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Effect of betamethasone, indomethacin and fenoterol on neonatal and maternal mononuclear cells stimulated with *Escherichia coli*

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ABSTRACT

Despite considerable progress in the field of perinatal care, infectious diseases, especially when caused by gram negative bacteria, remain a major reason for neonatal morbidity and mortality. Notably infants born prematurely and those with very low birth weight are at risk due to their immature and deficient immune system and their prolonged hospitalization which promotes nosocomial infections. In case of impending preterm birth, betamethasone is given to induce lung maturation and tocolytic agents like indomethacin or fenoterol are administered to suppress premature labor. The aim of this study was to analyze the effects of these drugs on the immune system of mothers and neonates. Therefore, mononuclear cells from cord blood and peripheral maternal blood were stimulated with *Escherichia coli* and incubated with betamethasone, indomethacin and fenoterol. Subsequently the effect of the treatment on cytokine production was determined. Betamethasone alone and in combination with tocolytic agents inhibited the production of pro- and anti-inflammatory cytokines. Not only does betamethasone dampen the immune response by reducing the production of cytokines, it also has a variety of other detrimental short- and long-term effects on the neonate. In conclusion we would recommend using biological markers to determine if premature labor actually leads to preterm birth and subsequently administer betamethasone only to mothers giving birth prematurely.

1. Introduction

Around 40% of the worldwide neonatal mortality is caused by infections [1]. Despite modern treatment and advances in medicine, sepsis remains a major reason for the high mortality rate, with gram negative bacteria being the most dangerous group of pathogens [2,3]. Neonatal sepsis can be distinguished between early onset sepsis (EOS), which occurs in the first week after birth, and late onset sepsis (LOS) which occurs within 28 days after birth [2]. While Group B streptococcus (GBS) is the most common pathogen (43%) causing EOS, the second most common pathogen (29%), *Escherichia coli* (*E. coli*), is the major cause for mortality [4,5]. In preterm and very low birth weight (VLBW) infants *E. coli* is responsible for the majority of infections and EOS [3,5]. These premature infants also generally suffer from a high mortality rate during sepsis [6,7].

A major factor for the susceptibility of newborn infants to infections is the immature and deficient immune system. Newborns feature a higher immature neutrophil count and display impaired neutrophil functions [8,9]. They have deficient monocytes, macrophages, dendritic and natural killer cells as well as lower complement levels [10,11]. To make matters worse, the adaptive immune system is even more deficient. T cells are predominantly of a naïve phenotype, the T cell response is skewed towards Th2 and less efficient against a variety of pathogens [12]. B cells show diminished class switching and impaired response to T-cell-dependent antigens. The defects and impairments are even more numerous and pronounced in preterm and VLBW infants who also frequently acquire nosocomial infections during their prolonged hospitalization [7]. Furthermore, the majority of maternal immunoglobulin (Ig)G transfer occurs in the later stages of the third trimester [13], resulting in a decreased protective effect in preterm infants.

Abbreviations: MNC, mononuclear cells; EOS, early onset sepsis; LOS, late onset sepsis; GBS, Group B streptococcus; *E. coli*, *Escherichia coli*; VLBW, very low birth weight; Ig, immunoglobulin; PBS, phosphate buffered saline; BMI, body mass index; rcf, relative centrifugal force; FBS, fetal bovine serum; ELISA, Enzyme Linked Immunosorbent Assay; TNF, tumor necrosis factor; IL, interleukin; MIP, macrophage inflammatory protein; SD, standard deviation; IFN, interferon; LPS, lipopolysaccharide; HPA, hypothalamic–pituitary–adrenal

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Table 1
Demographic and clinical characteristics of mothers (n = 30) and corresponding neonates including mean (bold).

Age	Smoker [y/n]	BMI (before pregnancy)	Parity	Gestational age [weeks + days]	Neonatal weight [g]
19	n	21.9	1	40 + 0	3120
19	n	22.5	1	38 + 1	3340
22	n	21.5	2	40 + 6	3460
22	n	27.1	1	39 + 2	2980
23	n	27.7	1	41 + 3	3710
25	n	15.2	1	40 + 6	2945
25	n	21.1	1	40 + 4	3770
25	n	19.7	1	38 + 3	2980
26	n	20.0	1	39 + 3	3645
26	n	24.0	2	40 + 1	2700
27	n	24.4	1	38 + 1	3105
27	n	23.5	3	40 + 0	4900
29	n	29.6	1	41 + 1	3260
30	n	18.7	2	40 + 3	3245
30	n	21.5	2	40 + 5	3630
30	n	23.3	1	39 + 5	3590
31	n	24.6	1	38 + 2	2730
32	n	21.7	2	39 + 2	3790
32	n	24.9	1	39 + 1	3270
32	n	26.4	2	40 + 5	4340
33	n	32.4	2	40 + 4	3120
34	n	26.2	2	40 + 5	3435
34	n	19.1	2	40 + 0	3490
34	n	25.4	2	39 + 5	3710
35	n	19.0	3	36 + 2	2870
35	n	25.0	2	39 + 6	3770
36	n	25.1	1	36 + 4	2750
37	n	19.1	1	39 + 5	3070
39	n	32.0	3	40 + 2	4100
39	n	24.8	1	39 + 1	3075
29.6		**23.6**	**1.6**	**39 + 5**	**3396.7**

Table 2
Demographic and clinical characteristics of adult non-pregnant controls (n = 25) including mean (bold).

