Abstract

Objectives: The aim of this study was to determine the effect of digested whole human milk (HM; first sample available after birth from mothers of premature infants) on inflammation, oxidative stress, and cytotoxicity in Caco-2 human intestinal epithelial cells stimulated with lipopolysaccharides (LPS) or tumor necrosis factor (TNF) to mimic the potential *in vivo* insults facing the premature infant's gastrointestinal tract.

Methods: Fully differentiated Caco-2 cells were exposed to digested HM (n=10; samples from 10 different individuals) prior to stimulation with LPS, TNF, or no stimulation overnight. Inflammation was determined by production of interleukin 8, (IL-8), oxidative stress by levels of F_2-isoprostane, and cytotoxicity by released lactate dehydrogenase.

Results: HM significantly suppressed IL-8 production and cytotoxicity in TNF-stimulated cells, while also suppressing cell death under baseline conditions. Individual HM samples differed widely in their ability to modulate cellular responses.

Conclusions: Results from this study provide evidence that digested HM can reduce both an exaggerated inflammatory response and intestinal damage that contribute to the pathogenesis of necrotizing enterocolitis.

Key words: necrotizing enterocolitis (NEC); inflammation; oxidative stress; intestine; epithelium

What is known:

- Necrotizing enterocolitis is associated with increased inflammation and intestinal damage/necrosis.
- Human milk feedings reduce risk for necrotizing enterocolitis.
- Various individual human milk bioactive components and partial human milk fractions decrease inflammatory responses in human intestinal epithelial cells.

What is new:

- Digested whole early lactation stage human milk from mothers of premature infants protects human intestinal epithelial cells from cytotoxicity under unstimulated conditions to support cell expansion, and suppresses TNF-induced IL-8 production and cytotoxicity.

There is individual variation among different human milk samples in protective effects**.**

Introduction

Necrotizing enterocolitis (NEC) is a severe inflammatory bowel disease resulting in bowel necrosis that affects up to 7% of very low birth weight premature infants, with 50% requiring surgical intervention and mortality rate up to 24%. [1] While the exact pathogenesis of NEC remains to be determined, the condition is characterized by inflammation, oxidative stress, and necrosis, which increase with severity of disease. [1-4] Possible mechanisms for NEC development include dysbiosis, which can result in an increase in lipopolysaccharide (LPS) burden leading to increased inflammation, and ischemia/reperfusion injury, which can increase tumor necrosis factor (TNF) production, resulting in inflammation and oxidative stress. [5,6] The use of human milk (HM) in premature infants reduces the risk of developing NEC. [7-12] Although several bioactive HM components, and partial HM fractions (the majority isolated from term HM or later in lactation preterm HM) alter inflammation *in vitro* [13-16], it is difficult to extrapolate these *in vitro* responses to how each component may exert its effects when combined with others as a whole in HM. Moreover, once HM is introduced into the infant's gastrointestinal tract, it is mixed with digestive enzymes prior to entry into the small intestine, further making interpretation of current *in vitro* findings difficult to extrapolate into clinical applications. To our knowledge, only one group of investigators consistently used digested whole HM samples on intestinal epithelial cells to examine their antioxidant capacities; however, HM samples used in these studies have been from mothers of preterm infants at mature milk lactation stage, which contains lower amounts of bioactive HM components. [17,18]

The aim of this study was to determine the effect of digested whole HM (first HM sample available after birth of premature infants) on inflammation (determined by production of interleukin 8; IL-8), oxidative stress (F2-isoprostane), and cytotoxicity (lactate dehydrogenase; LDH) in Caco2 human intestinal epithelial cells, stimulated with LPS or TNF, thus mimicking the potential *in vivo* insults facing the premature infant's gastrointestinal tract.

