Imbalance between inflammatory and regulatory cord blood B cells following pre-term birth

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Abstract

Preterm birth (PTB) is one of the most frequent pregnancy complications. It affects millions of babies each year worldwide and is associated with increased morbidity and mortality.

PTB-associated alterations in the maternal immune response may have a direct effect on the developing fetal immune system. Having recently shown that B regulatory (Breg) cells are decreased in number and functionally impaired in maternal blood from women delivering preterm, we now addressed the question whether the adaptive immune system is also altered in cord blood (CB) after the onset of PTB.

PTB was associated with increased concentrations of IL-6, TNF-α and IL-21 in CB and enhanced IL-6, but decreased IFN-γ and IL-4 in amniotic fluid (AF) samples compared to term delivery (TD). We found no differences in the frequency of CD19+ B cells, CD4+ T cells or CD4+Foxp3+CD25+ T regulatory (Treg) cells in CB cells in PTB vs TD. The frequency of CD86+ B cells was increased, while the percentage of $CD24^{hi}CD38^{hi}$CD19+ Breg and $CD1d^{hi}$CD5+ Breg cells and the ability of B cells to convert into Breg cells was diminished in PTB compared to TD. CB B cells from PTB secreted more IL-6, TNF-α, IL-9 and IL-2 compared to B cells obtained from term samples.

We conclude that, after PTB onset, a shift from immunoregulation towards inflammation takes place in CB cells that are reportedly representative of the fetal compartment. B cells have a substantial contribution herein. This phenomenon might account for the observed enhanced mortality and morbidity in prematurely born infants. Further studies will clarify how to employ this easy-to-obtain information for closely monitoring newborns at risk.

Keywords: preterm birth, B cells, pregnancy, interleukin-6, B regulatory cells

1. Introduction

Preterm birth (PTB) is defined as birth before 37 completed weeks of gestation. PTB is a syndrome with multiple etiologies. It might be the consequence of spontaneous onset of preterm labor or iatrogenic (Goldenberg *et al.* 2008). Inflammatory processes related to premature cervical ripening, premature rupture of membranes (PPROM) and infections often trigger PTB (Goldenberg *et al.* 2008).

Premature delivery is the most common cause of neonatal death worldwide. Surviving infants are at a greater risk to suffer from short- and long-term health problems than those born at term. Following delivery, PTB infants have an enhanced risk to develop infections (Picone *et al.* 2014, Steiner *et al.* 2019) and are prone to develop infectious and non-infectious respiratory syndromes and necrotizing enterocolitis. Long-term morbidity includes cerebral palsy, epilepsy, cognitive impairment, developmental co-ordination disorder, sensory impairment (hearing and visually impairment) and hypoxic ischemic encephalopathy (Platt 2014).

Maternal immunity plays a superordinate role in pregnancy. In order to support and nourish the semi allogeneic fetus, the maternal immune system first induces a pro-inflammatory environment that permits implantation and supports placentation. Later on, the immune system shifts to an anti-inflammatory, paternal-specific immunosuppressive stage in order to allow fetal growth, which is relevant to avoid immune activation in the uterus thus leading to an immune reaction towards the pregnant mother (Mold *et al.* 2008). At the end of pregnancy maternal immunity shifts back to a pro-inflammatory state to promote labor and birth (Mor *et al.* 2017). Meanwhile, the fetal immune system begins to develop and mature, with the subsequent appearance of macrophages, T cells, B cells, T regulatory cells (Treg) and B regulatory cells (Breg) between week 4 (macrophages) and 25 (Breg cells) of pregnancy (Zhang *et al.* 2017). The maturation of the fetal immune system continues after delivery and during infancy (Zhang *et al.* 2017).

Immune cells in cord blood (CB) differ from those found in adult blood (D'Arena *et al.* 1998, Paloczi 1999). Even if it is controversially discussed if all cells found in CB are of fetal origin, its composition is similar to that of peripheral blood of the fetuses during pregnancy (Mold *et al.* 2008) and with neonates shortly before and after normal birth (Mold *et al.* 2008) (Alexeev *et al.* 1996, Zamaraeva *et al.* 1997). In CB, the majority of the T cells are naïve, with only few activated and memory T cells (Nygaard *et al.* 2017), while Treg cells are present in higher frequency (Michaelsson *et al.* 2006). The number

of B cells in CB is increased compared to adult blood (Motley *et al.* 1996, Atanasova *et al.* 2014). The phenotype of B cell populations in CB also differ, the majority of B cells in CB express CD5 (Amu and Brisslert 2011, Chirumbolo *et al.* 2011, Lundell *et al.* 2015). Thus, CB predominately consists of immature B cells, mainly $CD24^{hi}CD38^{hi}$ transitional B cells (Glaesener *et al.* 2018). The frequency of $CD24^{hi}CD38^{hi}$ Breg cells in CB ranges between about 20% and 50% (Esteve-Sole *et al.* 2017, Lundell *et al.* 2017). In addition, CB naïve B cells exhibit the capacity to produce IL-10 (Esteve-Sole *et al.* 2017). This is indicative of their immunosuppressive or tolerogenic function. The frequency of memory B cells expressing CD27 is rare due to the fact that during pregnancy only exposition to maternal antigens occurs (Chirumbolo *et al.* 2011, Quinello *et al.* 2014). Moreover, compared to adults, the expression of B-cell receptors such as CD40, CD80 and CD86 is reduced, according to their immature or tolerogenic profile (Basha *et al.* 2014). Maturation and differentiation of B cells continues after birth. There is growing evidence that the phenotype of CB or fetal immune cells changes drastically in the event of PTB. Although the mechanisms underlying these changes are poorly understood, one can speculate that hormonal changes contribute to the phenotype shift, likewise the functional withdrawal of maternal progesterone that is linked to birth induction (Vannuccini *et al.* 2016, Mendelson *et al.* 2017) and the increase fetal cortisol due to the PTB-stress or due to corticosteroids administration for fetal lung maturation (Kemp *et al.* 2016). This has been recently well documented for T cells found in the amniotic cavity by Gomez-Lopez (Gomez-Lopez *et al.* 2019). However, so far no differences were reported for CB B cells among early PTB, late PTB and TD (Quinello *et al.* 2014). In addition, there is evidence suggesting that infections do not alter the number of B cells in the CB of premature newborns (Juretic *et al.* 2001). While the number of total B cells does not seem to be affected, it is unclear whether PTB is associated with alterations in the functionality of B cells or in the frequency of B cell subpopulations, such as Breg cells.

