Breast Milk Pro-Inflammatory Cytokines Are Suppressed During Infant Infection and Show Heterogeneous Recovery in Convalescence

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SUMMARY

Breast milk plays a pivotal role in maternal-infant immune communication. We examined cytokine dynamics in 45 asymptomatic breastfeeding mothers: 29 nursing infants with acute infection and 16 nursing healthy infants. All infants were born at full term, with high Apgar scores, and the mothers had no clinical or laboratory signs of inflammation. Milk samples were collected 3–5 days after the onset of infant illness and 4–6 weeks later during convalescence. Cytokine concentrations (IFN-γ, IL-1β, IL-17A, IL-4, IL-6, and TNF-α) were measured in the aqueous phase of breast milk using Human HS ProcartaPlex™ Mix&Match assay. Only IL-6, IL-1β, and TNF were consistently detectable. In the mothers of ill infants, pro-inflammatory cytokines, particularly IL-6, were markedly suppressed during acute infection and correlated with elevated infant C-reactive protein levels. Cytokine recovery during convalescence was heterogeneous: IL-6 rebounded in younger mothers and in infants with milder leukocytosis, but recovery was blunted in older mothers and in cases of pronounced infant inflammation. TNF restoration was more frequent in early lactation, suggesting an influence of lactation stage. These findings reveal that breast milk cytokine responses are dynamically regulated by both infant inflammatory status and maternal characteristics, highlighting an adaptive mechanism of immune communication during lactation.

KEYWORDS

Breastfeeding; *Infant*; *Infection*; *Cytokines*

Introduction

Breastfeeding is a universal biological mechanism of nutrition among all mammals, and human breast milk has evolved to optimally meet the nutritional and immunological needs of the human infant[1,2]. Its composition varies depending on the gestational and postnatal age of the child[3–5], the health[6–12] and diet[4] of the mother, and environmental factors[13], thereby enabling individualized support for infant growth and development. In addition to essential nutrients, breast milk contains numerous bioactive components[14], including immunoglobulins[3], growth factors[4], cytokines[5,15], and cells, such as leukocytes and stem cells[16], which contribute to the maturation of the infant's mucosal and systemic immunity[17–19], particularly during early life, before the closure of the intestinal barrier[20].

Cytokines are signaling proteins primarily secreted by immune cells and they play a crucial role in shaping immune responses. In human milk, various classes of cytokines have been identified — anti-inflammatory (TGF-β, IL-10, IL-7, IL-18, G-CSF) and pro-inflammatory cytokines (TNF-α, IL-6, IL-8, IL-12, IL-2, IFN-γ) — with their concentrations influenced by the stage of lactation, maternal health, diet, mode of delivery, and gestational age of the newborn[5]. Colostrum contains the highest levels of cytokines[3,12], which generally decline in mature milk in the absence of inflammation. Leukocytes in milk, including neutrophils, monocytes/macrophages, and lymphocytes, not only exert their effects through phagocytosis and immunoglobulin secretion but also secrete cytokines that contribute to immune defense and the development of immune tolerance in the infant.

Previous studies have shown that maternal infections (e.g., mastitis[10], respiratory[21], and other organ-specific infections[10]) significantly increase the number of leukocytes in milk. A comparable immune response has also been observed during infant infections, even when mothers remain clinically asymptomatic[22]. In such cases, retrograde ductal milk flow during breastfeeding is considered a possible mechanism for pathogen transfer from the infant's oral cavity to the mammary gland[18], possibly triggering local immune activation and alterations in the milk's cytokine composition. However, while leukocyte and cytokine fluctuations in human milk have been well-characterized in the context of maternal infections[8,10,23,24], autoimmune conditions[7] or allergy[4,6], data on cytokine changes in the milk of asymptomatic mothers breastfeeding sick infants remain limited.

We aimed to investigate whether alterations in pro-inflammatory cytokine levels occur in breast milk in the absence of overt maternal clinical inflammation during the nursing of a sick infant, in order to better elucidate mother–infant immunological communication throughout lactation. We hypothesized that pro-inflammatory cytokines would be modulated in breast milk in response to the infant's illness, reflecting a dynamic maternal immune adaptation during lactation.

Participants and methods

This prospective, controlled observational study was conducted following approval from the Ethics Committee of University Hospital Centre Osijek (approval no. R2-7990/2021), the Ethics Committee of the Osijek-Baranja County Health Centre (approval no. 03-1489-1/21), and the Ethics Committee of the Faculty of Medicine Osijek (class: 602-04/21-08/07, no. 2158-61-07-21-156). The entire study was carried out at the DNA Analysis Laboratory of the Faculty of Medicine Osijek and the Department of Transfusion Medicine Diagnostics, University Hospital Osijek. All participants were provided with detailed written information about the study and signed informed consent forms prior to enrolment.