Methods

Human Milk. Ten HM samples (first HM sample available from each subject; ranged from days 2 – 8 postpartum – representing colostrum or transitional milk) from ten different mothers of premature infants admitted to Rush University Medical Center between 2009 – 2010 were digested to simulate the premature infant digestion as previously established and described by Friel et al. [17] The digestion protocol simulates the premature infant digestion with higher gastric pH and lower digestive enzyme secretions compared with adult digestion. HM samples were snap frozen in liquid nitrogen after collection and kept at -80°C until used for this experiment. Briefly, the HM samples were diluted with water (200 μL HM + 2.2235 mL de-ionized water yielding 8.25% HM in starting volume) followed by digestion with pepsin (80 mg pepsin [≥250 units/mg] + 2 mL de-ionized water) titrated to pH 4.0 with hydrochloric acid and kept at 37°C for 30 minutes with agitation at 100 rpm. Following the first digestion, the pH was adjusted to 6.0 with sodium bicarbonate and incubated for another 30 minutes at 37°C. Additional digestion was done with pancreatin and bile salts (20 mg pancreatin [4X USP] + 120 mg bile salt [international units unspecified by manufacturer] + 10 mL 0.1M sodium bicarbonate) with titration of sodium bicarbonate to a pH of 7.0 and incubated at

37°C for 2 hours. Heat treatment at 90°C for 15 minutes was applied in the end to stop the enzymatic reaction. The final HM concentration after completion of digestive procedure was 6.36% HM. Prior to use for cell treatments, digested HM samples were diluted to 2% volume/volume (v/v) concentration with culture medium.

Intestinal Epithelial Cell Cultures. Caco-2BBe (a gift from Dr. Christopher Weber, University of Chicago) cells were grown and maintained at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% nonessential amino acids (Gibco) in T-25 cm^2 flasks. Cultures were split with 0.25% (w/v) Trypsin- 0.53 mM EDTA when confluence was reached. For individual experiments, cells were seeded at a density of 40 X 10^3 cells/cm^2 on type I collagen (Sigma–Aldrich, USA) coated 48-well plates. Cells were grown for 15 – 20 days after confluence to obtain fully differentiated cells to mimic characteristics of adult small intestinal epithelium and fetal ileal epithelial cells. [19] Passages 16 – 18 were used.

Experimental Conditions. Caco-2 cells were cultured as above before pretreatment with 2% (v/v) digested HM or no pretreatment (control – continue incubation with culture medium to maintain usual cell growth) for one hour. Pretreated cells were then stimulated overnight with either 100 μg/mL LPS (from *Escherichia coli* 0111:B4, Sigma, St Louis, MO), 100 ng/mL recombinant human TNF (PeproTech, Rocky Hill, NJ) or left unstimulated. All experimental conditions were done in triplicates.

Interleukin 8 and F2-isoprostane Measurement. Following overnight incubation, supernatants were collected from each well. Samples were used without dilution for measurement of IL-8 as a marker of inflammation using ELISA (R&D Systems,

Minneapolis, MN), and F2-isoprostane levels as a marker of oxidative stress, using ELISA (Cayman Chemical, Ann Arbor, MI).

Cytotoxicity Measurement. During each experiment, three additional wells of cells were maintained overnight. On the following morning during supernatant extraction, lysis buffer was added to these three control wells for 45 minutes to create maximum lactate dehydrogenase (LDH – a cytosolic enzyme used as a marker for cytotoxicity) release controls for calculations. All samples along with these three maximum LDH control samples were then measured for LDH using a colorimetric assay (Pierce Biotechnology, Rockford, IL). Percent cytotoxicity for each sample was then calculated as: (treated samples LDH – control LDH) / (maximum LDH – control LDH) X 100.

Statistical Analyses. Data were entered and analyzed using SPSS (IBM Corp. Released 2013. Version 22.0. Armonk, NY). Descriptive analyses were first conducted for all data and central tendency determined. All IL-8 and F2-isoprostane levels were adjusted for cytotoxicity. Wilcoxon signed-rank test was used to compare the differences in IL-8, F2-isoprostane, and LDH levels between Caco-2BBe cells without stimulation and those stimulated with either LPS or TNF. The change produced by each HM pretreatment under each stimulation condition was first calculated as *difference scores* (e.g., $X_{\text{HM pretreatment + LPS stimulation}} - X_{\text{no HM pretreatment + LPS stimulation}}$). Analysis of variance (ANOVA) was then used to compare the effects of stimulation condition (i.e., LPS, TNF, or no stimulations) on IL-8, F2-isoprostane, and cytotoxicity difference scores associated with HM pretreatments.

