Pentoxifylline modulates LPS-induced hyperinflammation in monocytes of preterm infants *in vitro*

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BACKGROUND: Pentoxifylline (PTX), a methylxanthine derivate with immunomodulating properties, has been used as adjunctive treatment in severe neonatal sepsis. The aim of the study was to investigate the anti-inflammatory effects of PTX on Lipopolysaccharides (LPS)-stimulated monocytes of preterm neonates *in vitro* compared with monocytes of term infants and adult controls.

METHODS: Whole cord blood samples and control adult blood samples were incubated with LPS and PTX. The expression of surface markers, phagocytosis, cytokine secretion, and Toll-like receptor (TLR)4 signaling of monocytes were assessed by flow cytometry. Changes of TLR4-messenger RNA (mRNA) levels were confirmed by reverse-transcriptase PCR.

RESULTS: The expression of CD14, CD11b, CD64, CD71, and CD80 was downregulated by PTX in a dose-dependent manner; the greatest effect was observed on CD14 and CD11b in preterm infants. PTX markedly downregulated LPS-induced tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 levels in all age groups. Early IL-10 production was significantly downregulated by PTX in term and preterm neonates, while remaining unchanged in adults. Moreover, PTX downregulated TLR4 expression of monocytes on cellular and mRNA level, decreased signaling, and suppressed phagocytosis.

CONCLUSION: PTX downregulated TLR4 expression and signaling, thereby leading to strong anti-inflammatory properties in monocytes. Age-dependent differences were identified for CD14 and CD11b expression and IL-10 production.

Neonatal sepsis is associated with significant mortality in preterm infants (1). Understanding of the pathophysiologic processes surrounding neonatal sepsis and specifically the individual immunologic responses to neonatal sepsis is yet to be elucidated. Compared with adults, the neonatal immune system is compromised both in its humoral and cellular components. Levels of receptor expression, cytokine, and mediator kinetics differ significantly between neonates and adults (2). Therefore, therapies that target adult sepsis-specific deficits may have a differential effect in neonates.

Neonatal antigen-presenting cells (APCs) demonstrate an impaired production of Th1-polarizing cytokines and an impaired upregulation of co-stimulatory molecules to most Toll-like-receptor (TLR) agonists as well as an impaired TLR signaling (3–5). Monocytes present antigens by means of expression of HLA receptors and secrete cytokines to amplify the immune response (6). Multiple studies have shown that following premature birth monocytes have diminished capacities for both these responses (7,8).

TLRs play a crucial role in sensing bacteria and, in the case of sepsis, stimulating a pathogenic response by the innate immune system. Recognition of bacterial products (e.g., LPS) by TLRs in monocytes/macrophages leads to the production of cytokines, chemokines, and nitric oxide (9). Among them, inflammatory cytokines such as tumor necrosis factor-α (TNF-α) play a pivotal role in the pathogenesis of early septic shock and organ dysfunction (10).

Pentoxifylline (PTX), a methylxanthin derivate and phosphodiesterase inhibitor, is a non-steroidal immunomodulating drug, which has been shown to be of potential benefit to the outcome of term and preterm neonates with sepsis and necrotizing enterocolitis. However, a current Cochrane review concluded that PTX, when used as an adjunct therapy to antibiotics in neonatal sepsis, might decrease mortality without any adverse effects (11). A recent randomized controlled trial on the use of PTX in late-onset neonatal sepsis showed no decrease in mortality, but it had several beneficial adjuvant effects in preterm infants (12). PTX is known to inhibit the synthesis of TNF-α (13) and other cytokines that play key roles in inflammation (14,15). The inhibition of cytokine production may be mediated through the inhibition of nuclear factor (NF)-kB and c-Rel activation (16). Yet, its detailed mechanism of action remains incompletely understood, and, to our knowledge, *in vitro* studies on the effect of PTX on monocytes of preterm neonates have not been published so far. Given the substantial burden of sepsis and necrotizing enterocolitis in preterm neonates and the

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potential benefit of PTX, PTX needs to be further evaluated in clinical trials and experimental studies. The purpose of this study was to assess whether PTX modulates the expression of various monocyte surface markers, cytokine production, phagocytic activity, TLR4 expression levels, and signaling in preterm and term infants compared with those in adults in an *in vitro* Gram-negative (LPS) sepsis model.

METHODS

Study Population and Blood Collection

Heparinized cord blood from term (37–42 weeks of gestational age) and preterm (24–32 completed weeks of gestational age) infants was obtained immediately after cesarean section (for infants' characteristics, see **Table 1**). Neonates with congenital malformations, signs of clinical infection, or elevated laboratory markers (elevated white blood cell count, CRP, and/or IL-8) were excluded. Additional exclusion criteria were clinical chorioamnionitis, maternal autoimmune disease, or the use of immunomodulatory drugs with the exception of prenatally administered betamethasone for the induction of fetal lung maturation during preterm labor. Peripheral blood was collected from healthy adult volunteers. The study was approved by the local ethics committee, and informed consent was obtained from pregnant women before delivery and from healthy volunteers.

Table 1. Neonatal characteristics

	Preterm neonates ($n = 14$)	Term neonates ($n = 13$)
Gestational age (weeks)	28.8 ± 3.14	38.78 ± 1.13
Infant body mass (g)	1,179 ± 427	3,340 ± 422
Male/female	8/14	6/13
Multiples	4/14	2/13
5-Min Apgar score	8.38 ± 0.65	9.92 ± 0.28

Data are presented as numbers or as mean values ± SD.