Data collection

A total of 45 breastfeeding mothers were included and divided into two groups. The first group comprised 29 mothers breastfeeding infants with clinical signs of acute infection (e.g., fever, irritability, diarrhea, excessive crying, rapid breathing, dyspnea, weakness, poor weight gain, rash, blotchy skin, cold extremities, poor circulation, or perioral cyanosis), confirmed by routine laboratory tests (differential blood count, C-reactive protein (CRP) concentration, erythrocyte sedimentation rate) and microbiological analyses (throat swab, urine culture, stool test). The second group included 16 mothers breastfeeding healthy infants without any signs of infection, willing to participate in follow-up. Exclusion criteria included maternal acute infection, chronic inflammatory conditions, or the use of immunomodulatory medications. Participants were randomly selected from the pool of eligible mothers recruited between March 28, 2022 and May 24, 2024 from the University Hospital Osijek, private pediatric practices, pediatric care units in the Osijek-Baranja County, and during routine visits by community health nurses. These sites were selected to capture a representative population of mothers and infants across hospital and community settings. Licensed pediatricians conducted recruitment and examined infants to ensure an accurate assessment of infection status. At recruitment, demographic and medical history data were systematically collected using a structured questionnaire, which included information on maternal age, parity, pregnancy course, delivery mode, and early postnatal history. Infants underwent routine capillary and venous blood testing, and microbiological analyses to assess infection status, while mothers underwent standard laboratory tests (complete blood count, differentials, erythrocyte sedimentation rate, and CRP) to rule out acute infection. A follow-up assessment six weeks later included a questionnaire to confirm full recovery in infants from the first group and continued absence of infection in the second group. Demographic, clinical, and biochemical characteristics of the mothers and their infants are provided in Table 1.

Breastmilk collection and processing

For the purposes of this study, the mothers provided 50–100 mL of breast milk, expressed during the second feed of the day, between days 3 and 5 following the onset of the infant's acute illness. The milk was collected in sterile plastic containers and stored in a portable refrigerator to maintain a cold temperature until delivery to the laboratory within 2 hours. Follow-up samples were collected 4–6 weeks later, during the convalescent phase. Milk samples were centrifuged at 600 × g for 15 minutes at 4 °C to separate the cellular, lipid, and aqueous (lactoserum) fractions. The aqueous fraction was aliquoted and stored at −80 °C until further analysis.

Multiplex cytokine quantification in human milk using the Luminex-based ProcartaPlex assay

Cytokine concentrations (IFN-γ, IL-1β, IL-17A (CTLA-8), IL-4, IL-6, and TNF-α) in the aqueous phase of human breast milk were measured using the Human HS ProcartaPlex™ Mix&Match 6-plex assay (Thermo Fisher Scientific, Vienna, Austria). After washing the magnetic beads, 25 µL of 1 × UAB buffer and 25 µL of the sample or standard were added to each well. Samples were incubated at room temperature for 30 minutes with shaking (500 rpm), and then stored overnight at 4 °C. Before proceeding with the next assay step, the samples were re-incubated at room temperature for 30 minutes with agitation at 500 rpm. Washing steps with 1 × UAB buffer were performed twice after each incubation. The Antibody Detection Mixture was added and incubated for 30 minutes protected from light. After washing, SAPE solution was added and incubated similarly.

Amplification Reagents 1 and 2 were sequentially added with incubation steps in between. Finally, Reading Buffer was added, and after a 5-minute incubation, samples were analyzed using the Luminex® 200™ system with xPonent® software (Luminex Corporation, Austin, Tx, USA). Data analysis was performed using the ProcartaPlex™ Analysis App (Thermo Fisher Scientific, Vienna, Austria).

Statistical data analysis

Categorical data are presented as ratios, and absolute and relative frequencies. The numerical data were summarized by the median and interquartile range. The Mann–Whitney test was used to compare two independent groups (continuous measurements). The difference between two proportions was assessed using Fisher's exact test (independent groups). All P values were two-tailed, with $P < 0.05$ considered significant unless otherwise stated.

Censored observations

Cytokine measurements often have a high rate of non-detections, or results below the method's detection limit (censored observations). We addressed this issue with two analytical solutions. In the simplest case, the zero-inflated censored variables (i.e., milk analytes) were dichotomized into Bernoulli variables according to the lower limit of quantitation (LLOQ), and then summarized using contingency tables.

For more informative measurements (IL-6), we employed a Bayesian formulation of hurdle regression, which uses two separate processes for modelling non-detects and non-zero (positive) values. The first part, the hurdle model, uses logistic regression to predict whether an observation will have a zero (< LLOQ) or a positive value, while the second part, the conditional model, uses a gamma distribution to predict non-zero values. We used the fixed-effect and linear structure for both parts, which allowed us to investigate the effects of the covariates separately, assuming that these two processes might be driven by different predictors. All models were fitted in the *Bayesian Regression Models using Stan* (brms) library[25] using default (flat or weakly informative) priors and Markov chain Monte Carlo simulations (4 chains, 8,000 iterations each; in total 16,000 post-warm-up draws, seed = 42). Posterior convergence was assessed by $\hat{R}$ statistics ($\hat{R}$=1.00), effective sample size measures, and a visual inspection of trace plots. No divergent transitions were observed. We assessed the goodness-of-fit using graphical posterior predictive checks (100 draws, bayesplot libray). The importance of the predictors was assessed by ROPE (Region of Practical Equivalence) values and 95% credible intervals that were either strictly positive or strictly negative. We interpreted the results as evidence against the null hypothesis if the 89% highest posterior density interval (HPDI) excluded ROPE (bayestestR package)[26–28]. For comparative purposes, we also provided the 95% HPDI.