Age	Smoker [y/n]	BMI
19	n	18.7
21	n	22.2
21	n	21.5
22	y	21.3
23	n	19.7
24	y	20.8
25	n	23.5
25	n	19.1
26	n	22.1
27	n	22.7
27	n	20.6
27	n	27.0
27	n	19.5
29	y	20.7
29	n	24.2
30	n	22.0
32	n	21.1
32	n	33.5
33	y	31.1
34	n	18.2
35	n	38.6
36	n	22.6
37	n	22.8
38	n	21.6
39	n	25.7
28.7		**23.2**

Especially these vulnerable premature infants receive glucocorticoids for lung maturation and tocolysis to block labor and prolong pregnancy. Since these neonates are less protected and more susceptible to infection and sepsis, it is of great importance that we determine if treatment with glucocorticoids and tocolytic agents dampen and/or inhibit the response of the neonatal immune system.

2. Material & methods

2.1. Ethic statements

Blood samples from umbilical cord (n = 30), corresponding mothers (see Table 1) (n = 30) and non-pregnant adult controls (n = 25) (see Table 2) were acquired with approval from the Ethics Committee of the University of Regensburg (permission no 11-101-0231). The cord blood samples were acquired from healthy neonates born on term. Peripheral blood samples were also taken from healthy individuals. All mothers and adult controls provided written informed consent. Mothers also permitted the use of the cord blood samples for scientific purposes. The experiments were performed in accordance with relevant institutional and national guidelines, regulations and approvals.

2.2. Bacterial strain and generation of bacterial lysate

The *E. coli* strain used for the experiments was a clinical isolate with the serotype O18:K1:H7, acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Leibniz Institut, Braunschweig, Germany). The bacteria stock was kept at −80 °C using the Microbank Bacterial Storage System (Prolab Diagnostics, Richmond Hill, Canada) according to the manufacturer's protocol. One day prior to sonication, 8% sheep blood agar plates were inoculated with Microbank beads and incubated overnight at 37 °C. The bacteria were scraped off, resuspended in phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA) and lysed using a Sonoplus HD70 sonicator (Bandelin, Berlin, Germany) at 50% intensity for 15 min and kept at 4 °C. To ensure that the lysate contained no living bacteria, 5 mg/ml Ampicillin were added. The bacteria solution was plated on 8% sheep blood agar plates before sonication and incubated overnight at 37 °C to determine the amount of living bacteria. Afterwards, the lysate was adjusted to 1×10^{10} lysed bacteria per ml and stored at −80 °C.

2.3. Preparation of mononuclear cells from cord blood and peripheral blood

Blood was drawn using a 21 G Safety-Multifly needle (Sarstedt, Nümbrecht, Germany) in combination with a 9 ml S-Monovette blood collection tube (Sarstedt) containing 1.6 mg EDTA/ml. The blood was mixed 1:1 with PBS, layered on Pancoll human separating solution (Pan Biotech, Aidenbach, Germany) and centrifuged at 600 relative centrifugal force (rcf) for 30 min at room temperature. The MNC that gathered in the buffy coat layer on top of the separating solution were collected using a serological pipette (Sarstedt) and transferred into PBS. Subsequently, the MNC were washed twice with PBS (centrifugation step: 300 rcf, 10 min, 4 °C) and resuspended in 2 ml RPMI (Sigma Aldrich) + 10% fetal bovine serum (FBS) + Ampicillin (100µg/ml). Afterwards, cells were counted using a Neubauer improved counting chamber (HGB, Giessen-Luetzellinden, Germany).

2.4. In vitro stimulation and treatment of MNC

For the in vitro stimulation, 2.0×10^6 MNC (1×10^6 cells/ml) were plated singly and incubated with either 80 ng/ml betamethasone (Celestan; essex pharma GmbH, Munich, Germany), 1 µg/ml indomethacin (Confortid; Alpharma, Copenhagen, Denmark), 800 pg/ml fenoterol (Boehringer Ingelheim, Ingelheim am Rhein, Germany) or a combination of betamethasone and either indomethacin or fenoterol. Cells were stimulated with *E. coli* lysate (5 lysed bacteria per MNC). Cells without stimulation and stimulated MNC without treatment served as controls. The volume in each well was adjusted to 2 ml with RPMI (Sigma Aldrich) + 10% FBS + Ampicillin (100 µg/ml) and

incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, cultured cells were centrifuged (300 rcf; 10 min; 4 °C) and supernatants were stored at −80 °C for cytokine analysis.

2.5. Cytokine detection in cell culture supernatant

The cytokine concentration in the cell culture supernatant of each sample was determined using the DuoSet Enzyme Linked Immunosorbent Assay (ELISA) Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol. Deviations from the protocol: 300 µl wash buffer instead of 400 µl, standard curve was prepared by diluting the highest standard 2.5-fold instead of 2-fold, standards and samples were incubated overnight at 4 °C instead of 2 h at room temperature. The cytokine levels of tumor necrosis factor (TNF), Interleukin (IL)-1β, IL-6, IL-8, IL-10 and macrophage inflammatory protein (MIP)-1α were determined. To measure the optical density an EMax precision microplate reader (Molecular Devices, Sunnyvale, USA) and SoftMax Pro Data Acquisition & Analysis software (Molecular Devices) was used.

2.6. Statistics

Statistical analysis was performed using GraphPadPrism (GraphPad Software, La Jolla, USA). All data are represented as mean ± standard deviation (SD), and were tested for statistical significance using Kruskal-Wallis test (one-way ANOVA), Friedman test (one-way ANOVA, repeated measures test) with Dunn's post-test, *t* test and Chi-squared test, as indicated in the figure and table legends.