Here, we aimed to compare the frequency and distribution of B cell subpopulations present in CB from PTB with CB from term pregnancies with special focus on Bregs, since Bregs play an important role in the balance between immunity and tolerance during pregnancy (Busse *et al.* 2019, Busse *et al.* 2020). For this, we recruited PTB and TB patients that delivered in our clinic and only included samples from women that were not at labour, so that cytokine data may not be influenced by term-intrinsic inflammation.

We found that newborns that suffered from respiratory insufficiency and/or temperature regulation disorder had a decreased frequency of IL-10-producing CD1d^{hi}CD5+CD19+ cells. We conclude that, after PTB onset, a shift from immunoregulation towards inflammation takes place in CB cells that are reportedly representative of the fetal compartment. Our observational study in a small population comprising heterogenous PTB individuals, suggest that CB analysis has a great diagnostic potential and this should be further studied in bigger cohorts.

2. Materials and Methods

2.1 Study cohort

The Ethics Committee of the Medical Faculty, Otto-von-Guericke University approved the study (EK28/08). Written informed consent was obtained from all enrolled in the study. Patients were recruited between April 2016 and September 2018. The demographic data of the patients are summarized in Table 1. Nine women undergoing planned cesarean section (CS) at term (term delivery, TD; mean gestational age (GA): 38.44 weeks, none of them in labour) and eleven women delivering preterm, also via caesarean section (preterm birth, PTB; mean GA 33.0 weeks; $p<0.0001$), volunteered to participate in the study (Table 1A). The low number of women enrolled in the study reflects the fact that we did only include samples from donors/patients that were not in labor. Both groups did not differ in maternal age, pregnancy numbers, parity numbers, APGAR scores, cord blood pH or base excess. Babies born preterm had a decreased birth weight ($p<0.0001$), a decreased head circumference ($p=0.0010$) and a decreased body length ($p=0.0025$). Reasons for preterm birth included IUGR, PPROM and preterm labor (Table 1B).

Up to 40ml umbilical cord blood was taken immediately after delivery and stored on ice in tubes containing EDTA. Blood was processed within the following hour.

2.2 Stimulation of UCBMCs

Umbilical cord blood mononuclear cells (UCBMCs) were separated by Ficoll-density gradient centrifugation and cultured in RPMI1640, supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). To document cytokine secretion, $2x10^6$ UCBMCs/ml were stimulated with PMA (50ng/ml) and ionomycin (500 ng/ml; both Sigma Aldrich, Taufkirchen, Germany) alone or combined with LPS (10μg/ml; Sigma Aldrich, Taufkirchen, Germany) or CpG ODN2006 (10μg/ml; Invivogen, San Diego, USA) for 5h at 37°C and 5% CO_2. Brefeldin A (Biolegend, San Diego, USA) was added to all wells, including medium control.

To analyze the ability of B cells to differentiate into IL-10-secreting Breg cells, $2x10^6$ UCBMCs/ml were stimulated with LPS (10μg/ml) or CpG ODN2006 (10μg/ml) alone or combined with human CD40L (1μg/ml; R&D; Minneapolis, USA) for 48h at 37°C and 5% CO_2. PMA (50ng/ml), ionomycin (500 ng/ml) and Brefeldin A was added for the last 5h.

2.3 Isolation and stimulation of B cells

B cells were isolated using the human B cell isolation kit II (Miltenyi Biotech, Bergisch Gladbach, Germany) as indicated by the manufacturer. Isolated B cells were stimulated with LPS (10μg/ml) or CpG ODN2006 (10μg/ml) alone or combined with human CD40L (1μg/ml) for 72h (for recovery of supernatants) at 37°C and 5% CO_2.

2.4 Cell staining and flow cytometry

3x10^5 PBMCs were stained for cell surface markers for 30 min at 4°C. The following anti-human antibodies were used: FITC-labeled CD19 (clone HIB19), APC-labeled CD24 (clone eBioSN3), PE-Cy7-labeled CD38 (clone HB7), PE-labeled CD5 (clone UCHT2) and APC-labeled CD1d (clone 51.1). To analyze the intracellular expression of IL-10, cells were fixed for 30 min. with Fix and Perm and stained with PerCP-Cy5.5-labeled IL-10 (clone JES3-9D7; all reagents were purchased from ebioscience, Waltham, USA).

T cells were analyzed as follows: FITC-labeled CD4 (clone RPA-T4) and PerCP-Cy5.5-labeled CD25 (clone BC96) were stained at the cell surface. Following a fixation for 30 min. with Fix and Perm, intracellular staining of APC-labeled Foxp3 (clone 236A/E7; all reagents: ebioscience, Waltham, USA) was performed for 30 min at 4°C. Flow cytometry measurements were performed using LSR Fortessa (BD Biosciences, Heidelberg, Germany) and Attune NxT (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Data were analyzed with FlowJo software (Ashland, Oregon, USA).

2.5 Cytokine detection in plasma samples and supernatants of isolated B cell cultures

Cytokines were quantified by the cytometric bead array (CBA) human Th1/Th2/Th17 Cytokine Kit from Biolegend following supplier's recommendation and using the Attune NxT flow cytometer.

2.6 Data analysis and statistics

Statistical analysis was performed using GraphPad Prism 8.0 software. Normality of distribution was determined by Shapiro-Wilk test. Data were analyzed by Mann-Whitney-U test or Student´s t test or Two-way ANOVA, followed Sidak's multiple comparison test when the column factor determined significance. A Spearman analysis was performed to determine a possible correlation between gestational week and

cytokine levels in amniotic fluid and CB. Significance was confirmed when p values were as follows: $p<0.05$ (*), $p<0.005$ (**), $p<0.0005$ (***) or $p<0.0001$ (****)

3. Results

3.1 PTB is associated with alterations in cytokine levels in cord blood and amniotic fluid

First, we wanted to determine whether PTB alters the cytokine profile in amniotic fluid (AF) and CB plasma. At the time CS was performed, no woman was in active labour. PTB cases that presented at the hospital with labour received the tocolytic drugs Partusisten (Fenoterol) or Tractocile (Atosiban) to prevent premature delivery while facilitating lung maturation using betamethasone. AF was collected during CS and CB was obtained immediately after delivering. The IL-6 level was also enhanced in the AF from PTB patients ($p=0.0121$; Fig. 1A) vs the TD women, while the levels of IFN-γ ($p=0.0455$; Fig. 1B), IL-4 ($p=0.0356$; Fig. 1C) and IL-17F ($p=0.0273$; Suppl. Table 1A) were lower in PTB AF samples as compared to TD AF. We detected increased CB plasma levels of IL-6 ($p=0.0392$; Fig. 1D and Suppl. Table 1B), TNF-α ($p=0.0135$; Fig. 1E) and IL-21 ($p=0.0058$; Fig. 1F) in CB from PTB as compared to TD CB. A further analysis revealed that the gestational week correlated with the levels of these cytokines (Fig. 1G).