All computational and graphical analyses were performed in R version 4.3.1 (https://www.R-project.org). The following packages were also used: see, insight, tidyverse, targets, tidybayes, scales, patchwork, ggtext, ggh4x, kableExtra, broom.mixed, marginaleffects, extraDistr, performance, data.table, table1, ggpubr, modelsummary, corrplot, DescTools, effectsize, Hmisc, and rstanarm. The codes that support the results and figures are available upon reasonable request to the corresponding author.

Results

Participant characteristics

The sample included 29 independent pairs of mothers and their ill infants and 16 pairs of mothers and their healthy infants. The demographic and clinical characteristics of the mother-infant dyads are presented in Table 1. In both groups, the majority of mothers were 30 years of age or older, and had similar leukograms and serum CRP levels. The ill infants were older than their healthy counterparts (min.–max. 1–15 vs 1–10 months, respectively). All of the children were born at full term (37–42 weeks) and most had Apgar scores of 10, except one infant in the acutely ill group (score 7 at 1 min, 8 at 5 min) and one healthy infant (score 9 at 1 min and 5 min). All mothers had uneventful postpartum recoveries and remained asymptomatic throughout the study. There were no significant differences in parity or mode of delivery between the groups (Table 1). Seven mothers (6 cases, 1 control) had a history of health issues (autoimmune thyroiditis, thrombophilia, asthma, psoriasis, hepatitis B, and essential hypertension) but there was no evidence of biased cytokine measurements (Supplemental Table 1). Eleven children (38%) had serum CRP levels >5 mg/L (max. 139 mg/L).

Table 1. Demographic, clinical, and biochemical characteristics of mothers and their infants

Variable	healthy (N= 16)	ill (N= 29)	*P**
Age, mother (yrs)	31.5 [30, 34]	32 [31, 34]	0.526
Age, infant (months)	3 [2.8, 4]	6 [4, 8]	0.004
Birth (vaginal/cesarean section, N)	11/5	22/6	0.492**
N, births (primiparous/multiparous)	13/1	15/9	0.087**
Smoking (Yes/No, N)	1/15	5/23#	0.392**
Leukocyte count, mother (x109/L)	6.6 [6, 7.6]	6.9 [5.8, 7.6]	0.991
Leukocyte count, infant (x109/L)	NA	9.7 [6.9, 11.9]	-
CRP, mother (mg/L)	2.25 [1.18, 3.33]	1.4 [0.5, 2.8]	0.112
Fever, infant (Yes/No)	NA	16/12	-
CRP, infant (mg/L)	NA	2 [0.4, 17.5]	-

NA = not available; CRP = C-reactive protein; * Man–Whitney test; ** Fisher's exact test
Continuous data are presented as a median [interquartile range]. # One mother in the "ill" group did not provide information regarding smoking status.

Pro-inflammatory cytokines are suppressed in breast milk during infant infection and show heterogeneous response upon recovery

Data analysis focused on IL-6, IL-1β, and TNF, as other measured cytokines were only sporadically detectable above the LLOQ. Roughly, one-third to one-half of the milk samples from mothers nursing healthy infants had IL-1β and IL-6 levels above the LLOQ, with no significant variation in repeated measurements (Table 2). The distribution and temporal dynamics of IL-1β and IL-6 levels were different in mothers nursing sick infants, showing close association with the infants' inflammatory status. More specifically, the proportion of milk samples testing positive for IL-1β and IL-6 in the early stage of the disease (the acute phase) was lower in mothers of sick infants than in the control group (Table 2). Only one of the 29 samples had an IL-1β level greater than the LLOQ, while IL-6 was successfully quantified in approximately one-fifth of the milk samples (Figure 1). For the latter, a proportionate shift in infant serum CRP concentrations was observed: undetectable milk IL-6 coincided with significantly higher acute-phase CRP levels in affected children (3.4 (0.5–25.3) vs 0.35 (0.18–1.93) mg/L; n = 23 vs 6; IL-6– vs IL-6+, $P = 0.031$, Mann–Whitney test, simultaneous measurements). The difference was the strongest for the IL-6–/IL-6+ dichotomy, but the detected (IL-6+) values also followed suit: the lower the observed IL-6, the higher the infant CRP (Figure 2).