3. Results

3.1. Maternal MNC show highest production of cytokines

Before analyzing the effect of treatment on cord blood MNC (CBMC), and MNC from corresponding mothers and non-pregnant controls, the cytokine production of stimulated MNC from all three groups was compared to determine the effect of the birth/pregnancy on CBMC and maternal MNC, and how naivety and deficiencies of CBMC affected the immune response. The magnitude of the activation of stimulated MNC was measured by their production of the cytokines TNF, IL-1β, IL-6, IL-8, IL-10 and MIP-1α. We found that maternal MNC produce higher amounts of pro- and anti-inflammatory cytokines than CBMC and adult MNC (see Fig. 1) with the exception of TNF (Fig. 1A). Since various factors affect the cytokine production, age, body mass index (BMI) and smoking status of mothers and adult non-pregnant controls were compared (see Table 3) and a significant difference in the smoking status was detected.

3.2. Effect of treatment

The main goal of this study was to determine the effect of betamethasone and the tocolytic agents indomethacin and fenoterol on the cytokine production of stimulated MNC, which served as readout of the impact of these drugs on the immune response.

3.2.1. Pro-inflammatory cytokine production reduced in CBMC by betamethasone

When focusing on CBMC, we found that betamethasone alone and in combination with either indomethacin or fenoterol reduced the release of the pro-inflammatory cytokines TNF (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C) and the chemokines IL-8 (Fig. 2D) and MIP-1α (Fig. 2F) but not the anti-inflammatory cytokine IL-10 (Fig. 2E). The tocolytic agents alone had no effect on the secretion of the cytokines, analyzed in this study.

3.2.2. Cytokine production generally reduced in maternal MNC by betamethasone

Maternal MNC were also affected by betamethasone. Here, the glucocorticoid alone and in combination with fenoterol or indomethacin reduced the release of all cytokines analyzed (Fig. 3). The only exception was the secretion of TNF, which was not affected by betamethasone when indomethacin was present (Fig. 3A). This effect was also seen in CBMC (Fig. 2A) and adult MNC (Fig. 4A).

3.2.3. Cytokine production of adult MNC least affected by treatment

The cells where cytokine production was the least affected by treatment were MNC from adult controls (Fig. 4). Here, IL-6 release was not altered by treatment at all (Fig. 4C). TNF (Fig. 1A) and MIP-1α (Fig. 4F) levels were only inhibited by the combination of betamethasone and fenoterol, and IL-8 production was only diminished by betamethasone and indomethacin combined (Fig. 4D).

4. Discussion

To determine the effect of different treatments on the immune response and to compare the response from CBMC, maternal and adult MNC the production of various cytokines was analyzed. Cytokines were used as readout since they are involved in numerous physiological processes, including initiation, regulation and termination of inflammatory and immune responses.

Before analyzing the effect of the treatment on cytokine production, we compared CBMC and MNC from corresponding mothers and non-pregnant adult controls to determine if pregnancy and natural birth affect cytokine production and if the immature CBMC show pronounced differences compared to mature MNC. Maternal MNC displayed higher secretion of cytokines and chemokines compared to CBMC and adult MNC. This is most likely due to priming of the leukocytes. Labor is an inflammatory event, where leukocytes migrate into myometrium, cervix and fetal membranes and increased amounts of cytokines are produced in the myometrium and cervix [14]. The reproductive tissue expresses TNF, IL-1β, IL-6, IL-8 and interferon (IFN)γ during labor [15,16]. IFNγ is not only known to induce Th1 immune responses, but also to prime monocytes which greatly enhances their expression of TNF after lipopolysaccharide (LPS) stimulation [17]. TNF in turn can induce IL-1β and IL-6 expression [18]. IFNγ and LPS have a synergistic effect on IL-8 production of human monocytes [19]. IL-6 activates the MCP-1 production in peripheral blood monocytes [20]. IL-10 has a primarily anti-inflammatory effect resulting in the suppression of immune responses [21]. It is therefore used to terminate immune responses, and a higher amount of pro-inflammatory cytokines, produced by maternal MNC naturally results in higher levels of IL-10 to counteract their effect. Based on these findings, a major factor of the increased cytokine production after stimulation of maternal cells is likely a result of priming of especially monocytes by the elevated levels of pro-inflammatory cytokines during labor. A possible contributing factor could be the time point of the blood sampling. Blood from adult controls was primarily taken in the morning while cord blood and maternal blood was sampled at various points in time. Periodic systemic fluctuations of cytokine levels which occur during the day [22] may have influenced the isolated MNC and CBMC enough to alter cytokine production. While adult MNC donors included significantly more smokers, it is debatable if this fact is primarily responsible for the markedly different cytokine profile. The literature shows inhibitory as well as pro-inflammatory effects of cigarette smoke [23,24]. However, smoking could still be a contributing factor and should not be discarded entirely.