3.2 Dysregulated Breg cell numbers in PTB

Next, we quantified the frequency of B and T cell subpopulations in both groups, PTB and TD. The frequency of CD19+ B cells was unaffected by the occurrence of PTB (Fig. 1H), while the percentage of $CD24^{hi}CD38^{hi}$ Breg cells was decreased in PTB, albeit not statistically significant ($p=0.1060$; Fig. 1I) compared to TD. The frequency of $CD1d^{hi}CD5+$ Breg cells in PTB CB was significantly diminished compared to TD CB ($p=0.0252$; Fig. 1J). No alterations were detected in the expression of the co-stimulatory molecule CD80 in B cells (Fig. 1K), but more CB B cells expressed CD86 in PTB samples compared to TD CB ($p=0.0332$; Fig. 1L). Neither the frequency of total CD4+ T cells (Fig. 1M) nor the percentage of Treg cells within this population (Fig. 1N) were different in CB from PTB newborns compared to TD CB. In addition, the concentration of progesterone (P4) did not differ between PTB and TD CB (Fig. 1O).

3.3 B cells from PTB CB samples secreted mainly pro-inflammatory cytokines

Next, we isolated B cells from CB of TD and PTB patients and cultured them for 72h in the presence or absence of LPS or CpG, alone or combined with CD40L to analyze

the cytokines secreted to the culture supernatant. Several cytokines were differentially secreted in PTB samples, namely increased compared to samples from TD, among them IL-2 ($p=0.0138$), IL-4 ($p=0.0209$), IL-6 ($p<0.0001$), IL-9 ($p=0.0044$), IFN-γ ($p=0.0384$) and TNF-α ($p=0.0002$; Suppl. Table 2). Further analysis indicated that B cells present in CB from PTB patients secreted higher amounts of IL-6 following stimulation with LPS ($p=0.0025$) as well as LPS+CD40L ($p=0.0066$) compared to TD CB B cells (Fig. 2A). In LPS-treated B cells from CB, the concentrations of TNF-α ($p=0.0031$; Fig. 2B) and IL-9 ($p=0.0269$; Fig. 2C) were higher compared to TD. In addition, the concentration of IL-2 was higher in CpG+CD40L-treated B cells from CB compared to TD CB B cells ($p=0.0349$; Fig. 2D).

3.4 Both, the frequency of Breg cells and the conversion of B cells into Breg cell populations is disturbed in PTB

Next, we addressed the question whether the pro-inflammatory B cell profile observed after the onset of PTB is associated with a decreased frequency of Breg cells and the inability of B cells to convert into Breg cells. To quantify the percentage of B cells able to produce IL-10 directly *ex vivo*, UCBMC were stimulated for 5h and the percentages of two different IL-10-producing Breg subsets, namely $CD24^{hi}CD38^{hi}$ or $CD1d^{hi}CD5+$ Breg cells were documented. The stimulation protocol was adopted according to the suggestions of Iwata et al. (Iwata *et al.* 2011) who used PMA/ionomycin/Brefeldin A (PIB) alone or combined with the TLR agonists LPS or CpG. We found a lower percentage of IL-10+$CD24^{hi}CD38^{hi}$CD19+ Breg cells ($p=0.0210$; Fig. 3A) and IL-10+$CD1d^{hi}$CD5+CD19+ Breg cells ($p=0.0019$; Fig. 3B), with diminished IL-10 expression in samples from PTB compared with TD following PMA and ionomycin stimulation ($p=0.0315$).

Based on the results published by Iwata et al.(Iwata *et al.* 2011) who stimulated CB cells with several TLR agonists for 48h and detected that LPS or CpG alone or combined with and CD40L induced the highest frequencies of IL-10+ B cells, we used this protocol to determine the ability of CB B cells from both groups to convert into IL-10-secreting Breg cells. The frequency of IL-10+$CD24^{hi}CD38^{hi}$CD19+ Breg cells ($p=0.0005$; Fig. 3C) was diminished in CB samples from PTB following stimulation with LPS + CD40L ($p=0.0308$) or CpG ($p=0.0111$) when compared to CB samples obtained from TD. PTB CB IL-10+$CD1d^{hi}$CD5+CD19+ Breg cells ($p=0.0001$; Fig. 3D) were

detected at a lower percentage in unstimulated (p=0.0410) and CpG stimulated (p=0.0354) conditions compared to what CB obtained following TD.

3.5 Association between Breg cell values and development of complications in the newborns

Term and preterm born infants differed in several parameters, such as the length of stay in hospital, the occurrence of respiratory insufficiency (RI) and problems to regulate their body temperature (temperature regulation disorder, TRD) following birth (Fig. 4A). We next tested whether the presence of these health problems is associated with the frequency of IL-10-producing Breg cells. We found that the occurrence of RI was associated with a decreased percentage of IL-10+CD1d^{hi}CD5+CD19+ Breg following 5h stimulation with PIB (Fig. 4B) and 48h after CpG+PIB (Fig. 4C). Both cell populations were also decreased in newborns suffering from TRD (Fig. 4D and Fig. 4E).

4. Discussion

We have recently unveiled that preterm birth (PTB) is associated with an enhanced proportion of inflammatory B cells while the frequency of Breg cells in maternal blood is reduced (Busse *et al.* 2020). In addition, Breg from patients undergoing PTB were impaired in their IL-10 production (Busse *et al.* 2020). Here, we show that the levels of pro-inflammatory cytokines are increased in cord blood (CB) obtained after PTB when compared to CB samples from TD patients. Additionally, we provide evidence that B cells from CB are responsible for the secretion of pro-inflammatory cytokines in the onset of PTB. The frequency of Breg cells producing IL-10 and the potential of B cells to convert into Breg cells was significantly reduced in the event of premature delivery. One of the most devastating disorders associated with PTB is neonatal sepsis or FIRS (fetal inflammatory response syndrome). The capacity to produce IL-10 is reduced in neonates compared to adults, which may result in a predisposition for organ damage in severe sepsis (Schultz *et al.* 2004). In this clinical picture, IL-10 might serve as a promising diagnostic marker (Sherwin *et al.* 2008, Zeitoun *et al.* 2010). Preterm infants with a low birth weight have a 3–10 times higher incidence of infection than infants born full-term and with normal birth weight (Shane *et al.* 2017). The relevance of B cells is underestimated. An imbalance between pro-inflammatory B cells and immunoregulatory Breg cells, or the interruption of transplacental maternal IgG passage might increase infection the risk of infection. The aim of our study was to evaluate the B cell status in CB from PTB neonates compared to TB. Furthermore, we analyzed cytokine secretion by B cells and their ability to convert into Breg. We sought to understand whether the data obtained using CB, an easily available sample at the earliest time point after birth, may be predictive of neonatal complications related to inflammatory conditions.

