ORIGINAL ARTICLE

Effects of extremely preterm birth on cytokine and chemokine responses induced by T-cell activation during infancy

Dhanapal Govindaraj[1,a] , Georg Bach Jensen[1,2,a] , Khaleda Rahman Qazi[3],
Eva Sverremark-Ekström[3] , Thomas Abrahamsson[1,2,b] & Maria C Jenmalm[1,b]

[1]Division of Inflammation and Infection (II), Department of Biomedical and Clinical Sciences (BKV), Linköping University, Linköping, Sweden
[2]Crown Princess Victoria Children's Hospital, Linköping, County of Östergötland, Sweden
[3]Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

Correspondence

D Govindaraj, Division of Inflammation and Infection, Department of Biomedical and Clinical Sciences, Building 420 Lab Ettan, Entrance 68, Campus US, Linköping University, 581 83, Linköping, Sweden.
E-mail: dhanapal.govindaraj@liu.se

[a]Shared first authorship.
[b]Shared senior authorship.

Abstract

Objectives. Extremely preterm (EPT; gestational week $< 28 + 0$, < 1000 g) neonates are vulnerable to infections and necrotising enterocolitis, important contributors to mortality and morbidity. However, knowledge regarding their immune maturation remains limited. We here investigated the longitudinal development of functional T-cell capacity in EPT infants. **Methods.** Peripheral blood mononuclear cells were isolated at 14th and 28th day (D) and at gestational week $36 + 0$ (Gw36) from EPT infants, participated in a randomised, double-blind, placebo-controlled study of *Lactobacillus reuteri* DSM 17938 probiotic supplementation. Blood collected from 25 full-term (FT) infants at D14 was used as control. The secretion of immune mediators was determined through comprehensive Luminex panels after stimulation with human T-cell activator CD3/CD28 beads. **Results.** The levels of many mediators were low in EPT infants at D14, whereas the secretion of several chemokines was higher in EPT than in FT infants. Furthermore, Th2:Th1 cytokine ratios were higher in EPT than in FT infants. Progressively elevated secretion of, for example, IFN-γ, TNF and IL-17A in EPT infants was observed from D14 to D28 and then at Gw36. Elevated levels were observed for many proinflammatory mediators at D28. Probiotic supplementation or perinatal factors (e.g. clinical chorioamnionitis, preeclampsia and delivery mode) did not influence the cytokine and chemokine responses. **Conclusions.** Immune mediators induced by T-cell activation in EPT infants were mainly reduced at D14 and Th2 skewed compared to those in FT infants, but mostly recovered at Gw36, indicating immune maturation. Increased proinflammatory responses at D28 may be related to the heightened risk of severe immune-associated complications seen in EPT infants.

Keywords: chemokines, cytokines, neonatal immunity, preterm neonates, T-cell activation

INTRODUCTION

Preterm birth (before gestation week 37) affects approximately 5.5–6% of pregnancies in Sweden and 10–15% worldwide. It constitutes a significant global concern, with an annual incidence of approximately 15 million cases worldwide.[1] Extremely preterm (EPT) infants, born before 28 weeks of completed gestation and representing approximately 4% of all preterm births,[2] have a particularly increased risk of morbidity and mortality. Although modern neonatal care has considerably increased the survival of preterm neonates, the mortality rates of EPT infants are still as high as 23% in affluent countries such as Sweden.[3]

Preterm infants, and EPT infants in particular, are highly susceptible to various infections, highlighting the immaturity of their immune system compared to that of full-term (FT) infants.[4,5] For early-onset sepsis (< 72 h), representing vertical mother-to-infant transmission, *Streptococcus agalactiae* (GBS) and *Escherichia coli* are the most common causes. For late-onset sepsis (> 72 h), commonly linked to organisms acquired from the hospital environment, coagulase-negative staphylococci are the most common pathogens, although a broader spectrum of bacteria, including *Staphylococcus aureus*, GBS, *Enterococcus*, *E. coli*, and other Gram-negative bacteria, is also encountered. Moreover, neonates are susceptible to systemic viral (e.g. herpes simplex virus and enterovirus) as well as fungal infections.[6,7] Preterm infants lack robust adaptive responses to infections, and they often display exaggerated inflammatory responses associated with common neonatal conditions such as sepsis, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP) and necrotising enterocolitis (NEC), the latter being an inflammatory disease of the gut associated with hyper-inflammatory immune response to enteric bacteria.[8,9] Additionally, preterm infants have reduced innate immunity compared to term infants.[10] The proinflammatory responses of monocytes to pathogens are reduced in preterm infants,[11] and they have an increased risk of hospitalisation because of infections after infancy as well.[12] While some differences in immune responses between preterm and term infants resolve with age,[13] certain immune discrepancies may not be alleviated but further exacerbated 3 months after birth.[14] We have previously observed notable differences in T-cell frequencies in EPT infants, with several differences that persisted up to Gw36.[15,16] However, the functional capacity of T cells to respond to activating signals has yet to be determined in EPT infants.[17–19]

The composition of the gut microbiota during the initial months after birth critically influences immune maturation,[20] and an impaired gut microbiota development has been observed in preterm infants.[21] Hence, promoting the establishment of a beneficial gut microbiota early in life may improve health outcomes in this vulnerable population. A recent Cochrane meta-analysis on probiotic supplementation in preterm infants demonstrated reduced NEC development.[22] The use of probiotics in very preterm, low birthweight infants was evaluated but systematic review and network meta-analysis, showing encouraging results that supplementation reduces feeding intolerance[23,24] and late-onset sepsis,[22,24] however, stated insufficient data with regard to the benefits and potential adverse effects in EPT infants. In preterm infants (excluding EPT neonates), *Lactobacillus reuteri* DSM 17938 supplementation has been observed to reduce sepsis,[25] duration of hospital stay and feeding intolerance[23,26] and increase the levels of the anti-inflammatory cytokine IL-10 in faeces, while decreasing CXCL8 levels. We recently reported that *L. reuteri* DSM 17938 supplementation to EPT infants promoted the growth of head during the first month of life.[27] Probiotic supplementation may promote T-cell homeostasis by inducing regulatory T-cell (Treg) responses and suppressing inflammatory responses.[28] However, the potential immunomodulatory effects in EPT infants remain largely unknown.