The neonatal immune system is immature and features a variety of deficiencies resulting in neonates being more vulnerable to infections [25,26]. Hence, it is not surprising that cytokine responses in neonates are weaker compared to adults in both term and preterm infants [27]. Therefore, one would have expected that our experiments show significantly lower cytokine levels produced by CBMC after stimulation

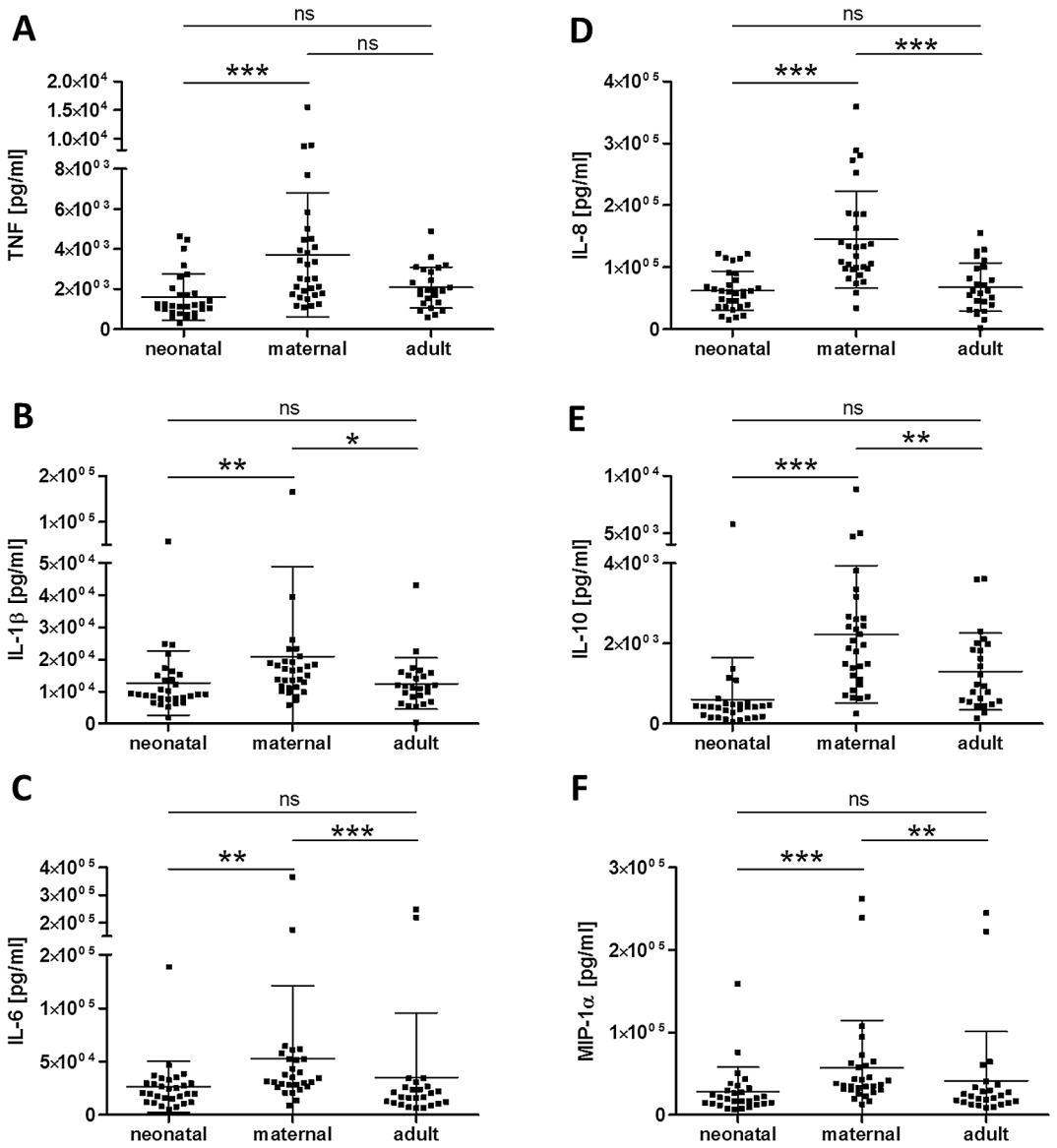

Fig. 1. Comparison of cytokine production between MNC from cord blood (neonatal, n = 30), corresponding mothers (maternal, n = 30) and non-pregnant adult controls (adult, n = 25) after stimulation with *E. coli*. Concentration of TNF (A), IL-1β (B), IL-6 (C), IL-8 (D), IL-10 (E) and MIP-1α (F) in cell culture supernatant of stimulated MNC in pg/ml. Statistical significances were calculated using a Kuskal-Wallis test with Dunn's post-test (not significant: ns; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Each dot represents one sample.

Table 3
Comparison of mothers (n = 30) and non-pregnant adult controls (n = 25).

	Age mean/ median	BMI mean/ median	Smoking status (smoker/ non-smoker)
Mothers	29.6/30	23.6/23.8	0/30
Adult controls	28.7/27	23.2/22.0	4/21
p-value	0.5476 (ns)	0.3064 (ns)	0.0371 (*)

Age & BMI: *t* test; smoking status: Chi-squared test; (not significant: ns; *: $p < 0.05$).

compared to adult cells. The fact, that both produced similar amounts in our setting could be due to priming of the CBMC during birth. Directly after birth, neonates have significantly elevated levels of pro-inflammatory cytokines in their peripheral blood compared to adults [28]. These pro-inflammatory cytokines could prime CBMC leading to higher cytokine production after stimulation. This hypothesis is supported by a study by Mohamed et al., which showed that upon stimulation with *E. coli*, neonatal CBMC produced similar amounts of TNF and IL-1β, and even higher levels of IL-6 and IL-8 compared to adult cells [29]. The higher amounts of IL-6 and IL-8 could stem from differences in the experimental setup.