Although the causes of PTB in our study are multifactorial and the studied samples rather few, an inflammatory profile at the maternal-fetal interface, reflected by the increased production of pro-inflammatory cytokines, is a common link for several triggers of PTB. We detected enhanced level of IL-6, TNF-α and IL-21 in PTB CB. We and others previously found that these cytokines were also increased in maternal blood from women undergoing PTB (Shahshahan and Hashemi 2014, Herrera-Munoz *et al.* 2017, Park *et al.* 2018, Busse *et al.* 2020). In CB, enhanced levels of pro-inflammatory cytokines correspond with increased morbidities in PTB newborns (Torbe *et al.* 2007). Recently, Enhanced IL-6 and TNF-α in CB from preterm newborns was reported

(Frascoli *et al.* 2018). The level of IL-6 in CB was significantly higher in PTB patients suffering from early-onset neonatal sepsis than those without sepsis as well as following PPROM or PTB (Cernada *et al.* 2012, Steinberger *et al.* 2014). However, the presence of chorioamnionitis, present in the same percentage in PPROM and in spontaneous PTB cases (Yuce *et al.* 2014) and the way of delivery (Singh *et al.* 1996; Trevino-Garza *et al.* 2016) might also influence the CB IL-6 level. Labour was associated not only with enhanced IL-6, but also IL-8 and IL-10 in CB in low-risk term pregnancies (Chan *et al.* 2013). This point has to be taken into account when analysing data sets like ours since many PTBs were associated with preterm labour albeit having a CS, but planned term deliveries by CS occurred without spontaneous labour. We included samples from patients whose labour has been avoided by the use of tocolytics. IL-21, an inflammatory cytokine, the most influential cytokine for human B cells, can induce the activation and drive the differentiation of CB B cells into antibody-secreting plasma cells (Ettinger *et al.* 2005). We found enhanced IL-21 levels in PTB CB, suggesting that PTB B cells are more prone to be activated towards plasma cells than those of TD.

Here, we found that IL-6 was enhanced in AF obtained from PTB patients, while IFN-γ and IL-4 were decreased. Son et al. suggested that IL-6 in AF might be a good marker for predicting a very early PTB in women with cervical insufficiency (Son *et al.* 2016). Gomez-Lopez et al. reported elevated IL-6 in AF in cases of suspected intraamniotic infection and/or inflammation (Gomez-Lopez *et al.* 2017). The pro-inflammatory cytokine IFN-γ, mainly produced by NK cells and T cells, was not elevated in AF in patients suffering from chorioamnionitis (Revello *et al.* 2016). Interestingly, it has been shown that preterm infants overexpressed genes that are involved in the negative regulation of IFN-γ production (Olin *et al.* 2018). Moreover, IFN-γ was detected in AF of both labouring women and elective CS at term (Olah *et al.* 1996), suggesting that this cytokine is important in pregnancy and delivery but not at all indicative of inflammation related to premature delivery. IFN-γ increase as pregnancy progresses and is elevated around term and therefore decreased in AF samples of the PTB patients included in our study compared to TD. We found that the anti-inflammatory cytokine IL-4 was also diminished in the AF of PTB patients. Higher IL-4 levels were observed in AF of patients delivering at term with a planned CS that were not in labour when compared to patients at term in labour (Korkmaz *et al.* 2017), indicating that labour, mediated by a shift from anti- to pro-inflammation, decreased the levels of IL-

4. However, other reports indicate that women with infection-associated preterm labour and delivery had higher level of IL-4 in AF compared to controls (Dudley *et al.* 1996). Infection was rare in our study cohort and detected in only two PTB babies. Therefore, we decided to investigate whether altered frequencies of IL-10-producing CD1d^{hi}CD5+CD19+ Breg cells are associated with other morbidities, which are most common in newborns (Gouyon *et al.* 2012). We found decreased percentages of IL-10+CD1d^{hi}CD5+CD19+ Breg cells in respiratory insufficiency and thermal instability. According to a previous study, stress contributes to PTB, and PTB is associated with postnatal stress (Goedicke-Fritz *et al.* 2017) and might lead to inflammation and infection (Shapiro *et al.* 2013). In healthy term newborns, inflammatory cytokine responses towards an inflammatory insult are attenuated, while the anti-inflammatory responses are stronger (Kannan 2013). This regulation system might still not be present or active in PTB infants. In mice, stress reduced B and T cells, Treg cells as well as B1 cells, which are known to secrete IL-10 (Garcia-Flores *et al.* 2020). Whether such mechanisms exist also in humans remains to be clarified. Hypothermia was shown to enhance the production of pro-inflammatory cytokines by CB macrophages (Fairchild *et al.* 2000). Moreover, hypothermia might be associated with systemic immunosuppression (Jenkins *et al.* 2013).

We next addressed the question whether the obvious proinflammatory profile in PTB samples influences the frequency of immunoregulatory cell populations, in particular Treg and Breg cells. We previously found no differences in the frequency of CD4+ T cells and CD4+ Treg cells in maternal blood (Busse *et al.* 2020). Here, we observed that none of these cell populations was altered in CB in PTB samples. Contradictory results have been published so far (Dirix *et al.* 2013, Zahran *et al.* 2019). Here, the frequency of total B cells in CB was not altered by PTB, and these results go in hand with previously reported data by other colleagues (Juretic *et al.* 2000, Walker *et al.* 2011). In addition, it was determined that following the adjustment of factors such as the gestational age, the presence of neonatal infection, preeclampsia, betamethasone therapy or the mode of delivery, no differences in the number of CD19+ and CD20+ B cells were detected between PTB and TD (Kotiranta-Ainamo *et al.* 1999). However, lower B cell numbers in CB were determined in IUGR and enhanced the risk of IUGR-associated PTB (Xiong *et al.* 2012) and decreased CD5+CD19+ B cell counts in CB were associated with preeclampsia (Kotiranta-Ainamo *et al.* 1999). Although the absolute B cell number was not influenced, B cell subpopulations were affected by

PTB in our study. Based on $CD5^{hi}$ staining, Zhivaki et al. described so-called “nBreg” cells, which were more abundant in preterm and term CB than in adult blood, with more $CD5^{hi}$ nBreg cells present between gestational weeks 24-30 and gestational week 38 (Cerbulo-Vazquez *et al.* 2003). We detected a decreased number of $CD24^{hi}CD38^{hi}$ and $CD1d^{hi}$CD5+ Breg cells in CB following preterm delivery compared to full-term birth. A reason for that might be the development of Breg cells which begins around gestational week 25 and continues after birth (Zhang *et al.* 2017), therefore in PTB the abundancy in Breg cells was not reached. Another reason might be that the inflammatory pathway started too early because of PTB and results in a dysregulation of fetal Breg development in favor of pro-inflammatory B cells and at the expense of Breg cells.