Gaining further insights into immune system development in EPT infants is highly important. However, there is a limited number of prospective studies available because of difficulties in sampling adequate blood volumes.[29] In this study, we longitudinally assessed the functional capacity of peripheral T cells in a group of extremely preterm infants with a birthweight < 1000 g, participated in a randomised, double-blind, placebo-controlled study of probiotic supplementation. The levels of a comprehensive panel of cytokines and chemokines induced after activation with human T-cell activator CD3/CD28 beads were determined at postnatal D14 and D28, and at postmenstrual week 36 + 0 (Gw36), and their immune function at D14 was compared to
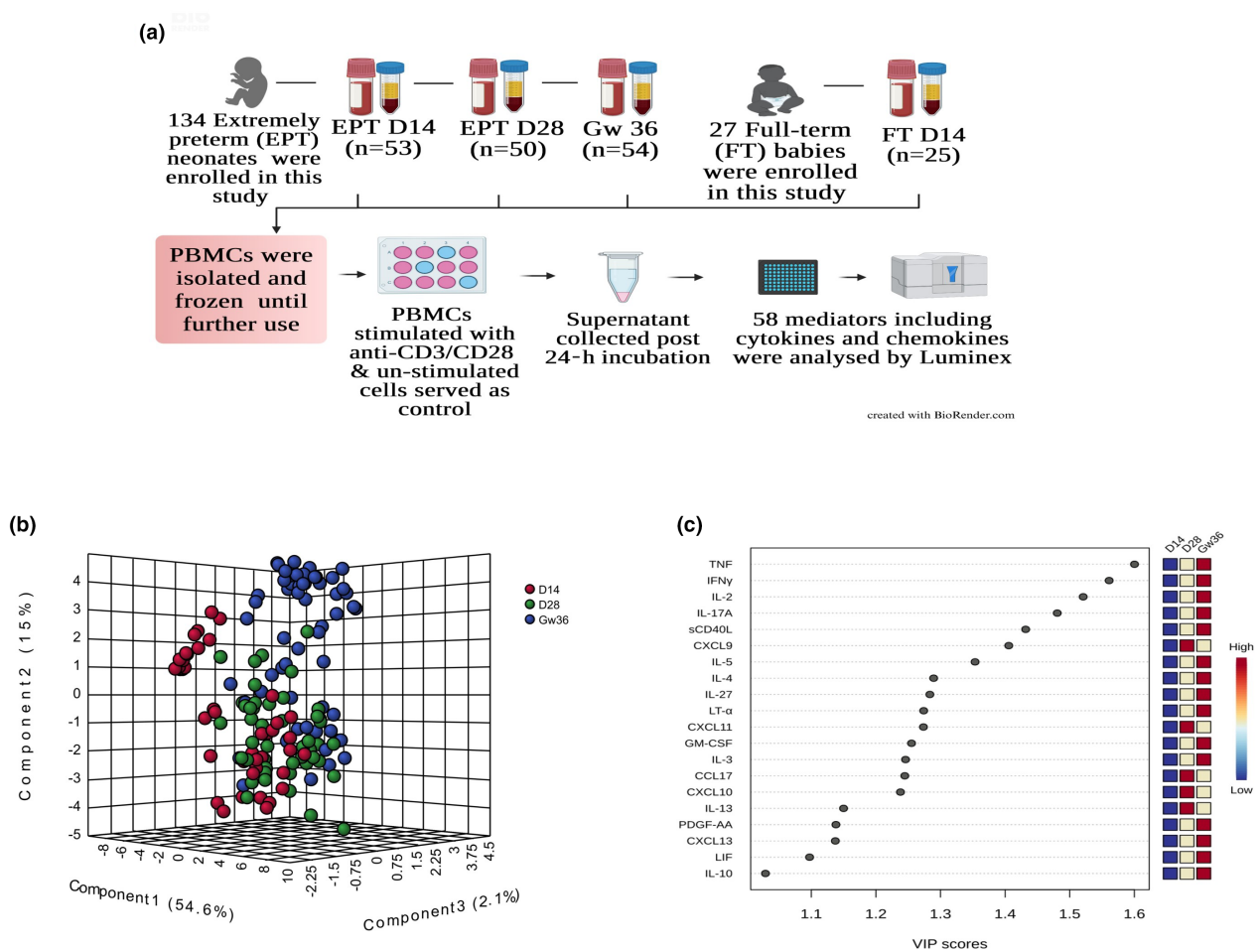
Figure 1. (a) Graphical diagram showing the number of subjects including time points for blood sampling and peripheral blood mononuclear cell (PBMC) separation. A total of 107 extremely preterm (EPT) infants and 25 full-term control subjects were included in this study. PBMCs from EPT infants were stimulated at D14 ($n = 53$), D28 ($n = 50$) and Gw36 ($n = 54$). PBMCs were stimulated by human T-cell activator CD3/CD28 beads for 24 h, and 58 cytokines and chemokines from the supernatant were analysed in different Luminex panels. **(b)** Partial least squares discriminant analysis (PLS-DA) model with 3D scores plot of anti-CD3/CD28-induced cytokine and chemokine responses in EPT at different postnatal time points. Each dot represents an infant at a given time point, D14 (red), D28 (green) and Gw36 (blue). The analysis included all 42 mediators, and the explained variances of each principal component (PC) are indicated in parentheses. The *P*-value based on permutation was $P < 0.001$. **(c)** Important mediators identified by PLS-DA depicted as variable importance in projection (VIP) scores. The 25 mediators with the highest VIP scores for separating EPT at different time points from D14 to Gw36 are depicted. The coloured boxes on the right indicate the relative concentrations of the corresponding mediator in each group. VIP scores > 1.0 were considered significant.

that of FT neonates at postnatal D14. The T-cell responses were also assessed in relation to *L. reuteri* DSM 17938 supplementation, perinatal factors and clinical outcomes.

RESULTS

Postnatal immune development in EPT infants

The PLS-DA model of anti-CD3/CD28-induced cytokine and chemokine levels was highly significant ($P < 0.001$) in separating EPT at different time points, even if some overlapping could be seen between groups (Figure 1b). TNF, IFN-γ and IL-2 were the most important cytokines in driving the difference in a chronological manner from D14 to Gw 36 (Figure 1c).

No association with probiotics or clinical variables

There were no significant changes or effects of the probiotic supplementation (Supplementary

figure 1). Further, there was no clear pattern of clustering at D14 or D28 when grouping for the perinatal factors of clinical chorioamnionitis, delivery mode, sex or small for gestational age (SGA; Supplementary figure 2). For these factors, univariate testing of cytokine or chemokine levels did not reveal any significant differences after adjusting for multiple comparisons. The same applied to preeclampsia (D14), preterm premature rupture of membranes (PPROM) (D14) and whether the infant had been postnatally treated with systemic betamethasone before the time of sampling (D14, D28 and Gw36). PCA plots did not show any clear patterns regarding culture-proven sepsis when comparing infants with preceding or future episodes of sepsis with infants having no sepsis episode at different time points in EPT (Supplementary figure 3). Analyses of individual cytokines and chemokines did not reveal any significant differences in infants with preceding sepsis onset at D28 or Gw36. The low number of NEC cases did not allow for further analyses of this diagnosis.

