Wnt signaling and thereby cellular proliferation (Figure 5B and D). Organoids supplemented with formula also showed decreased expression of those 3 negative Wnt regulators alongside down regulation of LZTS2,[38] but additionally had decreased expression of essential cell-cycle-promoting genes CTNNB,[39] TCF3,[40] and TCF7,[41] pointing to decreased cell cycle signaling (Figure 5C and D).

Human Milk Supplemented Organoids Have Distinct Patterns of Cytokines, Chemokines, and Immunomodulatory Genes

To determine if diet supplementation has immunomodulatory properties, we analyzed PM, DHM, and media after 5 days of growth or 3 days of differentiation (overall day 8) with Eve Technology's Human Cytokine/Chemokine 96-plex Discovery Assay, which additionally included the growth factors Epidermal Growth Factor (EGF) and fetal growth factor 2. There were no differences in the levels of EGF or fetal growth factor 2 between diet conditions (Figure 6A and B).

To identify if organoids segregated by their cytokine/chemokines expression, we graphed the data using a principal component analysis. The profile of organoids exposed to human milk was unique and these samples segregated from both those cultured under control conditions or those supplemented with formula (Figure 6C). Importantly, this was not driven by a particular case as there was uniform representation of all cases between the groups. There were numerous cytokines/chemokines with significantly higher concentration in the human milk group, including those involved in leukocyte recruitment such as Monokine Induced by Gamma Interferon (MIG/CXCL9), Macrophage-Derived Chemokine (MDC), Thymic Stromal Lymphopoietin (TSLP), C-C Motif Chemokine Ligand 28 (CCL28), and Myeloid Progenitor Inhibitory Factor 1; those involved in monocyte activation including Macrophage Colony-Stimulating Factor and interleukin (IL)-15; and those involved in plasma cell signaling such as A Proliferation-Inducing Ligand (APRIL) and B-cell Activating Factor (BAFF) (Figure 6D, Figure A5A–C). Conversely, there were several cytokines with lower concentrations in the human