One of the most important mechanisms that immunoregulatory cell populations employ is the secretion of IL-10. In pregnancy, constantly rising progesterone concentrations induce IL-10 production (Druckmann and Druckmann 2005) . Here, we detected no differences in the progesterone concentration in CB between PTB and TD, confirming results by others (Travers *et al.* 2018). Lee et al. demonstrated that progesterone promoted the differentiation of CB T cells into immunoregulatory Treg cells and further suppressed the generation of pro-inflammatory Th17 cells (Lee *et al.* 2011). In our study, we did not find differences in the frequency of Treg cells in CB between PTB and TD. We detected no alterations in IL-10 levels in CB or AF. However, after 5h or 48h culture, we could show that the B cell-specific IL-10 production and secretion as measured by flow cytometry (5h) or in supernatant (72 h) was diminished in B cells from PTB patients compared to TD. We used two different TLR agonists to stimulate fetal CB B cells, namely LPS and CpG. Both stimulants are known to induce Breg cells out of B cells (Iwata *et al.* 2011). As for the physiological relevance of using LPS or CpG to stimulate cells obtained from CB, Martinez et al. described higher concentrations of LPS in CB from PTB compared to full-term infants and correlated with the gestational age, birth weight, the CRP level and the presence of chorioamnionitis (Martinez-Lopez *et al.* 2014).

Besides TLR agonists, we added CD40L to the cultures with LPS or CpG. CD40 is the most important co-stimulatory molecule in B cells which becomes activated following binding to CD40L (Bishop 2004). The expression of CD40L was increased in CB platelets from PTB with underlying chorioamnionitis, associated with enhanced IL-6 (Sitaru *et al.* 2005). This indicates that other blood cells which were activated by the

intrauterine infection might also trigger the activation of B cells to secrete differentially IL-6 and less IL-10.

The ability of B cells to differentiate into pro-inflammatory or immunoregulatory B cells might be influenced by the pre-existing immune-stimulatory conditions: In the event of PTB, stimulation with LPS or CpG, alone or combined with CD40L, in already enhanced CB level of inflammatory mediators might further drive inflammation, suppressing the adequate conversion of B cells into of Breg cells.

We found that CB B cells from PTB had a higher expression of CD86 compared to term CB B cells, while the expression of CD80 remained unaltered. The co-stimulator CD86 is an activation-induced molecule, expressed by several B cell populations. A human antigen-presenting B cell subset with strong immunostimulatory properties is characterized by CD86 expression (Shimabukuro-Vornhagen *et al.* 2017). However, other reports indicate the presence of CD86 in Breg cells (Gallego-Valle *et al.* 2018) and it is proposed to be important for their suppressive capacity (Blair *et al.* 2010). Studies concerning CD86 expression after the occurrence of PTB are rare but it was shown that maternal dendritic cells from PTB CB have an enhanced CD86 expression (Frascoli *et al.* 2018). This was interpreted as an early activation of dendritic cells, resulting in the priming of T cells and their differentiation into effector T cells that in turn culminates in the breakdown of feto-maternal tolerance. Whether similar mechanisms exist for B cells and which functions CD86-positive B cells exert in PTB remains to be elucidated. The same is true for the question whether this mechanism is present at the maternal or fetal side.

Immune mechanisms relevant for PTB are mostly studied employing maternal material. In the present study, we concentrated on the fetal immune response employing CB obtained during birth that is representative of the peripheral blood composition of the newborn. Our results show the presence of inflammatory cytokines in CB from premature newborns. The total number of B cells was comparable between pre-term and controls but the numbers of the immunosuppressive population of Breg cells was diminished. Even though the number of included samples is rather low and the PTB cohort heterogeneous, a limitation we are aware of, the stringent differences between PTB and TB points out to the relevance these cells may have. The in vitro culture of B cells under different stimuli revealed that, in PTB, they have an inflammatory functional profile and are not prone to convert to Breg as cells from

controls do. These data reveal that B cells and in particular Breg cells are a central component of the fetal immune response and that, similar as in pregnant women, their function might be the maintenance of the balance between immunity and tolerance. Despite the fact that only two infants in our study developed an infection, other inflammatory disorders typically found in preterm born babies were associated with decreased Breg frequencies. These interesting results enable us to speculate that the functional characterization of CB immune cells may be a useful and quick tool to predict the risk of a premature newborn to develop infections because of their unbalanced proportion between Beff and Breg cells and the inability of B cells to convert into Breg. For this, studies recruiting a much larger number of samples and a narrower dissection of PTB ethiology is urgently needed. Identifying newborn babies with a risk to develop infections by just studying the composition of CB and performing *in vitro* non-invasive assays may help clinicians to better and more closely monitoring the newborns, prevent infections and reduce mortality.

Declaration of Interest

Funding

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Figure legends

Figure 1:

Cytokine levels in amniotic fluid (AF) and cord blood (CB) plasma and CB B cell and T cell frequencies and progesterone concentration at term and preterm

AF was obtained during Caesarean section; CB plasma was taken immediately after birth. Cytokine levels were determined using a Th panel multiplex bead-based assay. Shown are the level of AF IL-6 (A), IFN-γ (B) and IL-4 (C) and CB IL-6 (D), TNF-α (E), IL-21 (F). Cytokine levels changed depending on gestational week (G). The percentage of CD19+ B cells (H), $CD24^{hi}CD38^{hi}$CD19+ Breg cells (I), $CD1d^{hi}$CD5+CD19+ Breg cells (J), CD80+CD19+ B cells (K), CD86+CD19+ B cells (L), CD4+ T cells (M) and CD4+CD25+Foxp3+ Treg cells (N) as well as the level of Progesterone (P4; ng/ml; O) was determined in CB obtained following term or preterm delivery. Following Shapiro-Wilk test, data were analyzed either by Student´s t test or by Mann-Whitney test and presented as mean values and SD; *p<0.05; **p<0.005.

Figure 2:

Cytokine secretion by cord blood (CB) B cells from term and preterm patients

B cells were isolated from TD and PTB CB and stimulated with LPS or CpG alone or combined with CD40L for 72h. The supernatant was harvested and cytokine levels were determined using a Th panel multiplex bead-based assay. Mean values and SD from IL-6 (A), TNF-α (B), IL-9 (C) and IL-2 (D) are depicted. Data were analyzed by Two-way ANOVA with Sidak's multiple comparisons test; *p<0.05; **p<0.005.