Table 2. Dichotomized cytokine levels in the breast milk of mothers with sick infants

Cytokine	Phase	Status of the infant	< LLOQ (N)	> LLOQ (N)	Fisher's exact *P*	Average proportion of non-detects associated with infant's status
IL-6	AP	Healthy	8	8	**0.053**	0.3
		Ill	23	6		
	C	Healthy	8	8	0.76	0.09
		Ill	17	12		
IL-1B	AP	Healthy	10	6	**0.0054**	0.45
		Ill	28	1		
	C	Healthy	12	4	0.726	0.05
		Ill	23	6		
TNF	AP	Healthy	9	7	**0.021**	0.35
		Ill	26	3		
	C	Healthy	10	6	0.494	0.06
		Ill	22	7		

LLOQ = lower limit of quantitation; AP = acute phase; C = after convalescence

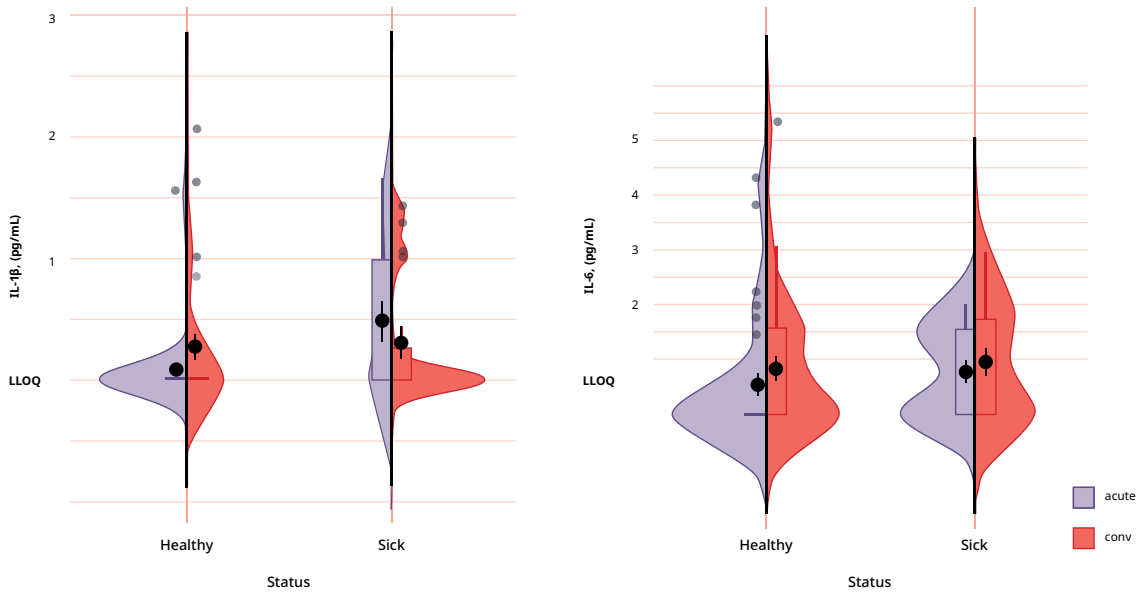

FIG. 1. Violin plots showing the distribution of IL-6 and IL-1β levels in breast milk according to disease status and phase. The y-axis represents natural log-transformed values, $\log^2(1+y)$. The boxplots are defined by the medians and their respective interquartile ranges (IQRs). Vertical lines extend to ±1.5×IQR. LLOQ = lower limit of quantitation.