Divergent T cell responses were observed in extremely preterm and full-term infants at D14

For the cytokines and chemokines, where spontaneous secretion was detectable (Supplementary table 2), the spontaneous release of most mediators was low in PBMCs from EPT infants at D14 and D28, but at Gw36, they were generally similar to background levels produced by FT infants at D14 (Supplementary figure 4). At D14, anti-CD3/CD28-induced cytokine and chemokine levels from PBMCs were generally lower in EPT than in FT neonates. However, four chemokines had more than a two-fold higher concentration in EPT than in FT infants at D14, namely CCL1, CCL2, CCL7 and CXCL8 (Figure 2a). From ROC analyses, several mediator ratios discriminated EPT from FT infants at D14, with IFN-γ/CCL2 ratio generating the largest AUC (0.998 [0.991; 1], $P < 0.001$; Figure 2b). IFN-γ had the largest AUC (0.96 [0.91; 0.99], $P < 0.001$) for a single mediator. While FT infants were clearly clustering in the PCA plot at D14, PCA did not reveal any clear pattern of clustering for different weeks of gestational age in EPT infants (Supplementary figure 5). PLS-DA clearly discriminated ($P < 0.001$) EPT from FT infants at D14 (Figure 2c). The top 25 cytokines and

chemokines that contribute most to this separation are shown in (Figure 2d).

T helper 1 (Th1) mediator levels were low at D14 in EPT infants but increased with postnatal age

Anti-CD3/CD28-induced IFN-γ, TNF, CXCL10 and CXCL11 levels were lower at D14 in EPT than in FT infants (Figure 3a–d). In EPT infants, IFN-γ, TNF, CXCL9 (Supplementary table 3), CXCL10 and CXCL11 levels then increased from D14 to both D28 and Gw36. The IFN-γ and TNF levels further increased from D28 to Gw36, while no significant changes between these time points were observed for the chemokines (Figure 3a–d).

Th2 mediator levels were low at D14 in EPT infants but they showed higher Th2:Th1 ratios than in FT infants

Anti-CD3/CD28-induced IL-4, IL-5 and IL-13 levels were lower at D14 in EPT than in FT infants (Figure 4a–c), while similar levels of the Th2-associated chemokine CCL22 were observed between the groups (Figure 4d). In the EPT infants, the IL-4, IL-5, IL-13 and CCL22 levels increased from D14 to both D28 and Gw36 (Figure 4a–d). The CCL22 levels then decreased from D28 to Gw36, while no significant changes between D28 and Gw36 were observed for the cytokines (Figure 4d). We next investigated how the ratios of Th2:Th1 mediators changed with postnatal age in EPT infants were compared to those in FT infants at D14. The ratios of the Th2 cytokines IL-4, IL-5 and IL-13 to the Th1 cytokine IFN-γ were higher at D14 in EPT than in FT infants (Figure 4e–g). In EPT infants, the IL-4/IFN-γ ratio then decreased from D14 to D28, while no significant differences were observed for the IL-5/IFN-γ and IL-13/IFN-γ ratios. However, all the ratios were lower at Gw36 than at D14 (Figure 4e–g). Correspondingly, the ratio of the Th2 chemokine CCL22 to the Th1 chemokine CXCL10 was higher at D14 in EPT than in FT infants and then decreased from D14 to Gw36 (Figure 4h).

Th17 immune mediators showed marked differences between EPT and FT infants at D14 and a clear trend of increasing levels with postnatal age in EPT infants

Anti-CD3/CD28-induced IL-17, CCL20 and GM-CSF, but not IL-6, levels were lower at D14 in EPT than
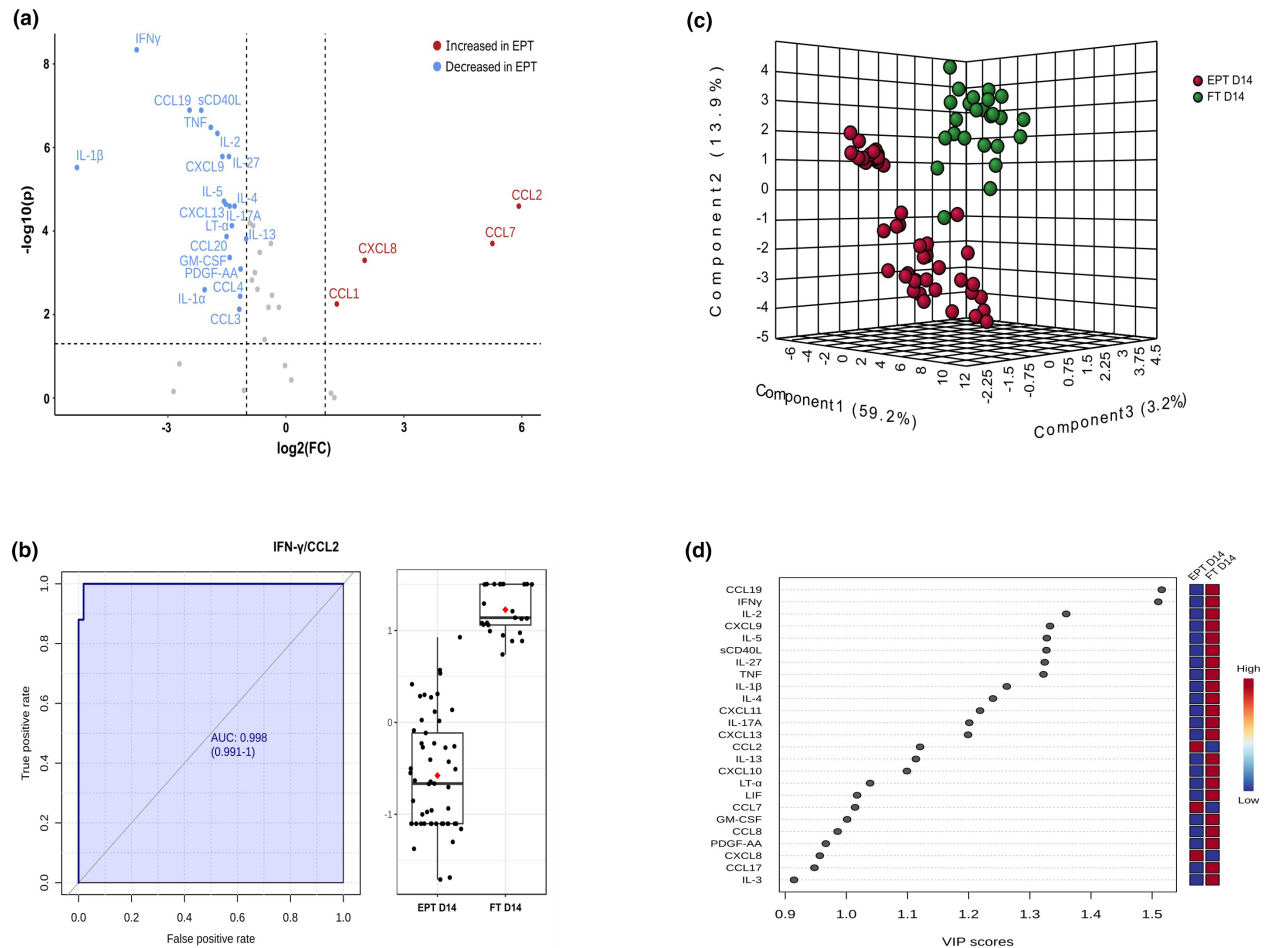
Figure 2. Cytokine and chemokine responses in extremely preterm (EPT) and full-term (FT) infants at D14. **(a)** Volcano plot of cytokine and chemokine levels in EPT infants compared to those in FT infants at D14. The *x*-axis represents $\log_2$-fold change (FC) and the *y*-axis represents $-\log_{10}$ (adjusted *P*-values). Increased mediator levels ($\log_2$FC > 1, adjusted *P*-values < 0.05) in EPT infants are shown as red dots, decreased mediator levels ($\log_2$FC < −1, adjusted *P*-values < 0.05) in EPT are shown as blue dots, and non-significant mediator levels as grey dots. The non-parametric Wilcoxon rank-sum test with the Benjamini–Hochberg false discovery rate. **(b)** Receiver-operating characteristic (ROC) analysis of the IFN-γ/CCL2-ratio in EPT infants compared to FT infants (left). Area under the curve (AUC) 0.998 [0.991; 1], *P* < 0.001. Box plot of the normalised concentration ratios with 95% CI around the median (right). The mean concentration ratio of each group is indicated with a red diamond. **(c)** Partial least squares discriminant analysis (PLS-DA) model with 3D scores plot of anti-CD3/CD28-induced cytokine and chemokine response in EPT infants (red) and FT infants (green). The analysis included all 42 mediators, and the explained variances of each principal component are indicated in parentheses. The *P*-value based on permutation was *P* < 0.001. **(d)** Important mediators identified by PLS-DA depicted as VIP scores. The 25 mediators with the highest VIP scores are depicted. The coloured boxes on the right indicate the relative concentrations of the corresponding mediator in each group. VIP scores > 1.0 were considered significant.