Figure 3:

Regulatory B cell populations in term and preterm cord blood (CB)

Umbilical cord blood mononuclear cells (UCBMCs) were obtained following delivery and stimulated for 5h in the presence of Brefeldin A alone ("Medium") or combined with PMA and ionomycin, partly added by LPS or CpG. Intracellular IL-10 production by $CD38^{hi}CD24^{hi}$CD19+ Breg cells (A) or $CD1d^{hi}$CD5+CD19+ Breg cells (B) was determined by flow cytometry. Data were analyzed using Two-way ANOVA with Sidak´s multiple comparisons test. Shown are mean values and SD. TD= term delivery; PTB= preterm birth; *p<0.05.

The maturation of IL-10-producing $CD38^{hi}CD24^{hi}$CD19+ Breg cells (C) and $CD1d^{hi}$CD5+CD19+ Breg cells (D) was analyzed following culturing in medium alone or following stimulation of UCBMCs with LPS or CpG alone or combined with CD40L for 48h. For the last five hours, Brefeldin A PMA and ionomycin were added to the cultures and cells were analyzed by flow cytometry. Data were evaluated using Two-way ANOVA with Sidak's multiple comparisons test, data is shown as mean and SD values. TD= term delivery; PTB= preterm birth; *p<0.05.

Figure 4:

Association between the development of complications in newborns and Breg populations

Birth-associated parameters including length of stay in hospital, the occurrence of respiratory adjustment disorder, respiratory insufficiency (RI), problems to regulate their body temperature (temperature regulation disorder, TRD), hyperbilirubinia and the occurrence of an infection were obtained in term and preterm born infants (A). An association between the occurrence of RI and a decreased percentage of IL-10+$CD1dh^{i}$CD5+CD19+ Breg following 5h stimulation with PIB (B) and 48h after CpG+PIB (C) and between the presence of TRD and a decreased percentage of IL-10+$CD1d^{hi}$CD5+CD19+ Breg following 5h stimulation with PIB (D) and 48h after CpG+PIB (E) was determined. Data were analyzed by the Mann-Whitney test and are shown as mean and SD values; *p<0.05.

Table 1:

Study cohort

Nine cord blood (CB) samples obtained at term (TD= term delivery) and eleven CB samples following preterm delivery (preterm birth, PTB) were included in the study. Maternal characteristics included age of the mother, gestational age (GA; weeks), number of pregnancies and parities. Neonatal features included birth weight (grams, g), head circumference (cm), body length (cm), APGAR scores at 1min., 5 min. and 10min. after birth, the CB pH value and CB base excess (A). The underlying individual diagnosis of the eleven PTB cases are shown in (B). PPROM= preterm premature rupture of membranes; IUGR= Intrauterine growth restriction

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Table 1

A

characteristics	TL N=9	sPTB N=11	p
maternal			
age (years)	31.44±6.444	28.55±3.446	0.2512
GA (weeks)	38.44±1.509	33.00±2.490	<0.0001
pregnancy	2.889±1.167	3.364±3.171	0.5254
parity	2.444±1.236	2.273±1.555	0.5893
neonatal			
birth weight (g)	3428±566	1896±447	<0.0001
head circumference (cm)	35.17±1.323	30.04±2.504	<0.0001
body length (cm)	50.67±3.041	42.88±3.852	0.0001
APGAR 1min.	8.875±0.641	7.846±1.519	0.1007
APGAR 5min.	9.500±1.069	9.077±0.954	0.1947
APGAR 10min.	9.750±0.707	9.615±0.507	0.3359
pH (cord blood)	7.319±0.038	7.343±0.052	0.1547
base excess	-1.363±1.390	-1.400±1.393	0.8181
gender (female)	33.3%	53.85%	0.4149

B

PTB case #	diagnosis
1	PPROM; preterm labour; IUGR
2	PPROM; preterm labour
3	PPROM; preterm labour
4	PPROM; preterm labour
5	PPROM; preterm labour
6	PPROM; preterm labour
7	preterm labour; placental insufficiency
8	IUGR; preterm labour
9	IUGR; preterm labour
10	IUGR; gestational diabetes
11	preterm labour

Study cohort
Nine cord blood (CB) samples obtained at term (TD= term delivery) and eleven CB samples following preterm delivery (preterm birth, PTB) were included in the study. Maternal characteristics included age of the mother, gestational age (GA; weeks), number of pregnancies and parities. Neonatal features included birth weight (grams, g), head circumference (cm), body length (cm), APGAR scores at 1min., 5 min. and 10min. after birth, the CB pH value and CB base excess (A). The underlying individual diagnosis of the eleven PTB cases are shown in (B). PPROM= preterm premature rupture of membranes; IUGR= Intrauterine growth restriction

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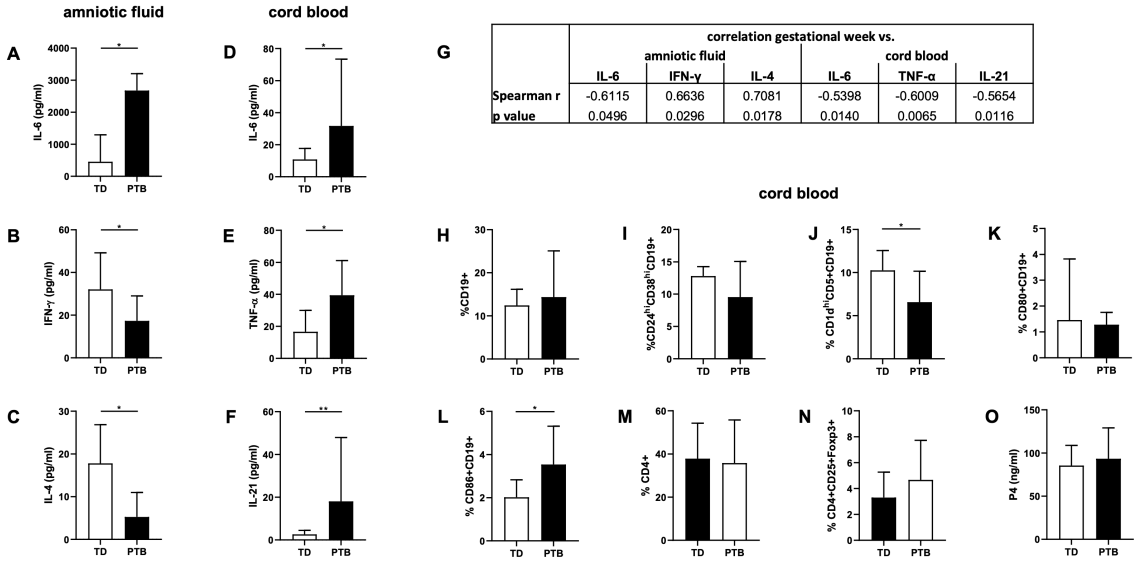
Figure 1