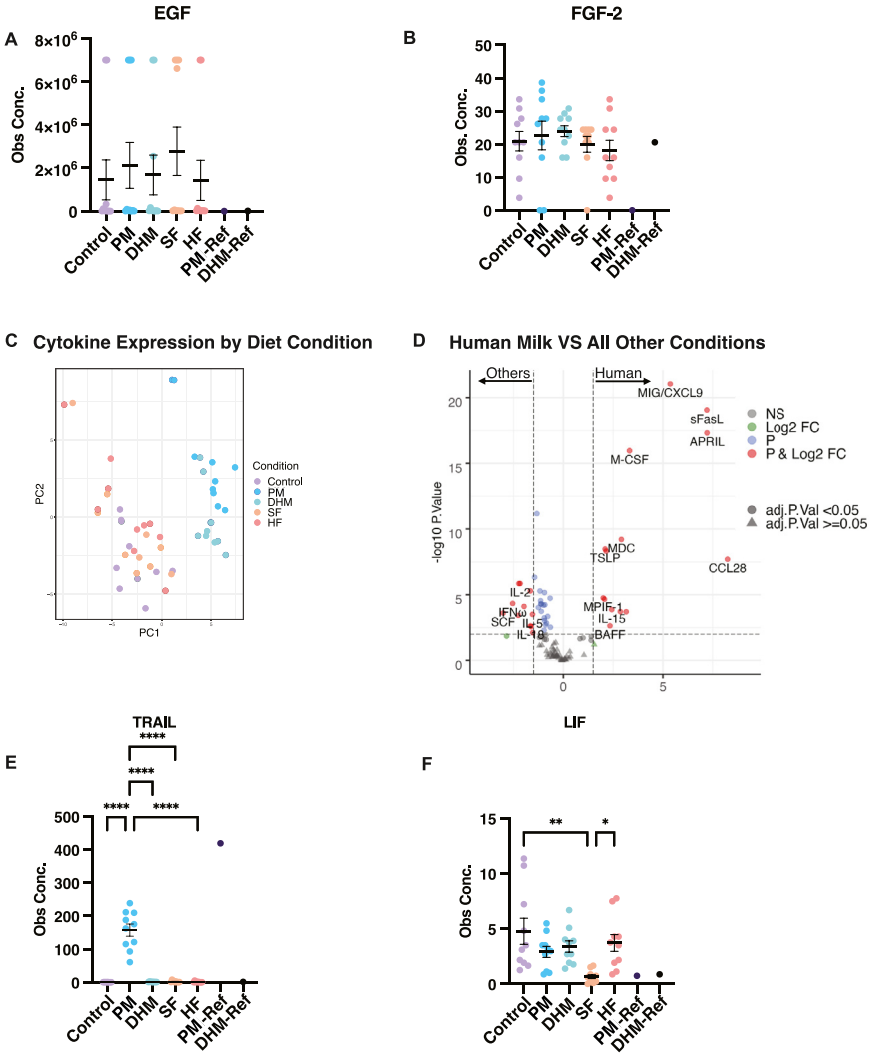

Figure 6. Human Milk Increases Immunomodulatory Cytokines. (A) Concentration of EGF, out of range (OOR) > values set to 7,000,000. (B) Concentration of FGF-2. (C) PCA plot of cytokine concentration levels in media. (D) Volcano plot showing DGE of HM supplemented organoids. (E) Concentration of TRAIL. (F) Concentration of LIF. Media pooled from day 5 of growth and day 3 of differentiation (day 8). OOR < set to 0. Graphs display mean and standard error of the mean (SEM). One-way ANOVA with Tukey's correction for multiple comparisons in individual cytokine analysis. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. PCA, principal component analysis.

milk exposed group, including inflammatory genes such as IL-18.

When we analyzed the cytokines/chemokines individually, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was found to be uniquely present in the PM exposed group with significantly higher concentration in PM compared to all other diet conditions, including DHM (Figure 6E). Fractalkine (C-X3-C Motif Chemokine Ligand 1 [CX3CL1], a potent chemoattractant for intraepithelial lymphocytes), IL-7 (lymphocyte growth factor), and C-X-C Motif Chemokine Ligand 10 (chemoattractant for leukocytes) were also upregulated in PM compared to all other groups (Figure A5D–F). Finally, leukemia inhibitory factor (LIF) was found to be present in lower concentrations in SF compared to all other conditions, including HF (Figure 6F).

DEG and Cytokines Upregulated by Human Milk are Reduced in NEC

To further validate our results, we compared our data to transcriptomic data (both bulk RNA sequencing and single cell RNA-sequencing of intestinal epithelial cells) from neonatal and NEC small intestinal patient samples published by Egozi et al.[42] We specifically evaluated genes and cytokines that were found to be upregulated in our human milk exposed organoids compared to formula and control organoids.

Analyzing the bulk RNA sequencing data first, we found transcripts for both PIGR and HHLA2 to be upregulated in neonatal samples compared to NEC samples, recapitulating our RNA bulk seq results that these genes are induced by human milk exposure and are downregulated in NEC (Figures 5B and 7A). Similarly, the genes for cytokines IL7 and TNFSF10 (TRAIL) were upregulated in neonatal samples compared to NEC samples (Figure 7A). The cytokine analysis of the organoid media exposed to human milk align with this, demonstrating significantly higher abundance of TRAIL and IL-7 in PM -exposed organoids (Figures 6E and A5E).

We then analyzed the single cell RNA sequencing data for any genes that we observed to be upregulated with human milk exposures in the organoids. Multiple genes were found to be upregulated in neonatal samples compared to NEC samples that correlated with genes upregulated in our human milk organoids, including HHLA2, CPT1A, DEK, PLIN2, SLC11A2, ERO1A, MGLL, EFHD2, FAM3D, and GRPEL1(Figures 5B and 7B). Notably, the transcript for IL15 was also found to be upregulated in neonatal compared to NEC samples, which matches our cytokine analysis results that demonstrated an increase of IL-15 in human milk organoids compared to other groups (Figure 6D and A5B).