Results

Effect of LPS and TNF. As expected, stimulation with LPS significantly increased IL-8 production ($p=0.002$; Figure 1a) and F2-isoprostane levels ($p=0.012$; Figure 1b) compared with controls without stimulation but significantly decreased cytotoxicity ($p=0.005$; Figures 1c). On the other hand, stimulation with TNF significantly increased IL-8 production ($p=0.002$; Figure 1a), F2-isoprostane levels ($p=0.017$; Figure 1b), as well as cytotoxicity ($p=0.005$; Figure 1c) compared with controls without stimulation.

Effect of pretreatment with HM. Exposure of Caco2 cells to HM significantly reduced production of IL-8 in response to TNF ($p=0.009$) but did not affect IL-8 production following LPS stimulation or under unstimulated conditions (Table 1, Figure 1a). Interestingly, pretreatment with HM significantly increased F2-isoprostane levels under each of the three conditions ($p<0.01$ for all conditions) (Table 1, Figure 1b). Pretreatment with HM also significantly decreased cytotoxicity in unstimulated Caco-2 cells ($p=0.016$), as well as in TNF-stimulated cells ($p<0.001$); however, HM did not decrease cytotoxicity after stimulation with LPS (Figure 1c).

There was a significant effect of stimulation conditions (LPS, TNF, and no stimulation) on IL-8 production difference scores; $F(2, 18) = 11.63$, $p = 0.008$. Planned contrasts indicated a significant difference between TNF and no stimulation; $F(1, 9) = 11.25$, $p = 0.008$ (Table 1). Furthermore, there was a significant effect of stimulation conditions on cytotoxicity difference scores; $F(2, 18) = 36.02$, $p <0.001$. Planned contrasts indicated significant differences between LPS and no stimulation; $F(1, 9) = 15.32$, $p = 0.004$; as well as between TNF and no stimulation; $F(1, 9) = 27.47$, $P = 0.001$ (Table 1).

Variations among individual HM samples. As shown in Figure 2a and 2b, there were wide variations among individual HM samples' ability to alter IL-8 production and cytotoxicity under all conditions. However, all but one HM samples suppressed TNF-induced IL-8 production, whereas the HM samples' effect on LPS-induced IL-8 production was inconsistent, resulting in an overall average non-significant change, as indicated in Figure 2a. Meanwhile, all but one HM samples suppressed LPS-induced cytotoxicity, whereas all HM samples suppressed TNF-induced cytotoxicity (Figure 2b).

Discussion

In this study, we examined the effects of digested HM samples from mothers of premature infants on human intestinal epithelial cells. We found that pretreatment with HM suppressed TNF-induced inflammation and reduced cytotoxicity in both unstimulated and in TNF-stimulated cells.

Pretreatment with HM significantly suppressed IL-8 production following TNF stimulation. In contrast, HM did not demonstrate an effect on LPS-stimulated inflammation. This may be in part due to the lower magnitude of LPS-induced IL-8 response, which was more than 7-fold smaller than the response seen to TNF stimulation. This differences in response is similar to the previous literature. [20] Given the level of IL-8 elevation seen with TNF stimulation, there may have been more margin for reduction. Additionally, differential mechanisms of induction of IL-8 by the stimuli used may account for the discrepant results. [21]

Expectedly, there was variability in the protective effects of individual HM samples since large variability in bioactive components of HM among mothers has been well documented. [22] However, despite the variations seen in the ability of individual

HM samples to alter cellular response, HM pretreatment consistently provided protective effects under a robust TNF-induced stimulation. This provides evidence that HM feeding in clinical practice can reduce the inflammatory response that may be result from various stimuli, including ischemia/reperfusion insult and cow's milk protein found in formula.