G

	correlation gestational week vs.					
	amniotic fluid			cord blood		
	IL-6	IFN-γ	IL-4	IL-6	TNF-α	IL-21
Spearman r	-0.6115	0.6636	0.7081	-0.5398	-0.6009	-0.5654
p value	0.0496	0.0296	0.0178	0.0140	0.0065	0.0116

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Figure 2

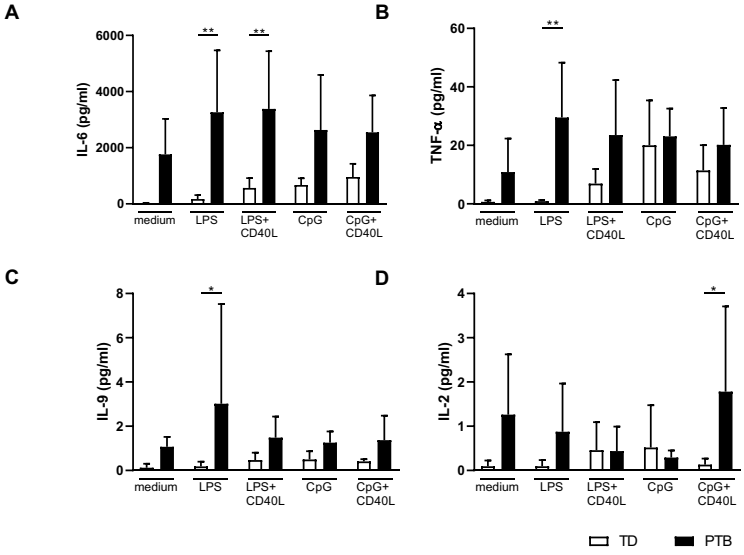

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Figure 3

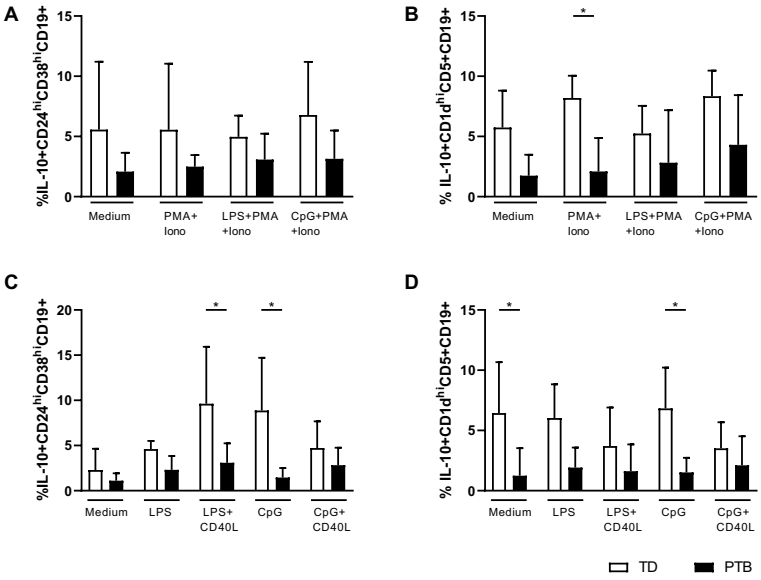

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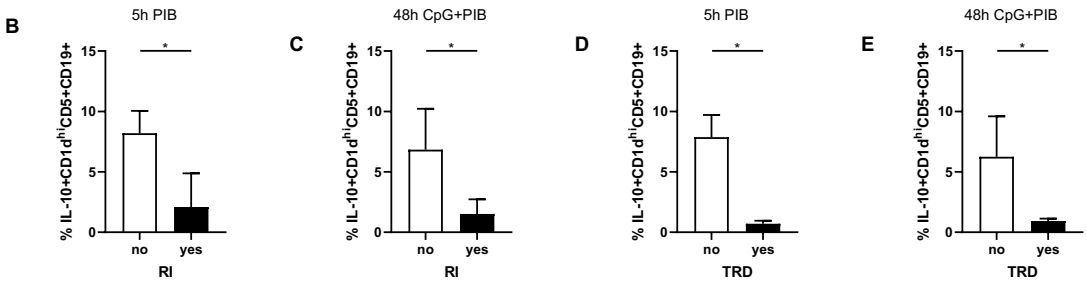
Figure 4

A

characteristics	TD	PTB	p
length of stay in hospital (days)	3.667 ± 1.000	23.38 ± 15.24	<0.0001
Respiratory adjustment disorder	11.1%	30.8%	0.2790
Respiratory insufficiency	0	46.2%	0.0169
Temperature regulation problem	11.1%	69.2%	0.0071
Hyperbilirubinia	11.1%	38.5%	0.1567
Infection	0	15.4%	0.2172



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Supplementary Table 1

A

amniotic fluid			
cytokine	**TD**	**PTB**	***p***
IL-2	2.021 ± 1.927	0.725 ± 0.849	0.2412
IL-4	17.79 ± 9.045	5.255 ± 5.734	0.0356 (*)
IL-5	20.89 ± 20.59	7.185 ± 8.7	0.2441
IL-6	453.0 ± 839.8	2675 ± 528.7	0.0121 (*)
IL-9	9.723 ± 12.35	3.190 ± 3.536	0.8273
IL-10	7.802 ± 7.090	2.981 ± 3.019	0.2351
IL-13	4.239 ± 5.229	1.863 ± 2.528	0.4224
IL-17A	2.791 ± 2.693	0.749 ± 0.501	0.1788
IL-17F	2.466 ± 1.886	0	0.0273 (*)
IL-21	4.643 ± 3.072	14.48 ± 20.70	0.5606
IL-22	2.205 ± 2.738	0.329 ± 0.480	0.1970
IFN-γ	32.30 ± 17.19	17.29 ± 11.74	0.0455 (*)
TNF-α	12.26 ± 18.56	1.167 ± 0.999	0.5212

B

cord blood			
cytokine	**TD**	**PTB**	***p***
IL-2	1.936 ± 1.424	2.261 ± 3.087	0.6920
IL-4	7.319 ± 5.379	13.11 ± 9.928	0.1346
IL-5	22.69 ± 12.21	20.16 ± 21.15	0.7546
IL-6	10.83 ± 6.831	31.76 ± 41.65	0.0392 (*)
IL-9	8.090 ± 7.652	9.722 ± 10.87	>0.9999
IL-10	4.582 ± 2.851	4.644 ± 4.650	0.9722
IL-13	5.584 ± 4.073	6.721 ± 6.046	0.6364
IL-17A	1.848 ± 1.085	2.201 ± 2.906	0.7098
IL-17F	1.214 ± 1.886	0.741 ± 1.517	0.7267
IL-21	2.628 ± 1.847	18.07 ± 29.85	0.0058 (**)
IL-22	2.218 ± 1.576	2.337 ± 1.897	0.8822
IFN-γ	24.24 ± 15.25	38.63 ± 23.67	0.1333
TNF-α	16.62 ± 13.33	39.38 ± 21.76	0.0135 (*)

Cytokines in term and preterm cord blood and amniotic fluid
The cytokine levels from term (TD= term delivery) and preterm delivering (preterm birth, PTB) patients in CB (A) and AF (B) are shown including mean values and SD. Following Shapiro-Wilk test, data were analyzed either by Student´s t test or by Mann-Whitney test; *p<0.05; **p<0.005.