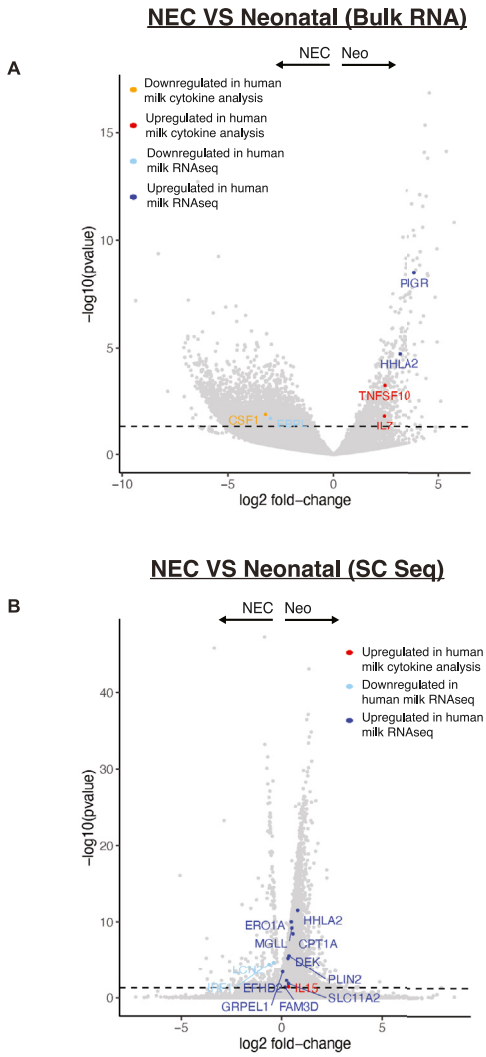

Figure 7. DEG and Cytokines Upregulated by Human Milk are Reduced in NEC. (A) Volcano plot showing DGE of neonatal samples compared to NEC samples in RNA bulk sequencing data from Egozi et al, 2023. Genes and cytokines up/downregulated in human milk organoids are highlighted. (B) Volcano plot showing DGE of neonatal samples compared to NEC samples in single cell sequencing data from Egozi et al, 2023. Genes and cytokines up/downregulated in human milk organoids are highlighted. $P < .05$ considered significant.

Discussion

NEC remains one of the leading causes of death in the neonatal intensive care unit, and therefore finding means of preventing this disease is of utmost importance. Here we demonstrate that fetal intestinal organoids cultured with human milk are more proliferative, more robustly differentiated, induce EEC differentiation, and primed for host protection with a decreased inflammatory profile when compared to control and formula supplemented organoids, suggesting that human milk may better support normal intestinal health and development in the preterm gut. Moreover, a number of DEG and cytokines that were upregulated upon exposure to human milk were downregulated in NEC suggesting that they might play an important role in NEC pathogenesis and should be evaluated further.

Our transcriptomics analysis, which revealed that human milk supplemented organoids had increased expression of