in FT infants (Supplementary figure 6). In the EPT infants, IL-6, IL-17 and GM-CSF levels increased from D14 to D28, whereas IL-17, CCL20 and GM-CSF also showed an increase from D14 to Gw36. The IL-6 levels peaked at D28, showing an increase from D14 to D28, followed by a subsequent decrease from D28 to Gw36 (Supplementary figure 6).

Notable differences in anti-inflammatory response patterns with postnatal age in EPT infants

Anti-CD3/CD28-induced IL-1RA and M-CSF levels were similar at D14 in EPT and FT infants (Figure 5a and c), while IL-10 levels were lower at D14 in EPT infants than in FT infants (Figure 5b).
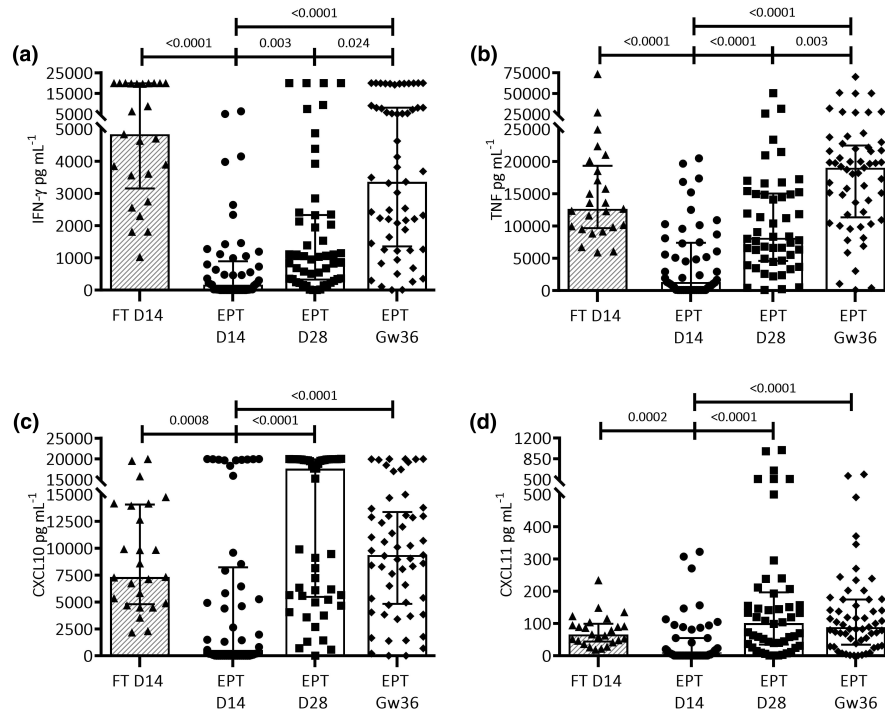
Figure 3. Th1-associated cytokines and chemokines induced by anti-CD3/CD28 stimulation of peripheral blood mononuclear cells (PBMCs) in extremely preterm (EPT) and full-term (FT) infants. The scatter plots show the levels of Th1 cytokines in picograms per millilitre **(a–d)** detected in PBMCs culture supernatants from EPT infants at D14, D28 and Gw36 compared to those from FT at D14. The bar plots show the median and interquartile ranges. The Kruskal–Wallis test with the Benjamini–Hochberg false discovery rate procedure for multiple comparisons was used to make group comparisons.