TNF and IL-1 are early response cytokines that propagate, amplify and coordinate the pro-inflammatory response [30]. TNF plays an important role in the protective immune response against pathogens and it is essential for survival [31]. The importance of this cytokine for fighting off infections can also be observed in patients receiving anti-TNF therapy. This therapy increases the risk of infection in general and also the risk of serious infections [32,33]. In both CBMC and maternal MNC, betamethasone alone and in combination with fenoterol caused a reduction of pathogen induced TNF production. In adult MNC only the combination of betamethasone and fenoterol reduced the release of TNF. Interestingly, the combination of betamethasone and indomethacin did not alter the production of TNF. The reason behind this could be that indomethacin upregulates TNF production in human MNC [34]. While we did not observe a statistically significant increase, we did see an increase in tendency, which might have been enough to offset the inhibitory effect of betamethasone.

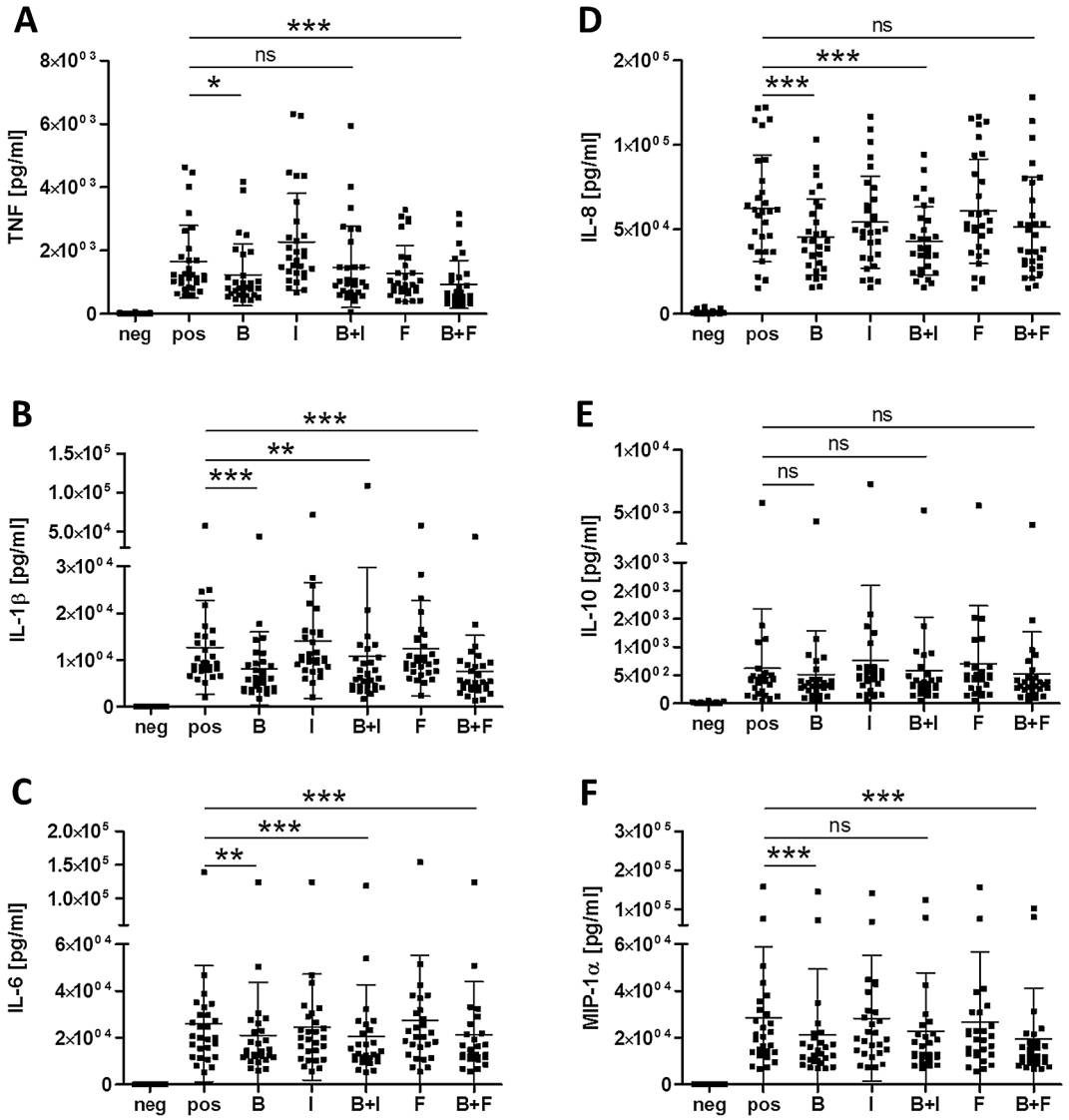

Fig. 2. Effect of treatment on the cytokine production of CBMC. Concentration of TNF (A), IL-1β (B), IL-6 (C), IL-8 (D), IL-10 (E) and MIP-1α (F) in cell culture supernatant of stimulated cells in pg/ml. CBMC were stimulated with *E. coli* except negative control (neg). Cells were treated with betamethasone (B), indomethacin (I), fenoterol (F) or a combination of betamethasone and indomethacin (B + I), or betamethasone and fenoterol (B + F). Statistical significances were calculated using a Friedman test with Dunn's post-test (not significant: ns; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Each dot represents one sample (n = 30).

IL-1β is a key mediator of the inflammatory response and crucial for the host response and resistance to pathogens [35]. The functions of IL-1β include the induction of prostaglandin E synthesis, nitric oxide production, induction of IL-6 and expression of adhesion molecules [36]. The physiological effects of IL-1β include fever, vasodilatation, and hypotension among others. In all three groups, betametasone alone and in combination with a tocolytic agent lead to a reduction in IL-1β production after stimulation.

IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties [37]. Its functions include the induction of acute phase proteins, platelet production, hematopoiesis, B cell differentiation and effector T cell development [37,38]. In both CBMC and maternal MNC, IL-6 production was inhibited by betamethasone alone and in combination with a tocolytic agent. However IL-6 production in stimulated adult cells was not affected by any of the treatments. An explanation for this phenomenon could be that CBMC and maternal MNC were both primed by pro-inflammatory cytokines, released during labor and birth. This priming could have made them more susceptible to the inhibiting effects of glucocorticoids. An example for diverging glucocorticoid sensitivity of MNC after different pretreatment before LPS stimulation can be seen in a study by Wirtz et al. [39]. Here the peripheral blood MNC from smokers showed a higher glucocorticoid sensitivity regarding their cytokine release compared to non-smokers. Of note here is the fact, that smokers have elevated levels of pro-inflammatory cytokines in their circulation.

IL-8 is a chemokine and primarily acts on neutrophils. It induces chemotaxis to the site of infection/inflammation and activates neutrophils as well as neutrophil functions e.g. exocytosis and respiratory burst [40,41]. In both CBMC and maternal MNC, IL-8 production was inhibited by betamethasone alone and combination treatment. In adult MNC, IL-8 levels were only affected by the combination of betamethasone and indomethacin. Therefore, despite the fact that glucocorticoids diminish IL-8 production [42] this effect or the concentration we used was not enough to impact adult MNC. Prostaglandin E, whose production is diminished by indomethacin, can induce IL-8 production, especially when combined with IL-1α [43]. While we did not measure IL-1α, it is likely that this cytokine was produced since LPS induces IL-1α production in myeloid cells [44]. Considering these findings, it is likely that the combination of the inhibition of Prostaglandin E production leading to a diminished IL-1α production together with the