genes involved in fatty acid metabolism and Wnt signaling, point to a potential mechanism behind the increased proliferation in the human milk supplemented groups. HMGCS2, which codes for the rate-limiting enzyme in the production of ketone bodies, is a target of Wnt signaling and has been found to be associated with intestinal stem cell replication and homeostasis while its loss has been shown to result in decreased intestinal stemness.[43,44] PLIN2 and CPT1A both play a role in fatty-acid metabolism and therefore energy generation through beta-oxidation, while AGPAT2 plays a direct role in the formation of phospholipids and triacylglycerols, which are needed for cellular replication.[45–47] Interestingly, CPT1A was downregulated in NEC compared to normal intestinal epithelial cells. Furthermore, our results revealed that human milk supplemented organoids had downregulation of negative Wnt regulators such as NOTUM, NKD1, and ZNRF3, which would result in the induction Wnt signaling and cell-cycle, consistent with increase in Ki67 staining observed in human milk exposed organoids. The Wnt pathway is well-known for its role in intestinal epithelial homeostasis, intestinal stem cell regeneration, and intestinal maturation.[48,49] NOTUM knockout organoids have been shown to have enhanced regenerative ability and Wnt signaling has been shown to be decreased in both mouse models of NEC and intestinal biopsies of infants with NEC.[35,44,50] Conversely, while formula supplemented organoids also had decreased expression of the same negative Wnt regulators as those supplemented with human milk, they also showed decreased expression of CTNNB1, the gene encoding β-catenin, and downstream effectors including TCF3 and TCF7, data suggestive of cell-cycle inhibition which correlates with our results showing smaller organoids and lower Ki67 staining in the formula supplemented group. However, there is a likely a delicate balance between uncontrolled proliferation and homeostatic proliferation and regeneration, which is essential in vivo to balance apoptosis at the villi tips. This balance may be especially important in the preterm gut that is not only undergoing normal development, but also at risk for diseases such as NEC.

Importantly, this study also revealed differences between SF and HF, with SF producing less proliferative and less differentiated organoids compared to HF. Additionally, we found that the cytokine LIF, or LIF, is present in lower concentrations in SF compared to all other groups. LIF is known to maintain intestinal stem cell function, and knockout LIF organoids have previously been shown to have a reduced ability to proliferate and decreased $Ki67^{+}$ cells.[51] The lack of LIF in our SF media aligns with our results and demonstrates that LIF plays a role in supporting vigorous organoid growth and suggesting that formula may be detrimental to intestinal health via suppression of LIF expression. Together with the suppression of Wnt signaling, these data demonstrate that there are likely multiple mechanisms suppressing proliferation in formula exposed organoids. We also observed increased epithelial apoptosis in the formula exposed group as measured by CC3, demonstrating that formula exposure can not only decrease epithelial growth but also induce apoptosis.

The early and robust differentiation observed in the PM supplemented organoids, even when compared to DHM organoids, may in part be explained by our cytokine data, which showed that TRAIL was present in higher quantities in PM compared to all other conditions. TRAIL, or TNF-related apoptosis inducing ligand, is best known for its role regulating apoptosis, but a study by Rimondi et al demonstrated that TRAIL is upregulated in the small intestine during culture conditions that prompt differentiation.[52] They found that exposure to TRAIL actually prompted expression of proteins that trigger growth arrest and induction of differentiated traits. Interestingly, TRAIL was absent from DHM potentially implying that it may be degraded by pasteurization. Upon comparison of RNA bulkseq data between NEC and neonatal samples, we observed that TRAIL was downregulated in NEC.

Our cytokine data also revealed that compared to all other diet conditions, both PM and DHM have significantly higher levels of many cytokines and chemokines involved in intestinal homeostasis, including Monokine Induced by Gamma Interferon, APRIL, BAFF, Macrophage Colony-Stimulating Factor, MDC, TSLP, CCL28, and IL-15. All of these molecules can be epithelially derived, with most of these factors, including TSLP, IL-15, APRIL, and C-X-C Motif Chemokine Ligand 10 being present in the original HM, suggesting that they are secreted by the mammary epithelial cells, while others such as CCL28 and MDC were present only in low levels in PM, suggesting that they are produced locally in the intestine in response to diet exposure. The transcript for IL15 was downregulated in intestinal epithelial cells in NEC. Factors involved in the regulation of plasma cells and IgA translocation into the intestinal lumen were increased in HM exposed organoids both at the protein level (including BAFF, APRIL, and TSLP that are known to induce antibody class switching[53]) as well as at the transcript level with an increase in IRF1 (IFNγ response gene that induces PIGR) and PIGR (transporter of IgA across the epithelial cells). Interestingly, PIGR was one of the most downregulated genes in NEC exposed intestinal samples. Additionally, factors involved in lymphocyte recruitment and lymphoid follicle inductions such as CXCL9, CCL28, and CCL23 (Myeloid Progenitor Inhibitory Factor) were found in the media of HM exposed organoids. In contrast, there was a reduction in IL-18 production upon milk exposure, a potent proinflammatory cytokine. Altogether, this pattern suggests that milk exposure promotes a homeostatic environment of intestinal epithelial immune interactions via induction of tertiary lymphoid structures and antibody IgA production with a reduction in inflammatory cytokines.