Figure 4. Th2-associated cytokines and Th2:Th1 ratio induced by anti-CD3/CD28 stimulation of peripheral blood mononuclear cells in extremely preterm and full-term infants. The scatter plots show the levels in picograms per millilitre for **(a)** IL-4, **(b)** IL-5, **(c)** IL-13 and **(d)** CCL22. Ratios between the Th2 cytokines **(e)** IL-4, **(f)** IL-5, **(g)** IL-13 over the Th1 cytokine IFN-γ and **(h)** CCL22 over CXCL10. The Kruskal–Wallis test with the Benjamini–Hochberg false discovery rate procedure for multiple comparisons was used to make group comparisons.
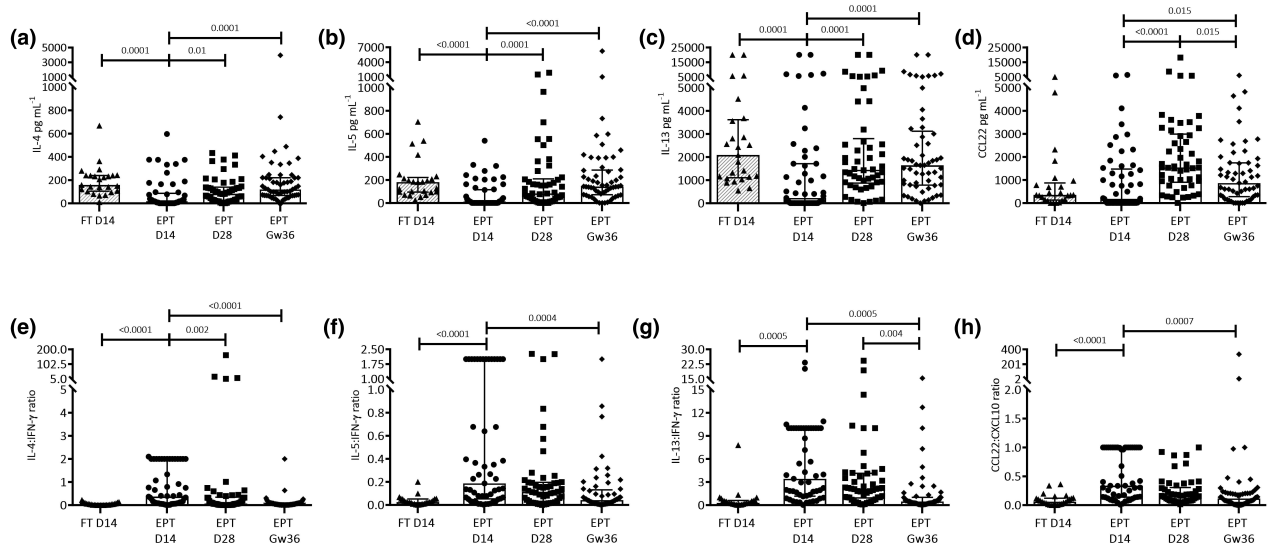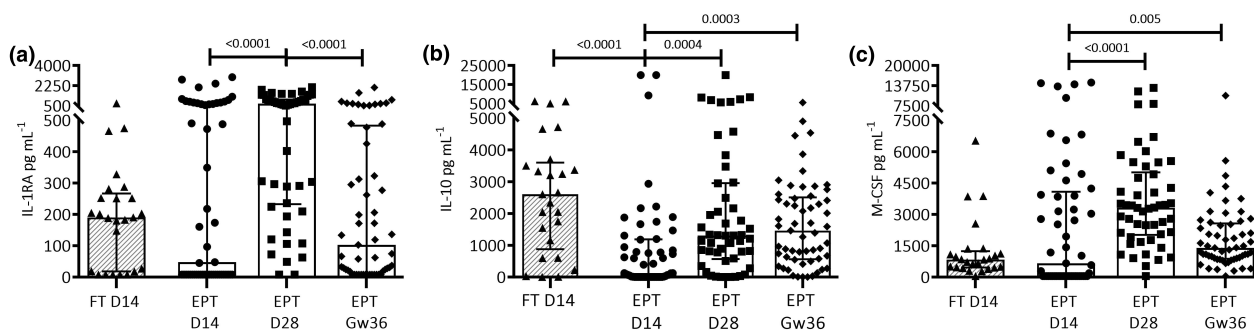
Figure 5. Anti-inflammatory cytokines induced by anti-CD3/CD28 stimulation of peripheral blood mononuclear cells in extremely preterm and full-term infants. The scatter plots show the levels in picograms per millilitre for **(a)** IL-1RA, **(b)** IL-10 and **(c)** M-CSF. Bars show medians with interquartile range. The Kruskal–Wallis test with the Benjamini–Hochberg false discovery rate procedure for multiple comparisons was used to make group comparisons.

In the EPT infants, IL-10, IL-1RA and M-CSF levels then increased from D14 to D28. Subsequently, the IL-1RA levels decreased from D28 to Gw36, while the levels of the other cytokines did not change significantly (Figure 5a–c).

Several chemokines showed higher response levels in EPT than in FT infants at D14

Anti-CD3/CD28-induced CCL1, CCL2, CCL7 and CXCL8 levels were higher at D14 in EPT than in FT infants (Figures 2a and 6a–d). They all demonstrated a peak level at D28, with an increase from D14 to D28, followed by a decrease from D28 to Gw36 (Figure 6a–d).

DISCUSSION

The understanding of how preterm birth affects immune development and function is currently limited,[30] and the specific effects of extremely preterm birth are particularly unclear. However, it is well recognised that the immune system of EPT/preterm infants differs from that of FT infants. In this trial, we made a comprehensive analysis of the functional immune development in a well-defined cohort of extremely preterm infants with a birth weight below 1000 g. In a population of immunologically compromised infants born at the very limit of viability, as early as 23–24 weeks of gestational age owing to proactive care in Sweden, we analysed the levels of cytokines and chemokines induced by anti-CD3/CD28 stimulation. To our knowledge, we are the first to report how the functional capacity

of T cells changes over time throughout the crucial early months of life in a large group of extremely preterm infants. Moreover, we explored the potential impact of several perinatal factors, such as preeclampsia, chorioamnionitis, delivery mode and sex on immune response. Additionally, we examined the potential effect of postnatal treatment with probiotics or systemic betamethasone and investigated whether alterations in cytokine or chemokine responses were associated with the occurrence of culture-proven sepsis.

We have previously reported on how conventional T-cell subpopulations are impacted by gestational age at birth, pointing at an immaturity of several aspects of the T-cell compartment in the early life of EPT infants.[16] In the present follow-up study, we found marked differences in the functional capacity of T cells to secrete cytokines and chemokines, although the T-cell activation might also have led to the secretion of some of these mediators by other cell types in the PBMCs. The diverging cytokine and chemokine responses induced after T-cell activation clearly emphasise the immature and deviating immune response in these vulnerable infants. Hence, most cytokine and chemokine levels were distinctly lower in EPT infants at D14 than in later postmenstrual ages (D28 and Gw36) and than in FT infants at D14. Both PCA and supervised PLS-DA models of the cytokine and chemokine profiles clearly demonstrated the separation between EPT and FT infants at D14, highlighting the distinct immunological differences in developmental stages between the two groups.
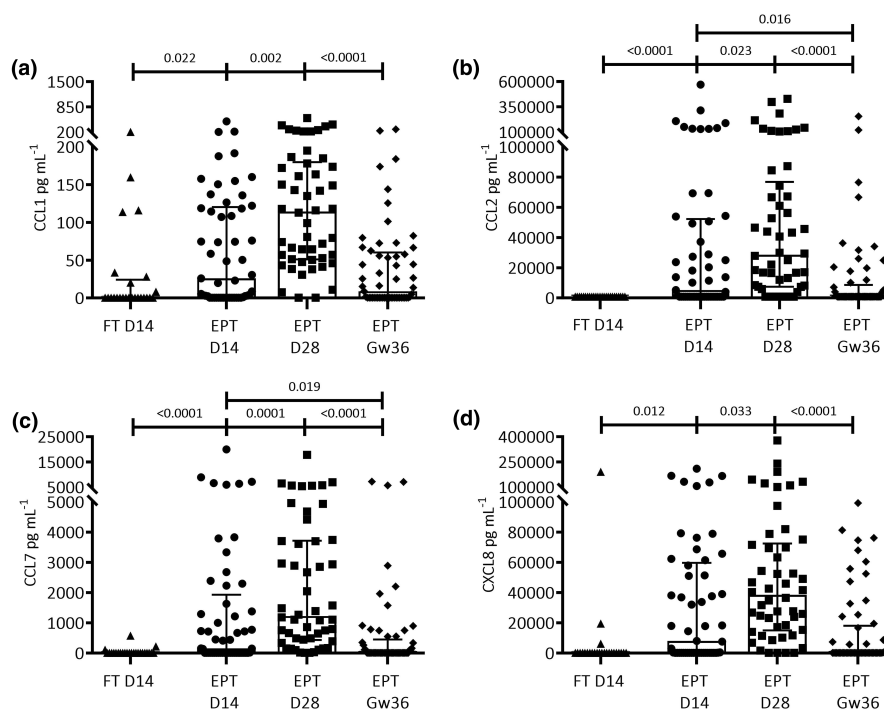
Figure 6. Chemokines in higher levels induced by anti-CD3/CD28 stimulation of peripheral blood mononuclear cells in extremely preterm compared with those in full-term infants. The scatter plots show the levels in picograms per millilitre for **(a)** CCL1, **(b)** CCL2, **(c)** CCL7 and **(d)** CXCL8. Bars show medians with interquartile range. The Kruskal–Wallis test with the Benjamini–Hochberg false discovery rate procedure for multiple comparisons was used to make group comparisons.