Supplementary Table 2

A

CB B cells	
cytokine	***p***
IL-2	0.0138 (*)
IL-4	0.0209 (*)
IL-5	0.1518
IL-6	<0.0001 (****)
IL-9	0.0044 (*)
IL-10	0.1334
IL-13	0.0594
IL-17A	0.1223
IL-17F	0.2283
IL-21	0.3234
IL-22	0.1611
IFN-γ	0.0384 (*)
TNF-α	0.0002 (***)

B

	IL-2	
	TD	PTB
Medium	0.095 ± 0.013	1.261 ± 1.363
LPS	0.095 ± 0.139	0.875 ± 1.090
LPS+CD40L	0.459 ± 0.633	0.438 ± 0.554
CpG	0.519 ± 0.957	0.293 ± 0.160
CpG+CD40L	0.133 ± 0.136	1.783 ± 1.925

	IL-4	
	TD	PTB
Medium	0.054 ± 0.066	0.281 ± 0.191
LPS	0.099 ± 0.103	0.265 ± 0.216
LPS+CD40L	0.208 ± 0.191	0.265 ± 0.152
CpG	0.151 ± 0.143	0.172 ± 0.133
CpG+CD40L	0.174 ± 0.178	0.350 ± 0.375

	IL-5	
	TD	PTB
Medium	0.329 ± 0.670	2.413 ± 2.177
LPS	3.015 ± 5.998	84.47 ± 185.7
LPS+CD40L	1.621 ± 1.382	58.63 ± 126.8
CpG	0.305 ± 0.615	5.090 ± 8.143
CpG+CD40L	0.280 ± 0.627	2.047 ± 1.720

	IL-6	
	TD	PTB
Medium	17.36 ± 17.69	1762 ± 1265
LPS	174.3 ± 136.5	3263 ± 2203
LPS+CD40L	568.6 ± 346.7	3385 ± 2051
CpG	673.3 ± 237.2	2633 ± 1958
CpG+CD40L	958.3 ± 470.1	2548 ± 1314

	IL-9	
	TD	PTB
Medium	0.126 ± 0.174	1.068 ± 0.452
LPS	0.186 ± 0.209	3.020 ± 4.507
LPS+CD40L	0.473 ± 0.328	1.482 ± 0.955
CpG	0.498 ± 0.370	1.256 ± 0.509
CpG+CD40L	0.408 ± 0.103	1.372 ± 1.105

	IL-10	
	TD	PTB
Medium	19.04 ± 22.03	0.083 ± 0.122
LPS	81.17 ± 175.4	1.427 ± 1.746
LPS+CD40L	17.34 ± 15.96	34.91 ± 22.90
CpG	92.75 ± 148.0	33.10 ± 11.63
CpG+CD40L	207.3 ± 233.4	101.4 ± 145.3

	IL-13	
	TD	PTB
Medium	0.084 ± 0.127	0.775 ± 0.762
LPS	0.233 ± 0.441	6.430 ± 12.74
LPS+CD40L	0.326 ± 0.517	4.766 ± 9.002
CpG	0.176 ± 0.394	1.0345 ± 0.999
CpG+CD40L	0	1.453 ± 1.223

	IL-17A	
	TD	PTB
Medium	0.010 ± 0.013	0.201 ± 0.288
LPS	0.006 ± 0.011	1.785 ± 3.977
LPS+CD40L	0.001 ± 0.003	1.697 ± 3.721
CpG	0.019 ± 0.025	0.040 ± 0.058
CpG+CD40L	0.090 ± 0.060	0.271 ± 0.547

	IL-17F	
	TD	PTB
Medium	0.092 ± 0.088	2.561 ± 4.457
LPS	0.098 ± 0.079	62.22 ± 138.6
LPS+CD40L	0.189 ± 0.191	11.95 ± 26.21
CpG	0.088 ± 0.091	0.483 ± 0.588
CpG+CD40L	0.138 ± 0.122	0.591 ± 0.618

	IL-21	
	TD	PTB
Medium	3.291 ± 3.093	6.478 ± 1.441
LPS	5.394 ± 1.317	3.734 ± 3.264
LPS+CD40L	5.643 ± 3.118	5.746 ± 3.196
CpG	5.490 ± 1.424	79.63 ± 164.4
CpG+CD40L	5.847 ± 0.495	3.672 ± 3.373

	IL-22	
	TD	PTB
Medium	0.034 ± 0.039	0.224 ± 0.167
LPS	0.059 ± 0.035	59.43 ± 132.6
LPS+CD40L	0.080 ± 0.093	45.97 ± 99.99
CpG	0.067 ± 0.057	0.434 ± 0.752
CpG+CD40L	0.057 ± 0.066	0.279 ± 0.249

	IFN-γ	
	TD	PTB
Medium	0.215 ± 0.240	1.443 ± 0.537
LPS	0.270 ± 0.377	201.4 ± 324.3
LPS+CD40L	0.977 ± 1.054	183.3 ± 245.2
CpG	0.539 ± 0.579	1.662 ± 1.869
CpG+CD40L	0.211 ± 0.434	3.668 ± 4.779

	TNF-α	
	TD	PTB
Medium	0.686 ± 0.488	10.88 ± 11.45
LPS	0.920 ± 0.425	29.54 ± 18.71
LPS+CD40L	6.943 ± 4.988	23.54 ± 18.77
CpG	20.03 ± 15.35	23.09 ± 9.500
CpG+CD40L	11.41 ± 8.649	20.17 ± 12.62

Cytokines in term and preterm isolated B cells

Isolated B cells from term (TD= term delivery) and preterm delivering (preterm birth, PTB) patients were cultured in medium or stimulated with LPS or CpG alone or combined with CD40L for 72h. The cytokine level in supernatant was analyzed using a Th panel multiplex bead-based assay. In (A), the *p* values of the Two-way ANOVA column factor is shown; *p<0.05; **p<0.005; ***p<0.0005; ****p<0.0001. In (B), the mean values and SD for each individual cytokine are presented.