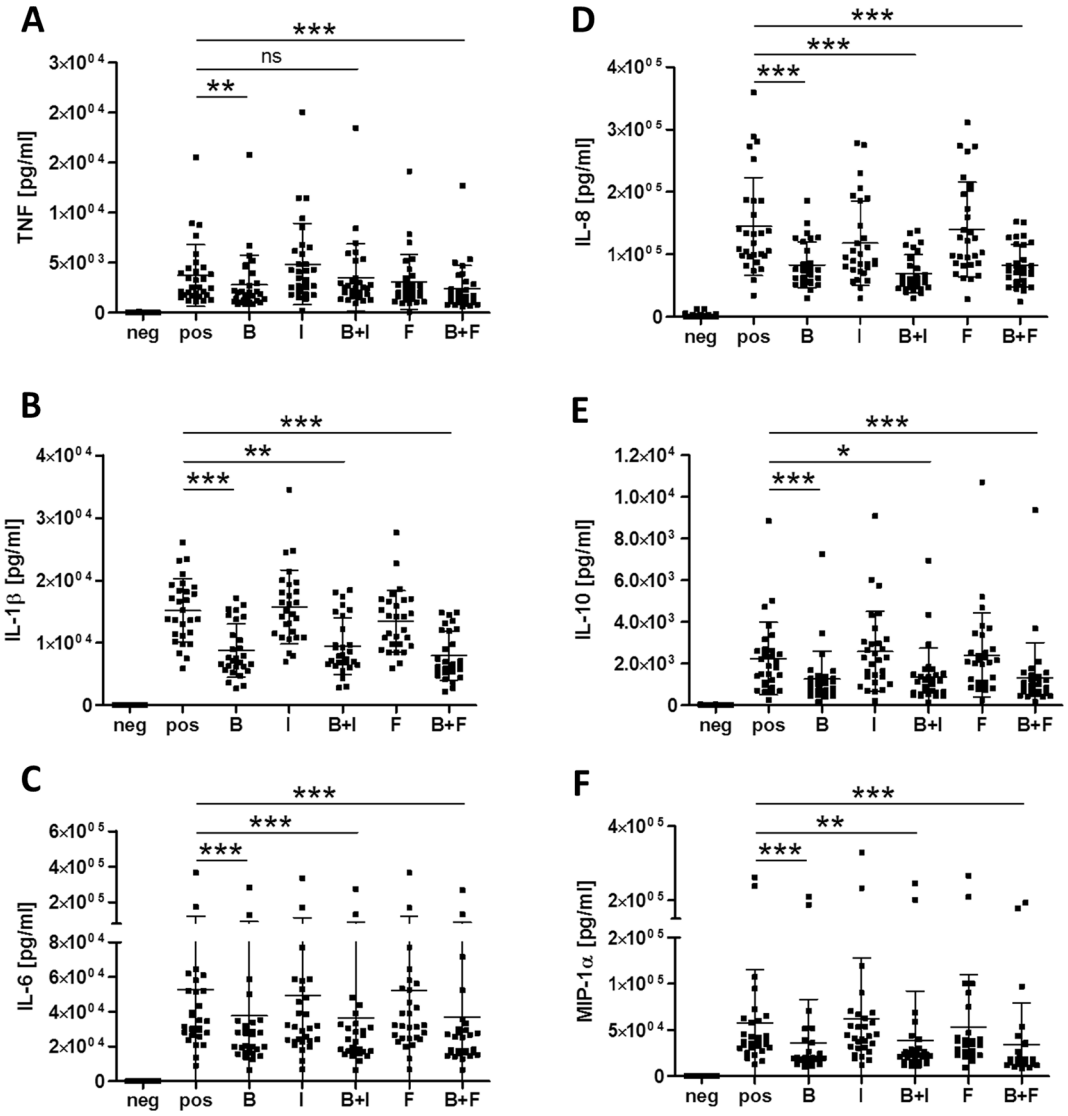

Fig. 3. Effect of treatment on the cytokine production of maternal MNC. Concentration of TNF (A), IL-1β (B), IL-6 (C), IL-8 (D), IL-10 (E) and MIP-1α (F) in cell culture supernatant of stimulated cells in pg/ml. Cells were stimulated with *E. coli* except negative control (neg). MNC were treated with betamethasone (B), indomethacin (I), fenoterol (F) alone or a combination of betamethasone and indomethacin (B + I), or betamethasone and fenoterol (B + F). Statistical significances were calculated using a Friedman test with Dunn's post-test (not significant: ns; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Each dot represents one sample (n = 30).

inhibitory effect of betamethasone was necessary for the reduced production of IL-8 by adult MNC.

IL-10 is a key regulator during the immune response. Its function is to terminate/dampen pro-inflammatory responses by regulating and repressing the expression of pro-inflammatory cytokines especially in the recovery phase of infections and to prevent tissue damage [45]. In our experiments, betamethasone alone, and in combination with a tocolytic agent, impaired the production of IL-10 after stimulation in maternal as well as adult MNC. However, treatment had no marked effect on CBMC, which usually produce markedly less IL-10 compared to adult cells [46]. In our experiments, we observed this phenomenon only in tendency. This deficient IL-10 production might be a contributing factor for the higher susceptibility of neonates to infection and sepsis since IL-10 has been shown to have a protective effect in a mouse model of endotoxic shock [47]. Similar to our findings however, Bessler et al. observed that glucocorticoids inhibit the production of IL-10 in adult MNC but not in neonatal CBMC [48]. Bessler et al. hypothesized that the regulatory mechanisms in neonatal cells might differ from those in mature adult MNC resulting in the diverging effect of glucocorticoids on IL-10 production.

MIP-1α is a chemokine that induces migration in monocytes, T and NK cells [49]. It also stimulates histamine release in basophils, degranulation of mast cells and production of pro-inflammatory cytokines. In maternal MNC and CBMC, betamethasone alone, and in combination with indomethacin or fenoterol, generally dampened the release of MIP-1α. In adult cells, this effect was only present when betamethasone was combined with fenoterol. The inhibition of MIP-1α by corticosteroids is not a surprising discovery [50]. While there is little evidence of fenoterol to directly affect MIP-1α expression, it is known, that fenoterol [51,52] and other β2-agonists [53] inhibit the expression of pro-inflammatory cytokines such as TNF, IL-1β and IL-6 in monocyte and macrophage cell lines after LPS stimulation. In addition, β2-agonists have been shown to inhibit LPS-induced MIP-1α production in human monocytes [54]. Why the combination of both inhibitory effects of fenoterol and betamethasone was necessary in adult cells, but not in maternal MNC and CBMC might be explained by the priming of those cells by the pro-inflammatory cytokines released during labor.

In summary, it is evident that betamethasone alone and in combination with tocolytic agents negatively affects the production of cytokines and chemokines. Due to the importance of these cytokines in