Notably, the chemokines CXCL8, CCL1, CCL2 and CCL7 all exhibited levels that were higher in EPT than in FT infants at D14. CXCL8 is an important effector of neutrophil function and, as such, may promote neonatal immunity by aiding in the prevention of infection. With many preterm infants born in the context of intrauterine infection, CXCL8 is likely to have significant relevance in the immune response in EPT infants. A study by Kamdar et al.[31] demonstrated that preterm infants born < 32 Gw, and experiencing infections, had reduced CXCL8 responses, and CD4^{+} T cells from preterm infants born as early as 23 Gw have been observed to produce CXCL8 in response to TCR stimulation.[32] CCL2 regulates monocytes and polarises macrophages towards an anti-inflammatory phenotype that migrates to inflamed tissues and infection sites.[33,34] Preterm infants < Gw30–36 with late-onset sepsis have previously been shown to have increased plasma levels of CCL2;[35,36] thus, CCL2 may function as an antimicrobial defence or a response to pathogens. Both CXCL8 and CCL2 increased at early postnatal age and elevated at D28. This observation

suggests that these chemokines may play a crucial role in compensating for a delayed maturation of specific humoral and cellular immune responses during infection or inflammatory stages in EPT infants.

Interestingly, ROC analysis found the IFN-γ/CCL2-ratio to be the strongest mediator combination in distinguishing EPT from FT infants at D14. While it is essential to view this finding as exploratory, it emphasises the potential significance of CCL2 as an important mediator in the early postnatal life of EPT infants, suggesting an encouraging direction for further investigation into its possible immune-modulating properties in preterm infants.

Longitudinal samples in EPT infants showed that many mediators were lower at D14 and peaked at D28. The majority of cytokine and chemokine levels had normalised by Gw36, indicating immune maturation. We observed a Th2-skewed cytokine milieu with elevated Th2/Th1 cytokine ratios at D14 in the EPT infants, pointing towards a postnatal persistence of Th2 bias. Our findings indicate significant changes in Th1,

proinflammatory and regulatory mediators during the neonatal period. Although many cytokine and chemokine responses were low after anti-CD3/CD28 activation of T cells *in vitro* in EPT infants at D14 after birth, we observed a progression of immune development and maturation, with increased cytokine and chemokine secretion at D28 and Gw36. Additionally, no significant differences were observed between the probiotic supplementation and the placebo group. Nor did we find evidence for any association with sepsis or any impact from the investigated perinatal factors.

In this study, the levels of the anti-CD3/CD28-induced Th1 mediators were lower in EPT infants than in FT infants at D14. Additionally, they significantly increased at later time points, which suggests a dynamic change in their potential role in the progressive development of cell-mediated immunity. An effective Th1 response promotes cell-mediated immunity and is important in the prevention of intracellular infections, including those caused by bacteria. Weak early Th1 differentiation in cord blood samples from FT neonates was observed compared to that in peripheral blood CD4^{+} T cells from adults.[37,38] Moreover, Olin *et al.*[39] demonstrated an overexpression of genes involved in the negative regulation of IFN-γ at 12 weeks of age in preterm infants born < 30 weeks gestational age. TNF is another essential factor in the immune response against infections and the induction of inflammation, and preterm infants (< 28–31 gestational weeks) with NEC have been found to have increased production of TNF.[40,41] In this study, we did not have enough NEC cases to further examine this.

Neonatal naïve CD4^{+} T cells have an intrinsic preference towards a Th2-biased cytokine production[42,43] because of impaired immunity to infections; moreover, human neonatal T cells seem to be preprogrammed to a type 2 phenotype in several ways. In EPT infants, there seems to be a further notable preservation of Th2 responses. In a previous study, conducted on the same cohort as the present study, we demonstrated a reduced ratio of T-bet to GATA3-expressing CD4^{+} T cells in EPT compared to that in FT infants.[16] This is in line with our present findings, as we noted a marked skew towards Th2 response in EPT infants and elevated Th2:Th1 ratios were particularly pronounced at D14, and subsequently showed a significant decrease in Gw36, suggesting a process

of postnatal adaptation and maturation. However, correlation analysis comparing Th1 and Th2 mediators with MFI of T-bet and GATA-3 transcription factors showed no significance (data not shown). Our findings provide additional support for a neonatal predisposition towards Th2 immunity that is even more prominent in EPT infants. As such, a high level of immune tolerance during the *in utero* stage of development is advantageous for the foetus. However, it may also, particularly when compromising Th1 immunity, as our observations suggest, contribute to an increased risk of infections and a shift towards a proinflammatory stage during postnatal development that potentially could be harmful to EPT infants.

Th17 cells are critical for the host defence against fungal and extracellular bacterial infections, particularly at epithelial and mucosal surfaces.[44] These cells are preferentially attracted by expression of CCR6, which is induced by the secretion of CCL20.[44] As we observed increasing CCL20 responses with age in the EPT infants, this may indicate increasing recruitment of IL-17-producing Th17 cells, although in this cohort we have previously observed a very low expression level of RORγt transcription factors for Th17 at all time points in EPT infants.[16] In parallel, IL-17 levels were low at D14 and exhibited a clear pattern of increase at later time point, which may indicate postnatal development of protective mucosal barriers that could aid in clearing extracellular pathogens.

In our study, IL-6 levels were elevated at D28, followed by a decrease at Gw36. Additionally, several other proinflammatory cytokines (IFN-γ, TNF, IL-17 and GM-CSF) and chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL10 and CXCL11) exhibited high levels at D28. This time frame coincides with a particularly vulnerable period in the life of EPT infants, characterised by the need for intensive care involving oxygen exposure, mechanical ventilation and an increased risk of complications such as sepsis and NEC. Moreover, this proinflammatory state of postnatal immune development may serve as a precursor to other prevalent inflammatory morbidities of prematurity, such as BPD,[45] ROP[46] and future neurodevelopmental impairment.[47] As mentioned, we did not find any association between IL-6 or other proinflammatory cytokines or chemokines and sepsis. It is important to note that the number of sepsis cases was relatively low and that

sampling time points independent of sepsis time of onset may have contributed to high variability in the data, which could have affected the observed results.