It is also important to note the lack of differences in EGF levels amongst diet conditions in our results. EGF has been proposed as a mechanism behind PM's protective effect against the development of NEC via inhibition of toll-like receptor 4 in the intestinal epithelium, and enteral administration of EGF has even been shown to decrease the incidence

and severity of NEC in a neonatal rat model.[54,55] However, our data indicates that the positive effect on organoid growth and differentiation in the human milk conditions cannot be explained solely by the presence of EGF or the related growth factor FGF-2 in our PM and DHM groups.

Taken together, our results indicate the presence of critical factors in PM, and to a lesser extent, DHM, that support intestinal epithelial proliferation, differentiation, and promote a homeostatic immune landscape when compared to control and formula supplemented organoids. Importantly, the causative agent behind this effect is likely a combination of many agents acting together, some of which may be altered by pasteurization. Our results recapitulate the findings by Lanik et al who demonstrated that PM, and to a lesser extent DHM, increased growth of human fetal organoids and increased Ki67 staining.[56] Similarly, in a 2D pediatric organoid model, Noel et al showed that human colostrum improved intestinal health as measured by reduced ion permeability, increased production of Paneth and goblet cell antimicrobials, reduced proinflammatory cytokines, and expression of proteins involved in tissue healing and mucosal homeostasis.[57] While both of these papers demonstrated the ability of human milk to positively modulate the growth and function of intestinal organoids, to our knowledge our study is the first to evaluate the effect of diet on organoid differentiation, to compare SF and HF, and to investigate cytokine/chemokine and transcriptional changes. There are some limitations to this study. We utilized PM from a single source, though our macronutrient analysis aligns with published standards (Table A2).[5,58,59] Similarly, though our DHM was sourced from a pooled milk bank, there may be regional variations that affect milk composition. Additionally, there is variability between organoid lines developed from different human samples; however, our results were consistent across our case replicates. We also used organoids oriented to have their apical cell surface facing within the 3D structure rather than monolayer or apical-out organoids. While this model does not fully recapitulate physiological feeding, it allowed us to measure organoid generation and initial growth, which would not be possible with monolayer organoid model. Apical out-organoids would be a more physiologically relevant model, but require large amounts of tissue to generate. Finally, it is important to note that all patient samples used in this study were from male fetuses. It would be insightful to include female tissue in future studies to evaluate for any sex-specific differences.

Conclusion

In conclusion, our data demonstrate that PM and DHM support greater fetal intestinal organoid growth and differentiation and cultivate a homeostatic environment of intestinal epithelial immune interactions when compared to both SF and HF. If the factors responsible for this effect can be identified, there could be significant clinical value in supplementing these components in DHM and formula to help prevent NEC and foster normal intestinal development in preterm infants.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2024.07.007.

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Ethical Statement:
Deidentified human milk was acquired from Yale New Haven Children's Hospital under approved IRB 2000031633. Intestinal tissue samples were provided by the Konnikova Lab Biorepository that were collected at the University of Pittsburgh with IRB approval.

Data Transparency Statement:
The transcriptional data is deposited in the GEO repository. Other data will be made available upon request. GSE253501- https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE253501.

Reporting Guidelines:
Not applicable for this study. This manuscript adheres to *Gastro Hep Advances'* ethical standards for responsible research.