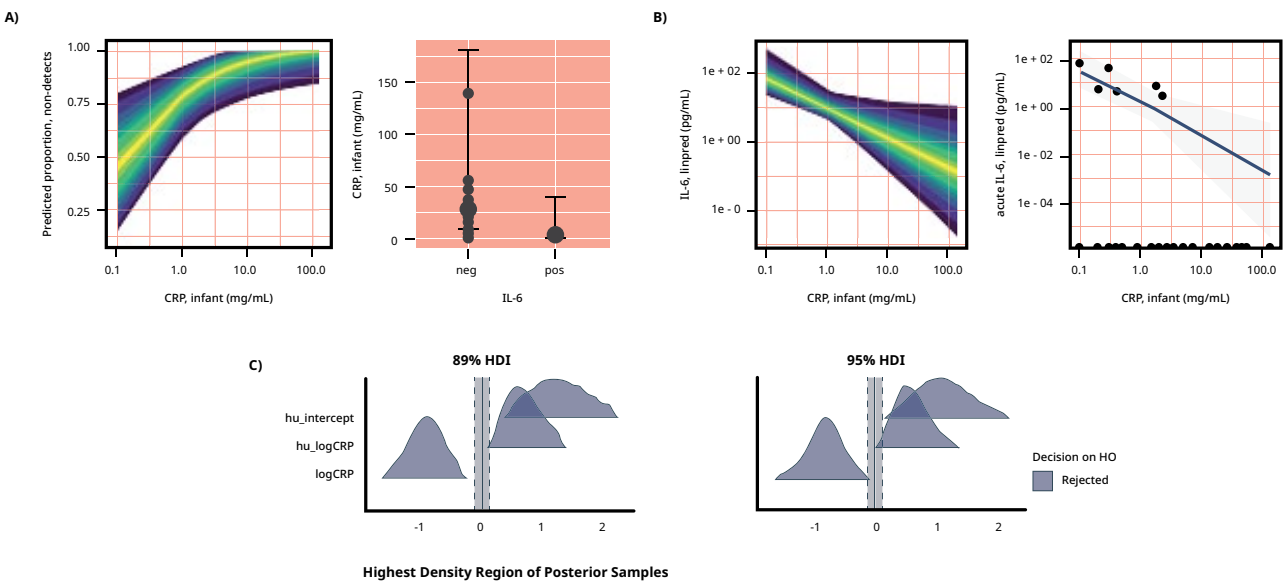

FIG. 2. Interleukin-6 (acute phase, breast milk, mothers nursing sick infants). The hurdle regression model shows the conditional effects of the predictors (the expectation of the posterior predictive distribution). The hurdle (hu) part, which predicts probability, and the non-hurdle part that predicts the magnitude of non-zero outcome values are shown in A) and B), respectively. The shaded area represents the median and (50%, 80%, 95%) credible interval. C) An equivalence test based on the highest density interval and the region of practical equivalence decision rule (Ref. 2). CRP = C-reactive protein.

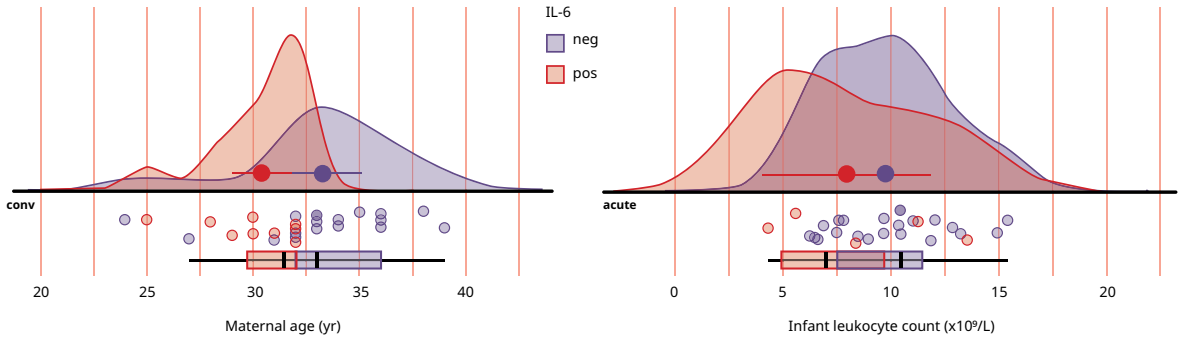

FIG. 3. Ridge plots, maternal age, and infant leukocyte counts (in venous blood), stratified by dichotomized IL-6 levels (ill infants and their mothers). *P* values are derived from the Mann–Whitney U test. Each dot represents an individual sample. (A) Dot color encodes group membership. Boxplots are defined by medians and their respective interquartile ranges (IQRs). Vertical lines extend to ±1.5×IQR.

During recovery, the proportion of IL-1β+ and IL-6+ milk samples increased among mothers nursing ill infants, approaching their respective distributions in healthy controls (Figure 1, Figure 3, Table 2). IL-6 reappeared more often in younger mothers (33 (32–36) vs 31.5 (29–32) yrs, n = 17 vs 12, IL-6– vs IL-6+, $P = 0.0031$) and in mothers whose infants had mild initial leukocytosis or normal leukocyte counts (10.5 (7.7–12.7) vs 7.7 (5.8–10.2) × 10^9/L, n = 17 vs 12, IL-6– vs IL-6+, $P = 0.027$). In contrast, older mothers and those whose infants experienced fever (Supplemental Table 2) or pronounced leukocytosis showed blunted IL-6 recovery during the control interval. The effect of maternal age extended across the entire range of observed and unobserved IL-6 values: older mothers were not only more likely to remain IL-6 negative but also had lower detected IL-6 values (Figure 4A). By contrast, the relation with infant acute-phase leukocyte count was more prominent for the IL-6–/IL-6+ dichotomy than for the absolute IL-6 levels (Figure 4B–C). These trends remained consistent even when we included a resampling step that controlled for infant age, though the changes were less pronounced (Supplemental Table 3).

TABLE 3. Dichotomized TNF (tumor necrosis factor) levels in breast milk during infant recovery, by lactation duration (mothers nursing sick infants)

Breastfeeding	TNF		Fisher's exact *P*
	< LLOQ (N)	> LLOQ (N)	
< 5 months	4	5	0.016
≥ 5 months	18	2	

LLOQ = lower limit of quantitation

The temporal profile of IL-1β paralleled that of IL-6. IL-1β rarely tested positive during acute illness and returned to near-control distribution during recovery (Figure 1, Table 2). However, despite similar aggregate dynamics, their expression patterns were not closely related. Co-detection of IL-6 and IL-1β in the same samples was rare, and only four of the 29 samples showed simultaneous reactivation during recovery. No specific maternal or infant characteristics were identified as predictors of IL-1β expression in milk.