IL-10, an anti-inflammatory cytokine that is recognised for its ability to inhibit proinflammatory cytokines and dampen inflammation, had increased levels at D28 and Gw36 than at D14 in EPT infants. This suggests the potential significance of IL-10 as a key mediator of postnatal immune balance in EPT infants since IL-10, along with TGF-β, induce Treg cells which help suppress inflammation and maintain tolerance. However, in the same cohort, we recently reported that Treg cell frequencies did not change across time points in EPT infants.[16] The regulatory mediators IL-1RA, which plays a role in homeostatic functions in children,[48] and M-CSF, shown to have protective functions against infections in EPT infants,[49] reached their peak levels at D28 in EPT infants. This may suggest an important counter-regulatory response to the heightened proinflammatory activity, as previously noted, occurring around this particular point in time among EPT infants.

In this trial, *L. reuteri* supplementation did not significantly affect immune development. This is consistent with our previous finding in the same cohort, where we did not observe any phenotypic changes of the conventional[16] or unconventional[15] T-cell compartment. It is important to note that the lack of effects of *L. reuteri* on induced T-cell response in peripheral blood does not rule out a local immunological impact on the small intestine, where probiotics are more likely to exert their effects.[16] Accordingly, we have previously observed an increased gut microbiota diversity in the first month of life after *L. reuteri* supplementation.[50]

CONCLUSIONS

In conclusion, the cytokine and chemokine responses induced by T-cell activation in extremely preterm infants were mainly reduced and Th2 skewed at D14 compared to those in full-term infants. Although we did not find any clear association with sepsis, this observation may still contribute to the increased susceptibility to postnatal infections that is generally observed in EPT infants. Most cytokine and chemokine levels had been recovered by Gw36, indicating immune maturation. Increased proinflammatory responses

at D28 coincides with a particularly vulnerable period in the life of EPT infants, characterised by the need for intensive care and an increased risk of complications such as sepsis and NEC. Consequently, this inflammatory stage of postnatal development signifies a critical period of vulnerability for EPT infants, potentially contributing to an elevated risk of later immune-associated complications such as NEC, BPD and ROP. Probiotic supplementation had no significant effects on immune parameters. The insights gained from this study contribute significantly to guiding future research aimed at improving the understanding, prediction and management of the diverse infectious and inflammatory conditions encountered in extremely preterm infants.

METHODS

The Prophylactic Probiotics to Extremely Low Birth Weight Premature Infants (PROPEL) trial was a double-blinded, randomised, placebo-controlled, multicentre study, conducted in 10 neonatal units in Sweden from June 2012 to November 2015 in the regions of Stockholm and Linköping, evaluating the effect of probiotic supplementation with *L. reuteri* DSM 17938 in EPT infants. The study was approved by the Ethics Committee for Human Research at Linköping University (Dnr 2012/28-31, Dnr 2012/433-32).

Participant enrolment

Infants born between gestational weeks 23 + 0 and 27 + 6 with a birthweight < 1000 g were eligible for enrolment. Written informed consent was obtained from parents within 3 days after delivery. Infants were followed up using thorough clinical data, including perinatal data, growth, feeding intolerance, treatment, antibiotics and mild-to-severe morbidities, collected daily in a study-specific case report form until Gw36. The study design has been previously described in detail.[27] In the original trial, a total of 134 EPT infants were enrolled, with PBMC samples available from 107 infants for the immune functional analysis. Additionally, the analysis of PBMCs from 25 FT infants at D14 were used as controls. The background characteristics of the 107 EPT infants included in this study are displayed in Table 1.

Lactobacillus reuteri supplementation

The active intervention, once-daily *L. reuteri* DSM 17938 (provided by BioGaia, Stockholm, Sweden), was provided in oil drops consisting of sunflower oil, medium-chain triglyceride oil and silicon dioxide. The daily dose was 1.25×10^8 bacteria (0.2 mL).[27] The placebo was maltodextrin provided in an identical oil suspension and having similar smell, taste and visual appearance as the

Table 1. Background and clinical characteristics of the study participants.

EPT (*n* = 107)	*Lactobacillus reuteri* (*n* = 58)	Control (*n* = 49)	*P**
Gestational age, weeks, mean (SD)	25.5 (1.2)	25.4 (1.3)	0.59
Birthweight, g, mean (SD)	720 (126)	727 (144)	0.79
Birthweight *z*-score, mean (SD)	−1.28 (1.21)	−1.13 (1.36)	0.54
Small for gestational age (weight < 2 SD), *n* (%)	18 (31%)	11 (22%)	0.32
Male, *n* (%)	27 (47%)	30 (61%)	0.13
Apgar at 5 min, median (IQR)	7 (4–9)	7 (5–8)	0.94
Infants from multiple pregnancies, *n* (%)	26 (45%)	14 (29%)	0.08
Antenatal corticosteroids, *n* (%)	57 (93%)	48 (99%)	1.00
Caesarean section, *n* (%)	44 (76%)	26 (53%)	0.01
Maternal smoking, *n* (%)	3 (5%)	5 (10%)	0.47
Preeclampsia, *n/N* (%)	7 (12%)	4 (8%)	0.51
Chorioamnionitis, *n* (%)	19 (32%)	7 (14%)	0.03
Preterm premature rupture of membranes, *n* (%)	20 (34%)	12 (24%)	0.26
Maternal antibiotics, *n* (%)	35 (60%)	24 (49%)	0.24
Inclusion site, Stockholm/Linköping, *n/n* (%/%)	39/19 (67%/33%)	32/17 (65%/35%)	0.83
Antibiotics during the first week, *n* (%)	58 (98%)	49 (100%)	1.00
Antibiotics during the second week, *n* (%)	50 (86%)	40 (82%)	0.52
Days on antibiotics, mean (SD)	29 (14.5)	26 (14.5)	0.33
Betamethasone treatment, *n* (%)	14 (24%)	16 (33%)	0.33
Culture-proven sepsis, *n* (%)	20 (34%)	14 (29%)	0.51
Days on mechanical ventilation, mean (SD)	20 (19.3)	18 (14.4)	0.51
NEC, Bells stages II–III, *n* (%)	6 (10%)	4 (8%)	0.75
Death before 360 days, *n* (%)	2 (3.4%)	0 (0%)	0.50

*The Student's *t*-test was used to compare means, the Mann–Whitney test was used to compare skewed distributions, the chi-squared test was used to compare frequencies, or Fisher's exact test was used if the expected count was less than five.

active product. The intervention was started within 3 days after birth and continued until Gw36 + 0. The drops were administered through a gastric tube, by flushing down at least 0.3 mL of breast milk after the administration in the gastric tube or by mouth (when the nasogastric tube had been removed) but were withheld during the periods when infants were nil orally. The quality of the study product was checked regularly by the manufacturer, and the concentration of *L. reuteri* was within the stipulated limits in all batches used in the trial. The product was stored in a refrigerator in the pharmaceutical room at the neonatal ward and handled by nurses as a pharmaceutical product in compliance with the instructions of the manufacturer.