In our model, pretreatment with HM increased F2-isoprostane levels regardless of experimental conditions. F2-isoprostane is a prostaglandin-like compound formed by peroxidation of arachidonic acid that reflects oxidative stress *in vivo.* [23] Our findings differ from previous studies that demonstrated reduced oxidative stress in intestinal epithelial cells treated with digested HM. [17,18] We postulate that oxidative stress measurements in our *in vitro* model may not accurately reflect the true effects of HM, since the observed increase in F2-isoprostane levels was seen in all experimental conditions. The oxygen exposure in the gastrointestinal lumen *in vivo* is relatively low (<1 mmHg) in comparison to the approximately 150 mmHg in a cell culture system, which provides a significant source of oxidants. [24] In addition, some common ingredients used in the cell culture medium are present as free ions and can act as pro-oxidants. [24] Furthermore, some of the compounds in HM may have undergone oxidation when they came into contact with the culture medium and generated additional reactive oxygen species, thus falsely elevating oxidative stress measured in the Caco-2 cells. [24] Specifically, we suspect that the arachidonic acids present in HM may have undergone peroxidation due to the relative oxygen-rich environment, as well as other free ions present in the culture medium, and subsequently releasing an abundance of F2-isoprostanes. Therefore, due to the possibility of artifacts, we are unable to reach a solid conclusion on the effect of HM on oxidative stress in our experimental system. Use of a

different experimental technique, such as 2',7'-dichlorodihydrofluorescein diacetate used by Friel et al., may have altered our findings. [17,18]

In our study, pretreatment with HM significantly decreased cytotoxicity in Caco-2 cells without stimulation. This is similar to results showing reduced cytotoxicity in unstimulated intestinal epithelial cells exposed to HM samples digested with a combination of lipase and protease. [25] Interestingly, in the study by Penn *et al.*, cytotoxicity was increased when cells were exposed to HM samples digested with lipase alone, indicating the importance of using a digestive process that includes all digestive components in an *in vitro* system to truly mimic the premature infant's digestive tract, such as the digestive process used in the present study. While the protective effect of HM from cytotoxicity in our study was lost when Caco-2 cells were stimulated with LPS, we nevertheless demonstrated a significant protective effect when Caco-2 cells were exposed to HM and then stimulated with TNF. It is important to consider that LPS stimulation alone did not increase, but rather decreased, cytotoxicity, thus we would not expect much additional protection from HM treatment under this condition. However, TNF stimulation alone induced an intense cytotoxic effect, and we showed that pretreatment with HM significantly protected Caco-2 cells from TNF-induced cytotoxicity.

In this model, LPS induced only a low magnitude of IL-8 response and did not induce cytotoxicity in Caco-2BBe cells. This finding corroborates previous research that Caco-2 cells are heterogeneous in TLR4 expression [26-29]; while some studies showed moderate to low TLR4 expression [27,29], other studies showed Caco-2 cells lack TLR4 expression. [26,28] In this study, LPS was chosen as one of the stimulus to simulate the gut flora of premature infants with the understanding that LPS with CD14 binds with

TLR4/MD-2 complex transducing signals through MyD88 downstream activating the NFκB pathway inducing inflammation. However, likely due to the heterogeneity in TLR4 expression in Caco-2 cells, LPS did not induce a robust inflammatory response with subsequent lack of impact on cytotoxicity in our model.

This was the first study to examine the effects of fully digested whole HM on intestinal epithelial cells using stimulation conditions that mimic the different potential insults in the premature infant's gastrointestinal tract and concomitantly evaluating inflammation, oxidative stress and cytotoxicity. The strengths of this study are that we digested whole HM samples to truly simulate what the infant would ingest. In addition, the first available HM samples from each mother was used for this study, making the samples as close to colostrum as possible to mimic the effect of feeding colostrum as first feeding exposure in premature infants. And the use of different mothers allowed us to examine the variability in effects. The limitations of this study included the technical aspects of the cell culture model and the assay used to quantify oxidative stress, which may have resulted in artifacts.

In the current study, HM was a strong suppressant of TNF-induced inflammation and cytotoxicity. While oxidative stress levels were increased following HM treatment, this was likely an artifact from either the cell culture conditions or the assay used for quantification. Future studies should explore the effects of digested HM, donor HM, fortified HM, different preterm infant formulas, and combined HM and formula on intestinal epithelial cells under similar conditions, as well as combined LPS and TNF stimulation to mimic the many possible feeding practices and stimulation exposure variations found in clinical practice. In addition, we observed variability among

individual HM samples in their ability to alter inflammation and cytotoxicity. Future studies should examine the effect of variables that may alter maternal immune response and stress levels, thus potentially affecting the type and amount of bioactive components in the mother's breast milk (such as presence of inflammatory diseases, acute illness [30], medical history or medications that can impact maternal immune response, body habitus [31,32], dietary quality [33], as well as environmental and social exposures [34,35]) to evaluate the impact of maternal factors on HM gut-protective properties.