TNF profiles followed a similar trajectory. During the acute phase, TNF was only sporadically detectable in milk from mothers nursing ill infants, contrasting relatively static control settings (mothers nursing healthy infants). By the recovery phase, TNF levels generally returned to control-like

distribution (Table 2), suggesting a dynamic landscape of human milk cytokines during infant illness. At this stage, the proportion of non-detects increased as lactation progressed: TNF reappearance was associated with shorter breastfeeding, i.e. a younger age of the infant (4 (3–6) vs 7 (5–10) months, n = 7 vs 22, TNF+ vs TNF–, $P = 0.026$), while persistent suppression of TNF was significantly more common in extended breastfeeding (Table 3, Figure 4). This suggests that late-stage milk TNF levels are strongly influenced by factors other than the child's health status or infectious challenge.

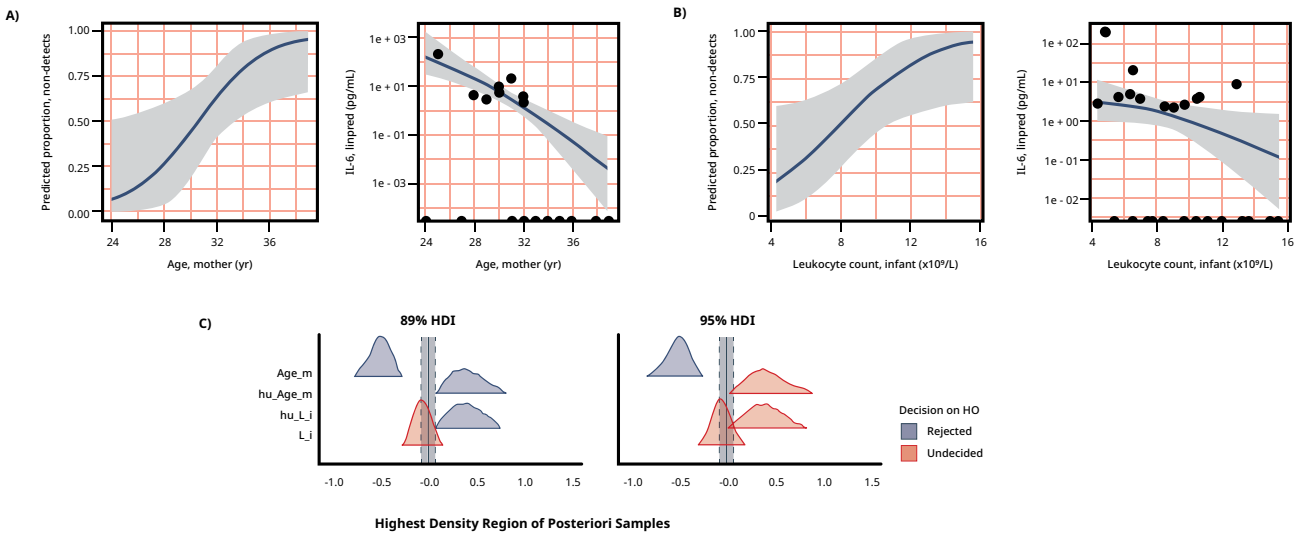

Fig. 4. Interleukin-6 (convalescent phase, breast milk, mothers nursing sick infants). A hurdle regression model shows the conditional effects of the predictors, the hurdle and non-hurdle parts that predict non-zero outcome values. Two predictors were evaluated: (A) maternal age and (B) infant leukocyte count (L_i). The shaded area represents the 95% credible interval. C) The equivalence test is based on the highest density interval and the region of practical equivalence decision rule (Ref. 2). Each dot corresponds to one measurement. The intercept was omitted from the plot to maintain readability.

Discussion

In this study, breast milk cytokine responses were examined in relation to infant infection and maternal-infant characteristics. Among the six measured cytokines, only IL-6, IL-1β, and TNF were consistently detectable. In mothers nursing ill infants, pro-inflammatory cytokines were markedly suppressed during the acute phase of infection, particularly IL-6, which was associated with elevated infant CRP levels. Cytokine recovery during convalescence varied: IL-6 levels rebounded in younger mothers and in those whose infants had milder leukocytosis, while recovery was blunted in older mothers and those with infants showing pronounced inflammation. TNF reactivation was more common in early lactation, suggesting a potential influence of lactation stage on cytokine restoration. These findings highlight a dynamic, individualized cytokine response in breast milk, shaped by both infant inflammatory status and maternal factors, such as age and lactation duration.

Human breast milk contains diverse bioactive molecules, including hormones, growth factors, cytokines (e.g., IL-1β, IL-2, IL-6, IL-8, TNF-α), and trace elements such as zinc and copper, which may actively stimulate infant immunity[29–31]. Breastfeeding, particularly in premature infants, is associated with lower infection rates compared to formula feeding, likely reflecting protection against pathogens such as *Bacteroides*, *Clostridium*, and *Escherichia coli*[29,31]. Due to ethical constraints, most studies on the immunomodulatory effects of breast milk have been conducted in vitro or in animal models. These consistently support a role for TGF-β, IL-10, IL-6, and sCD14 in promoting immune tolerance, with other cytokines and soluble receptors also being implicated[32].