Isolation of PBMCs

Venous blood (0.5 mL whole blood) was collected in collection tubes containing heparin (BD Biosciences Pharmingen, San Jose, California) on three occasions: Day (D) 14, D28 and Gw36 (Figure 1a). Blood from 25 full-term (FT) infants (born between weeks 38 and 42) was also collected at 14 days of age to use as a control for the study (Figure 1a). Peripheral blood mononuclear cells (PBMCs) were isolated with gradient separation using Ficoll-Hypaque (GE Healthcare Biosciences AB, Uppsala, Sweden) as previously described.[16] Isolated PBMCs were gradually frozen in a freezing medium containing 40% RPMI-1640, 50% fetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO) (both from Sigma-Aldrich, St Louis, Missouri) in Nalgene cryotubes (Thermo Fisher) and stored in liquid nitrogen until further analysis.

In vitro stimulation of PBMCs

In this study, PBMCs from 107 EPT infants were used for stimulation. Frozen PBMCs were thawed gently and washed with RPMI-1640 supplemented with 20 mm HEPES (GE Healthcare Life Sciences). The cells were counted and checked for viability with Trypan Blue staining. Subsequently, the cells were resuspended in a concentration of 10^6 cells mL^{-1} in a cell culture medium, consisting of RPMI-1640 supplemented with 20 mm HEPES, 100 U mL^{-1} penicillin, 100 ug mL^{-1} streptomycin, 2 mm L-glutamate (2 mm; all GE Healthcare Life Sciences) and 10% heat-inactivated FCS (Sigma-Aldrich). The cells were then harvested in 96-well flat bottom cell culture plates (Corning Incorporated, Corning, NY) at a concentration of 5×10^5 cells in 200 μL volume per well followed by stimulation with the human T-cell activator CD3/CD28 beads (Gibco by Life Technologies) at 2:1 (cell: bead) ratio or kept unstimulated. Cells were incubated for 24 h at 37°C and 5% CO_2. After 24 h of incubation, supernatants were collected, centrifuged and stored at −70°C for later cytokine and chemokine analyses.

Luminex analysis for cytokines and chemokines

The levels of cytokines and chemokines were analysed using Millipore's Milliplex magnetic bead-based detection kits (EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer's instructions. The Milliplex Panel 1

(8 plex), Milliplex Panel 2 (30 plex), Milliplex Panel 3 (14 plex) and Milliplex Panel 4 (6 plex) were used for the analysis of a total of 58 analytes (Supplementary table 1). For Panel 4, the samples were diluted 1:100 in RPMI media with 10% FCS. To the plate, 25 μL of standard or control and culture supernatant from each respective infants were added to appropriate wells. Plates were analysed using a Luminex 200 analyser with the MasterPlex CT control software and the MasterPlex QT analysis software (MiraiBio, San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by the manufacturer. The medium control values were subtracted from the stimulated values.

Statistical analysis

Among 58 analytes, 16 cytokines/chemokines were excluded from the analyses because of the low proportion ($< 40\%$) of samples with higher levels than medium control after stimulation (Supplementary table 1). Thereafter, 42 cytokines and chemokines were considered for the analysis. Their levels at different time points in EPT infants were compared using the Kruskal–Wallis test. Correction for multiple comparisons across time points for each cytokine and chemokine was performed with the Benjamini–Hochberg false discovery rate (FDR) procedure, and corrected Q-values < 0.05 were considered significant for all the analyses. The analyte levels in EPT and FT infants at D14 were compared using Mann–Whitney U-tests. The results in the figures are displayed as scatter plots with bars showing medians and interquartile ranges. GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for preparing the figures and statistical analysis. For perinatal factors and the outcome of culture-proven sepsis in EPT infants, the Mann–Whitney U-test was performed with SPSS V25.

Principal component analyses (PCA) were performed to demonstrate the separation between groups. Data were $\log_{10}$-transformed, and the correlation matrices were analysed. Varimax rotation was used. The validity of the PCA was checked with the Kaiser–Meyer–Olkin measure of sampling adequacy and the Bartlett test of sphericity. The factor scores were used for the PCA scores plots, and the two principal components (PC) explaining the most variance are presented. The explained variances from the individual PCs are shown in parentheses in the figures. The PCA was performed in SPSS V25. The graphics for the score plots were designed in GraphPad Prism 9.

The MetaboAnalyst 5.0 software[51] for multivariate partial least squares discriminant analysis (PLS-DA), based on R version 4.0.2 (R plsr and R caret packages), was used as a supervised method to further optimise the separation between groups demonstrated by PCA. $\log_{10}$ transformation and Autoscaling (mean-centred and divided by the standard deviation of each variable) were used for normalisation of the data (Supplementary figure 7). The PLS-DA model was evaluated with cross-validation. Permutation tests (number of permutations: 1000) were performed to assess the significance of class discrimination, and P-values < 0.05 were regarded as significant. Important cytokines and chemokines identified with PLS-DA are depicted with VIP. VIP scores > 1.0 were considered significant in driving the separation. Mediators were ranked according to VIP scores, and the coloured boxes on the right in figures indicate the relative concentrations in each group.

Volcano plot and receiver-operating characteristic (ROC) curves for individual cytokines and chemokines were performed with the MetaboAnalyst 5.0 software. As above, $\log_{10}$ transformation and Autoscaling were used. For the volcano plot, the non-parametric Wilcoxon rank-sum test was used. The Benjamini–Hochberg FDR was set at 5%. The fold change (FC) threshold was set at 2.0. For the ROC curves, the area under the curve (AUC) with 95% confidence interval was calculated. P-values < 0.05 were regarded as significant. In all figures, the following abbreviations are used: FT D14 (samples from FT infants at D14 after birth), EPT infants and samples at D14, D28 and Gw36 (postmenstrual week 36 + 0).

CONFLICT OF INTEREST

Thomas Abrahamsson has received honoraria for lectures and a grant for the present trial from Biogaia AB. Maria Jenmalm has also received honoraria for lectures from Biogaia AB. Eva Sverremark-Ekström has received honoraria for lectures and a grant for another research project from BioGaia AB. All other authors declared no competing interests.

AUTHOR CONTRIBUTIONS

Dhanapal Govindaraj: Data curation; formal analysis; investigation; methodology; writing – original draft. **Georg Bach Jensen:** Data curation; formal analysis; investigation; writing – original draft. **Khaleda Rahman Qazi:** Formal analysis; investigation; methodology; writing – review and editing. **Eva Sverremark-Ekström:** Conceptualisation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; writing – review and editing. **Thomas Abrahamsson:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing. **Maria C Jenmalm:** Conceptualization; formal analysis; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.