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Figure Legends

Figure 1a – c. Interleukin 8 (IL-8; pg/mL) production, F_2-Isoprostane levels (pg/mL) and percent cytotoxicity in Caco-2BBe cells ± digested human milk (HM; n=10), stimulated with lipopolysaccharide (LPS; 100 μg/mL), tumor necrosis factor (TNF; ng/mL), or no stimulation. $^{\#}$LPS and $^{\$}$TNF significantly increased IL-8 (both p=0.002) and F_2-Isoprostane (p=0.012 and p=0.017, respectively) without HM. †LPS significantly decreased (p=0.005) and ‡TNF increased (p=0.005) cytotoxicity without HM. $^{\$}$HM significantly decreased cytotoxicity in unstimulated cells compared with no HM (p=0.016). *HM significantly decreased IL-8 (p=0.009) and cytotoxicity (p<0.001) in TNF-stimulated cells compared with no HM, while increased F_2-Isoprostane in each stimulation condition compared with no HM (p<0.01).

Figure 2a – b. Individual digested human milk (HM) samples (n = 10) were used to pretreat Caco-2BBe cells before stimulation with lipopolysaccharide (LPS; 100 μg/mL) or tumor necrosis factor (TNF; 100 ng/mL). Interleukin 8 (IL-8; pg/mL) and lactate dehydrogenase (cytotoxicity) were measured and calculated against controls of the same stimulation to determine the effect of individual HM sample as percent difference (e.g. (HM sample 1 + LPS) – (no treatment control + LPS)/(no treatment control + LPS) X 100).

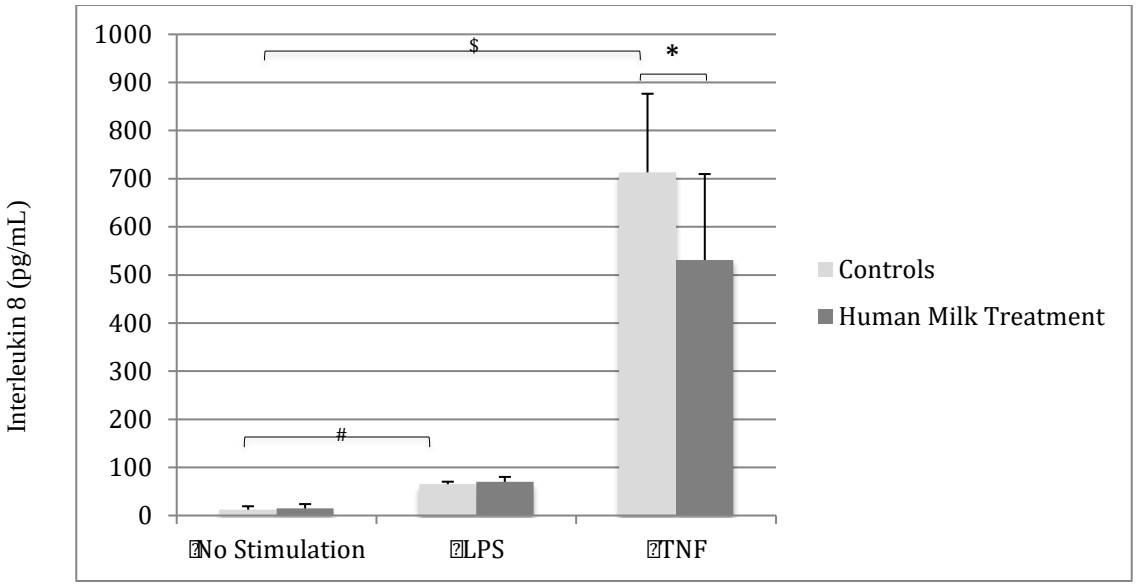
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Figure 1a.