Interestingly, we were able to detect targeted cytokines only in a portion of breastmilk samples collected in this study. This is in line with a previous study by Hawkes et al., which followed IL-1β, IL-6, TNF-α, TGF-β1, and TGF-β2 cytokine levels during the first three months postpartum. In their study, pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were detectable in only a subset of breast milk samples from healthy mothers breastfeeding healthy infants, with concentrations showing considerable variability across time points[33]. In contrast, anti-inflammatory cytokines TGF-β1 and TGF-β2 were present in substantial quantities in all samples, and there was little change in the mean concentration during 12 weeks of lactation[33].

Ustundag et al. reported that cytokine concentrations peak in colostrum and transitional milk, followed by a marked decline by day 21 and a further reduction by two months postpartum. Levels were additionally influenced by gestational age at delivery, with colostrum from mothers of preterm infants containing significantly lower cytokine concentrations than that of term mothers. These differences persisted into transitional and early mature milk, but were no longer evident by two months postpartum[31]. The inconsistent detection of cytokines in the present study may therefore be attributable to the exclusive analysis of mature milk samples and the inclusion of a wide range of lactation stages. As cytokine concentrations diminish substantially in later lactation, and given that temporal variation is further shaped by delivery timing, restricting sampling to mature milk is likely to obscure transient but biologically relevant peaks. This heterogeneity may have contributed to reduced sensitivity for detecting consistent cytokine patterns across participants. This is further supported by our finding that TNF reappearance in the recovery phase was associated with shorter lactation duration, while its persistent suppression correlated with late lactation, suggesting mammary gland maturity-related modulation.

Breast milk composition has been shown to adapt during infant infections, with reported increases in leukocytes — particularly macrophages — and TNF-α levels[34]. This aligns with the concept of breastfeeding as a dynamic, responsive immune defense that contributes to shaping neonatal immunity. In contrast to previous observations, the present study identified an unexpected pattern in which IL-6 and TNF-α levels were frequently suppressed and often undetectable in mothers nursing an ill infant, with concentrations returning to measurable levels in most cases following convalescence. Notably, IL-6 suppression was significantly associated with elevated CRP levels in the infants, suggesting a potential link between infant systemic inflammation and a reduction in breast milk cytokine content. This pattern may reflect an adaptive maternal mechanism designed to fine-tune the infant's immune exposure and mitigate excessive inflammation during acute illness. By modulating breast milk cytokine concentrations, the mother may contribute to maintaining the immune balance in the infant and reducing the risk of inflammation-related complications. Comparable regulatory effects have been observed in colostrum from mothers with peripartum infections, which exhibited significantly lower levels of several pro-inflammatory cytokines compared with those from healthy mothers[35]. Additionally, uncontrolled

variables such as maternal diet[36], psychological and physiological status (including depression or sleep disturbances)[37], the breast milk microbiome, and immune cell composition tailored to the infant's immune challenge may have influenced the observed cytokine suppression. These observations highlight the complexity of breast milk immune regulation and suggest that maternal responses to infant illness are context-dependent, potentially varying with the nature, severity, and systemic impact of the infection.

In our study, IL-6 and IL-1β levels tended to return toward control values during recovery, particularly in younger mothers and in cases where infants exhibited only mild leukocytosis or normal leukocyte counts. In contrast, older mothers and those with infants showing marked leukocytosis demonstrated a sustained suppression of IL-6. Previous research also supports an age-related influence on breast milk cytokine profiles. Shivani et al. reported mean colostrum IL-6 and TGF-β concentrations of 113.3 ± 73.1 pg/mL and 20.9 ± 23.6 pg/mL, respectively, with no significant correlation between maternal age and IL-6, but a significant positive correlation with TGF-β[31]. Together, these findings suggest that maternal age may differentially modulate specific cytokine pathways. The potential implications of such age-related variation, particularly when combined with lactation-stage effects, warrant further investigation to determine their impact on neonatal immune development and disease susceptibility.

Several limitations of our study should be acknowledged. The sample size was relatively small (29 acutely ill and 16 healthy mother–infant pairs), which may have limited the detection of subtle associations, especially for IL-1β and TNF. While the study was powered for large effect sizes, smaller or nuanced interactions between maternal and infant characteristics may have gone undetected. The cohort included only full-term infants with uncomplicated births from a single region, limiting generalizability to preterm or high-risk populations. Cytokine detection was constrained by assay sensitivity, and analyzing only mature milk likely reduced the ability to capture transient cytokine peaks. Finally, unmeasured factors, such as maternal diet and mental health, subclinical infections, or environmental exposures, could have influenced milk cytokine profiles. Despite these limitations, the study offers preliminary insight into dynamic, individualized breast milk cytokine responses during infant infection and may inform future, larger, multi-center studies.