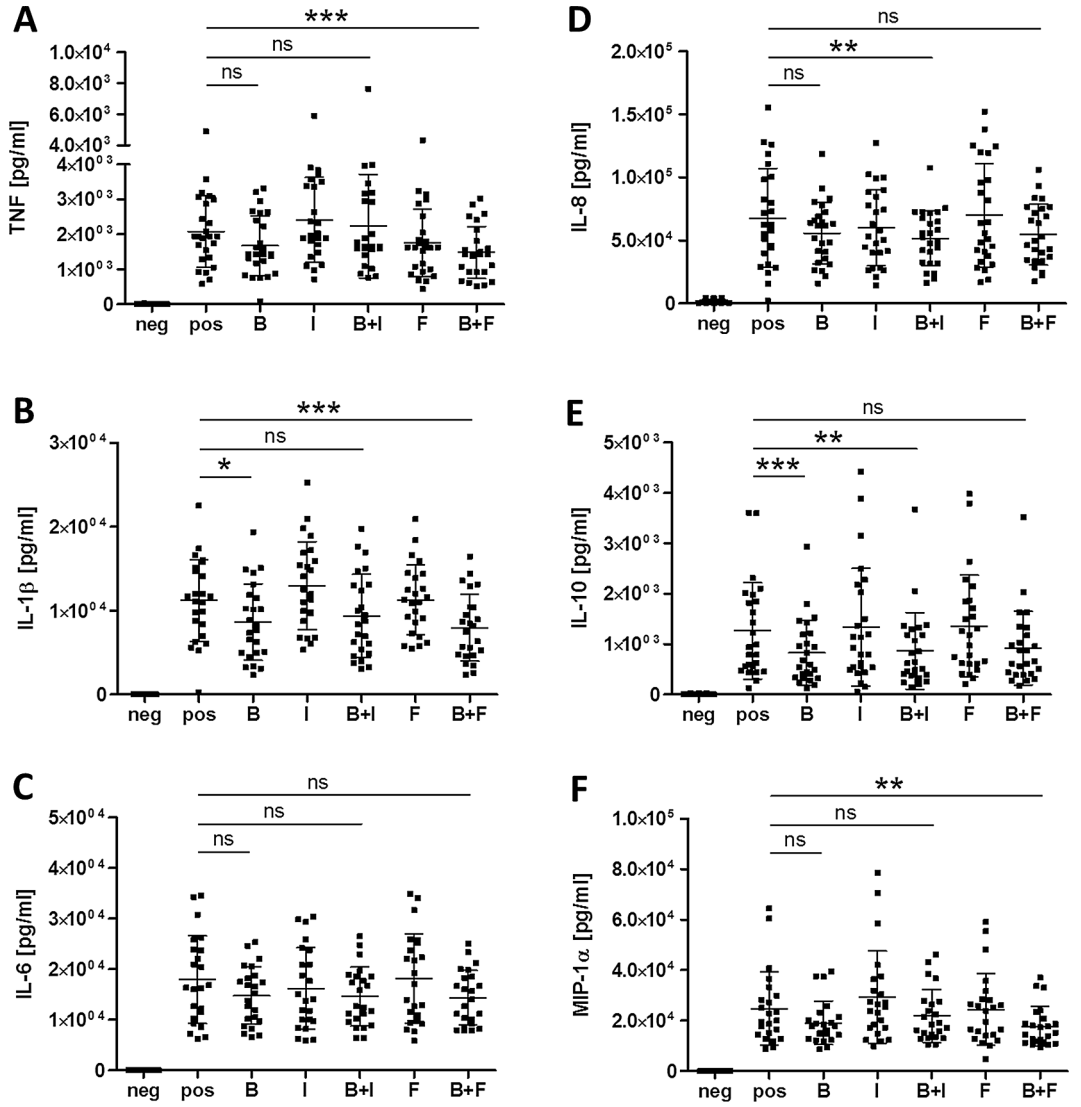

Fig. 4. Effect of treatment on the cytokine production of MNC of non-pregnant adult controls. Concentration of TNF (A), IL-1β (B), IL-6 (C), IL-8 (D), IL-10 (E) and MIP-1α (F) in cell culture supernatant of *E. coli* stimulated cells in pg/ml. MNC from negative control (neg) were not stimulated. Cells were incubated with betamethasone (B), indomethacin (I), fenoterol (F) or a combination of betamethasone and indomethacin (B + I), or betamethasone and fenoterol (B + F). Statistical significances were calculated using a Friedman test with Dunn's post-test (not significant: ns; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Each dot represents one sample (n = 25).

activating, regulating, orchestrating and terminating the immune response, it is safe to assume that this therapy negatively affects the immune response. This is especially concerning in neonates, since their immune system is far from fully developed, and is struggling to fight off infections even without being impaired by glucocorticoids. In addition, neonatal glucocorticoid therapy has other short-term effects including a direct effect on fetal circulation [55], alteration of the hypothalamic–pituitary–adrenal (HPA) response of the neonate after birth [56] and a reduction in thymus size in neonates due to apoptosis of thymic stroma cells [57] and double positive thymocytes [58]. Furthermore, it induces a transient decrease in lymphocyte proliferation and IL-2 production [59] and apoptosis in lymphocytes [60] indicating that there is a timeframe where the adaptive immune system is inhibited profoundly. Another short-term effect of betamethasone is a reduction of red blood cell and platelet count in mothers receiving antenatal glucocorticoid therapy [61]. Treatment with glucocorticoids can also have detrimental long-term effects which can be seen in preterm infants treated for chronic lung disease. Here, treatment resulted in significantly more learning problems and a higher need for special school education compared to untreated controls [62]. This effect most likely stems from an impairment of grey matter growth [63]. In case of chronic lung disease, glucocorticoids are administered over a longer period, compared to the 2 doses within 24 h that are given for lung maturation. However, it shows that glucocorticoids can negatively affect neurodevelopment. Antenatal betamethasone treatment (two doses of 12 mg, 12–24 h apart) can also have detrimental long-term effects on behavior (altered anxiety behavior [64], decreased motivation and learning [65]), brain development (cortical thinning [66], somatic growth retardation [67], programming the development of the HPA axis [68]) and physiology (increased risk for insulin resistance in adulthood [69]). Other long-term consequences affect the immune system: The glucocorticoid-induced apoptosis changes the T cell repertoire and thymocyte ratio [70] which in turn can influence the development of autoimmunity [71]. The negative long-lasting effects of antenatal glucocorticoid treatment can also be seen in the fact, that it increases the risk for developing allergic diseases [72].

While it is undisputed, that the antenatal glucocorticoid therapy is a necessary and cost-effective way to reduce the occurrence of respiratory distress syndrome and mortality of preterm infants [61,73], it is also clear that the therapy has detrimental short and long-term effects for

neonates. Interestingly, it seems that preterm labor does not necessarily lead to preterm birth. Depending on the study and the timeframe of the analysis, preterm labor only leads to preterm birth in roughly 50% of the cases [74–76]. Considering this fact, the necessity of glucocorticoid therapy to prevent respiratory distress syndrome in preterm infants and the side effects, the most beneficial cause of action would be to determine when preterm labor and/or other clinical signs of preterm birth result in preterm delivery. These infants benefit from betamethasone administration, whereas the infants, where preterm labor ceases spontaneously do not benefit but suffer from short and long-term side effects. To determine, in which case preterm labor actually leads to premature birth, certain biological markers can be analyzed. Suitable biological markers are cervicovaginal fetal fibronectin [77], estriol in maternal saliva [78] and placental alpha microglobulin-1 [79], among others.

Declaration of interests

The authors declared that there is no conflict of interest.

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