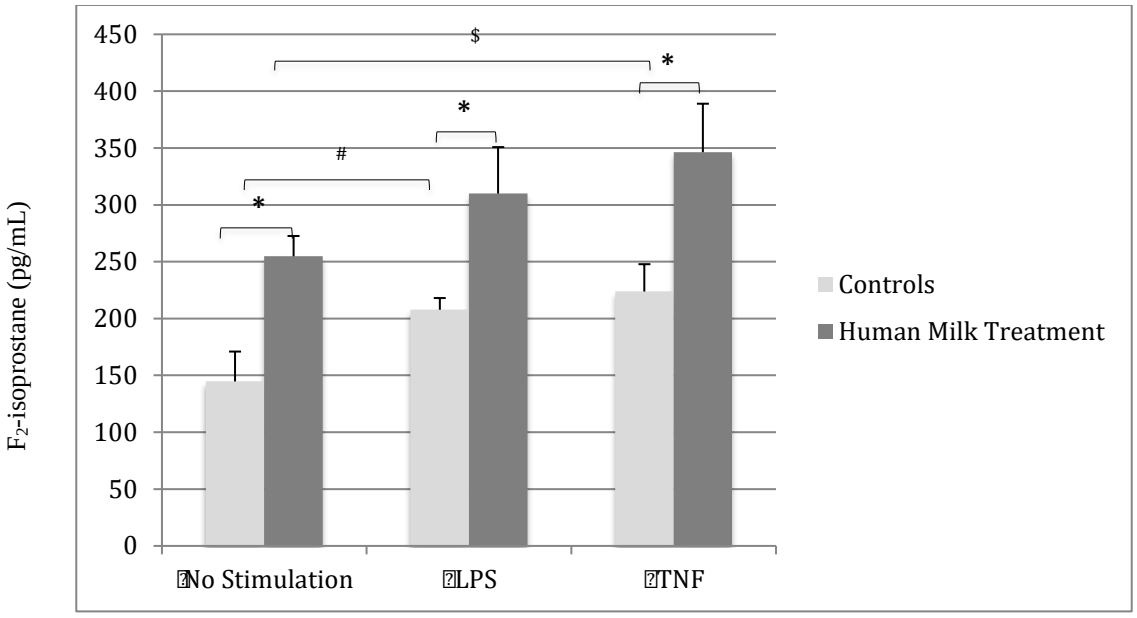
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Figure 1b.

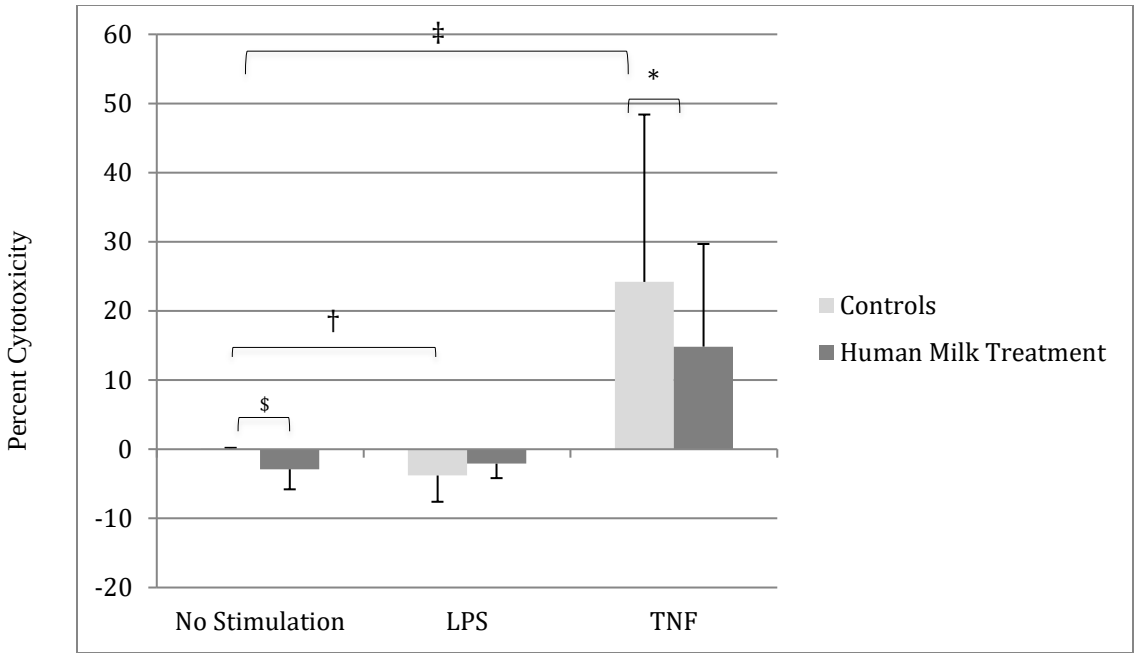
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Figure 1c.