Conclusion

Collectively, these findings demonstrate that breast milk immune composition is shaped by a multifactorial interplay of lactation stage, maternal age, and infant health status. The observed suppression of IL-6 and TNF-α during infant illness represents a departure from the previously assumed uniform upregulation of immune factors and suggests that maternal responses may be tailored to specific inflammatory contexts. Elucidating the mechanisms underlying such context-dependent regulation will be essential for understanding how breastfeeding shapes neonatal immunity and for developing targeted interventions to support vulnerable mother-infant pairs.

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DATA AVAILABILITY STATEMENT The data presented in this study are available upon request from the corresponding author. The authors do not have the consent of the participants for public distribution of the database.

CONFLICTS OF INTEREST The authors declare no conflicts of interest. ■

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SAŽETAK

Proupalni citokini u majčinom mlijeku smanjeni su tijekom infekcije dojenčeta te pokazuju neujednačen oporavak tijekom rekonvalescencije

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Majčino mlijeko ima ključnu ulogu u imunološkoj komunikaciji između majke i djeteta. Ispitivali smo dinamiku citokina u 45 asimptomatskih dojilja: 29 majki koje su dojile dojenčad s akutnom infekcijom i 16 koje su dojile zdravu djecu. Sva su dojenčad bila rođena u terminu, s visokim Apgar bodovima, a majke su bile bez znakova upale. Uzorci mlijeka prikupljeni su 3-5 dana nakon početka bolesti djeteta i ponovno 4-6 tjedana kasnije tijekom oporavka. Koncentracije citokina (IFN-γ, IL-1β, IL-17A, IL-4, IL-6, TNF-α) mjerene su u vodenoj fazi mlijeka pomoću Human HS ProcartaPlex™ Mix&Match testa. Dosljedno su bili mjerljivi samo IL-6, IL-1β i TNF. Kod majki bolesne djece razine proupalnih citokina, osobito IL-6, bile su snižene tijekom infekcije i povezane s povišenim CRP-om u dojenčadi. Oporavak citokina bio je heterogen: IL-6 se povećao kod mlađih majki i djece s blažom leukocitozom, dok je bio slabiji kod starijih majki i izraženije upale. Povratak TNF-a bio je češći u ranijim fazama laktacije, što ukazuje na utjecaj stupnja laktacije. Rezultati ove studije upućuju na dinamičku regulaciju sinteze citokina u majčinom mlijeku ovisno o upalnom statusu djeteta i majčinim osobinama, sugerirajući kontinuiranu prilagodbu imunološke komunikacije tijekom dojenja.

KLJUČNE RIJEČI

Dojenje; Novorođenčad; Infekcija; Citokini

Supplemental data

Supplemental table 1. Cytokine measurements in breast milk according to the mother's health status.

Cytokine (absent/present)	Measurement	History of chronic condition (Yes/No) Fisher's exact *P*
IL-6 (absent/present)	acute phase	0.66
	recovery	1
TNF	acute phase	0.648
	recovery	0.089
IL-1beta	acute phase	1
	recovery	0.678
IFNG	acute phase	1
	recovery	0.23

IL = interleukin; TNF = tumor necrosis factor; IFNG = interferon gamma

Supplemental table 2. Interleukin-6 (IL-6), acute phase measurements (breast milk), stratified by infant fever (sick children)

Fever	IL-6, acute phase		Fisher's exact *P*
	< LLOQ (N)	> LLOQ (N)	
Yes	16	0	0.0081
No	7	5	

LLOQ = lower limit of quantitation

Supplemental table 3. Hurdle-gamma regression (cytokines, breast milk): infant age-adjusted conditional estimates

Cytokine	Measure-ment	Group	proportion of non-detects (hurdle part) by group			response (hu part, pg/mL) by group		
			median	lower.HPD	upper.HPD	median	lower.HPD	upper.HPD
IL-6	AP	healthy	0.493	0.267	0.729	4	2	7.4
		ill	0.792	0.637	0.919	12.7	10	47.7
	C	healthy	0.462	0.267	0.729	13.1	4.1	38.7
		ill	0.606	0.424	0.778	7.9	3.6	15.4
IL-1b	AP	healthy	0.69	0.405	0.914	3.2	1.3	6.7
		ill	0.96	0.877	1	5.3	0.3	22.6
	C	healthy	0.743	0.5	0.929	2.4	1.2	4.4
		ill	0.798	0.642	0.931	3.1	1.8	5
TNF	AP	healthy	0.554	0.292	0.803	14	7.4	23.5
		ill	0.9	0.782	0.987	8.7	3.7	19
	C	healthy	0.693	0.447	0.9	10.8	8.6	13.5
		ill	0.745	0.576	0.895	7.7	6.1	9.5

Highest probability density (HPD) interval: 0.95; cytokine ~ Age(i)+Group; hu ~ Age(i)+Group
AP = acute phase; C = after convalescence