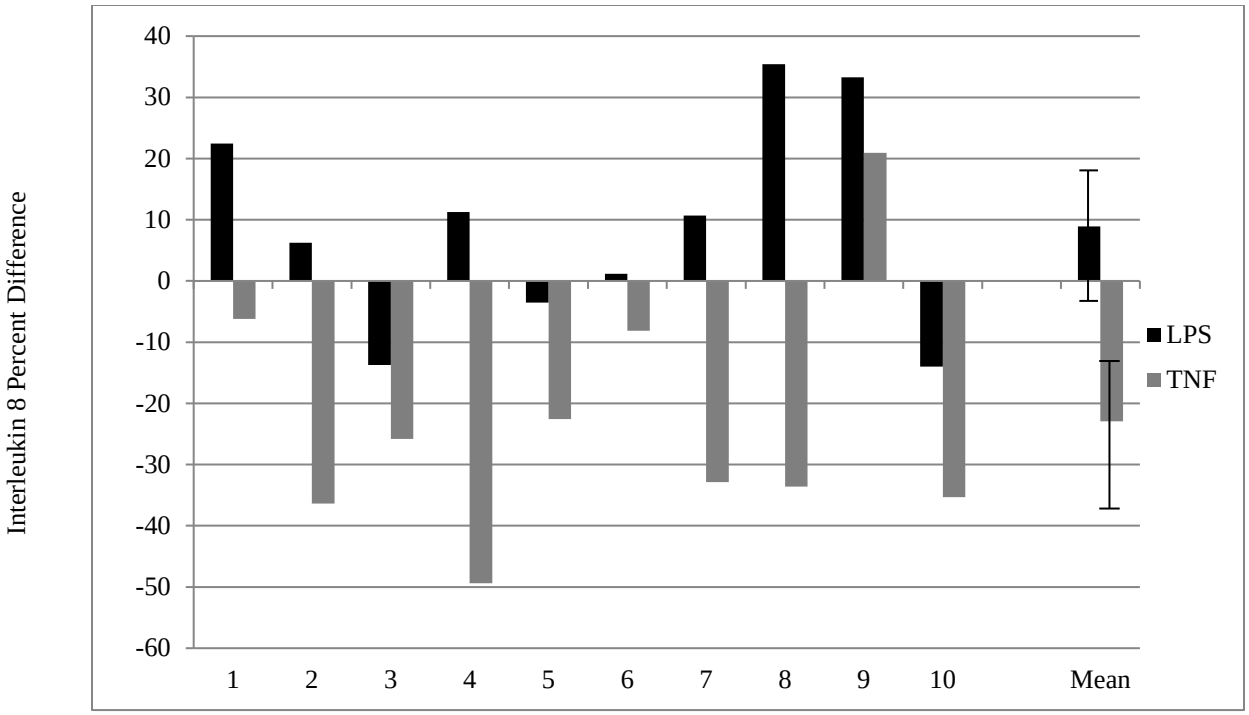

Figure 2a.

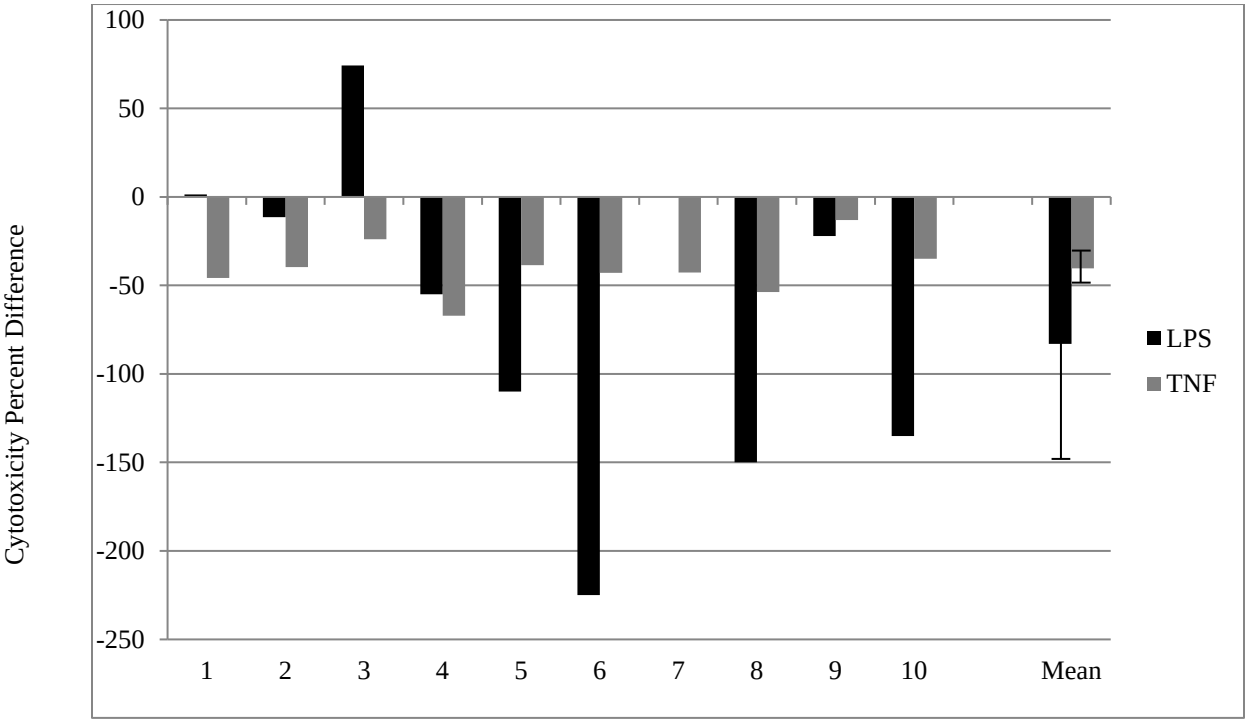

Figure 2b.

Table 1:

One-way analysis of variance of interleukin-8 (IL-8), F2-isoprostane, and cytotoxicity changes as a function of human milk treatments following lipopolysaccharide (LPS), tumor necrosis factor (TNF), or no stimulations.

				Overall ANOVA		Planned contrast (LPS vs No Stimulation)		Planned contrast (TNF vs No Stimulation)	
	No Stimulation M (SD)	LPS M (SD)	TNF M (SD)	*F* (2,18)	*p*[1]	*F* (1,9)	*p*[1]	*F* (1,9)	*p*[1]
IL-8	2.77 (1.83)	5.46 (3.47)	-162.16 (49.29)[2]	11.63	.008[1*]	0.61	.455	11.25	.008[#]
F2-Isoprostane	97.82 (11.64)	101.40 (16.22)	173.03 (51.37)	1.57	.243[1]	0.02	.880	1.61	.236
Cytotoxicity	-2.90 (0.98)	1.71 (1.03)	-9.36 (1.40)	36.02	.000[§]	15.32	.004[†]	27.47	.001[‡]

[1]Greenhouse-Geisser adjustment when the assumption of sphericity was violated.
[*]IL-8 concentration changes as a function of human milk treatment was significantly different among LPS, TNF, and no stimulations (p=0.008).
[#]IL-8 concentration changes as a function of human milk treatment was significantly different between TNF and no stimulations (p=0.008).
[§]Cytotoxicity changes as a function of human milk treatment was significantly different among LPS, TNF, and no stimulations (p<0.001).
[†]Cytotoxicity changes as a function of human milk treatment was significantly different between LPS and no stimulations (p=0.004).
[‡]Cytotoxicity changes as a function of human milk treatment was significantly different between TNF and no stimulations (p=0.001).