Reagents

Pentoxyfylline (Trental, 100 mg/5 ml) was purchased from Sanofi-Aventis Germany GmbH (Frankfurt, Germany) and was used at final concentrations of 20, 200, and 2,000 µg/ml based on previous *in vitro* studies (15), and blood concentrations were measured *in vivo* (17). LPS from *Escherichia coli*, Serotype R 1515 (Re) (liquid), was purchased from Alexis Biochemicals (San Diego, CA) and used at a concentration of 10 ng/ml.

PTX–Time and Dose–Response Studies

For most experiments, we used whole-blood assays to mirror a most physiological environment; no further serum was added. Samples (0.5 ml) were cultured in 24-well plates with LPS (10 ng/ml) and PTX (20, 200, and 2,000 µg/ml) for 4, 8, or 24 h at 37 °C, 5% CO_2. After each incubation period, supernatants were collected, centrifuged, and stored at − 80 °C until assayed for cytokine production.

Cord blood MNCs as wells as PBMCs were isolated from freshly drawn heparinized blood by Ficoll gradient centrifugation (Ficoll-Paque PLUS; Amersham, GE Healthcare Life Sciences, Little Chalfont, UK) and counted on a Sysmex XS800i (Sysmex Europe GmbH, Norderstedt, Germany). MNCs (2×10^6/ml) were cultured with RPMI 1640 supplement with 10% fetal calf serum (Invitrogen, Carlsbad, CA) without antibiotics. MNCs were stimulated with or without LPS (10 ng/ml) and with or without PTX (200 µg/ml) and incubated in a humidified 5% CO_2 environment at 37 °C.

Monoclonal Antibodies and Flow Cytometric Analysis

For surface staining, 50 µl samples of whole blood were stained with phytoerythrin (PE) mouse anti-CD40 (clone FC3), PE-Cy5 mouse anti-CD71 (clone M-A712), fluorescein isothiocyanate (FITC) mouse anti-CD64 (clone 10.1), APC-H7 mouse anti-CD80 (clone L307.4), PE-Cy7 mouse anti-CD86 (clone 2331), Pacific Blue mouse anti-CD11b/Mac-1 (clone ICRF44), and V500 mouse anti-HLA-DR (L243), all by BD Biosciences (Franklin Lakes, NJ). Anti-CD14 monoclonal antibody (mAb; My4-FITC) or Alexa Fluor 700 mouse anti-CD14 was used to define monocyte population (both from BD Biosciences). Mouse anti-CD 284 (Toll-like receptor 4) PE (clone HTA125) was purchased by eBioscience (San Diego, CA). Antibody incubation was performed on ice for 30 min. After cell staining, erythrocytes were lysed with VersaLyse (Beckman Coulter, Brea, CA), washed, fixed with formaldehyde, and analyzed within 1 h by flow cytometry (LSRII, BD Biosciences, San Diego, CA). Data analysis was performed with the FlowJo software (TreeStar, Ashland, OR).

Intracellular Cytokine Detection

For intracellular TNF-α detection, PBMCs were incubated for 5 h with LPS (10 ng/ml) or LPS and PTX (200 µg/ml) in the presence of Brefeldin A (eBioscience), washed with cold phosphate-buffered saline without Ca^{2+}/Mg^{2+}, and subsequently stained using IntraPrep (Beckman Coulter), according to the manufacturer's instructions. Monocytes were characterized by CD14 FITC (incubation for 15 min at room temperature), and intracellular TNF-α was detected with an anti-TNF-α PE mAb (clone MAb11; eBioscience). A corresponding isotype control was used in each experiment. TNF-α levels were calculated as fold change of MFI values by the formula ($MFI_{stimulated}/MFI_{unstimulated}$).

Phospho-Staining

Samples of 200 µl human whole blood were either treated with vehicle (RPMI 1640) or PTX (200 µg/ml) for 24 h. Thereafter, blood was stimulated with LPS (10 ng/ml) or left untreated at 37 °C in sterile pyrogen-free tubes. After stimulation, red cell blood cell lysis and fixation of phospho-epitopes were performed simultaneously using BD Phosflow Lysis/Fix Buffer for 10 min at 37 °C (BD Biosciences), followed by permeabilization with ice-cold Perm Buffer II (BD Biosciences, Franklin Lakes, NJ) for 30 min. Staining with anti-phospho-ERK1/2 (T202/Y204) PE and anti-phospho-NF-κB p65 (pS529) (both from BD Biosciences) was performed according to the BD Phosflow protocol (BD Biosciences). Briefly, cells were washed twice with washing buffer and phospho-specific mAbs were added. Anti-CD14 FITC was added simultaneously. The mixture was incubated for 30 min at room temperature. The MFI was used to determine the fold change on TLR–ligand stimulation ($MFI_{stimulated}/MFI_{unstimulated}$).

Cytokine Quantification in Supernatants after Whole-Blood Stimulation

A volume of 0.5 ml of whole blood was stimulated with LPS (10 ng/ml) or LPS+PTX (20, 200, or 2,000 µg/ml) as indicated. After 4, 8, and 24 h of stimulation, samples were centrifuged. Cell-free supernatants were collected and frozen at − 80 °C until further analyzed. The level of cytokines in the culture supernatants were determined using a Bead Array System (Human Inflammatory Cytokines Kit, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol (BD Pharmingen, Franklin Lakes, NJ). Samples were assayed using flow cytometry (LSRII, BD Biosciences). Results were analyzed using BD CBA software version 3.0. (BD Biosciences). The limits of sensitivity were 3.3 pg/ml for IL-10, 2.5 pg/ml for IL-6, 7.2 pg/ml for IL1-β, 1.9 pg/ml for IL-12p40, and 3.7 pg/ml for TNF-α.

Phagocytosis

Phagocytosis was determined by incubating 100 µl of whole blood for 30 min with LPS (10 ng/ml)+PTX (20, 200, or 2,000 µg/ml) at 37 °C with non-opsonized, FITC-conjugated *E. coli*, according to the

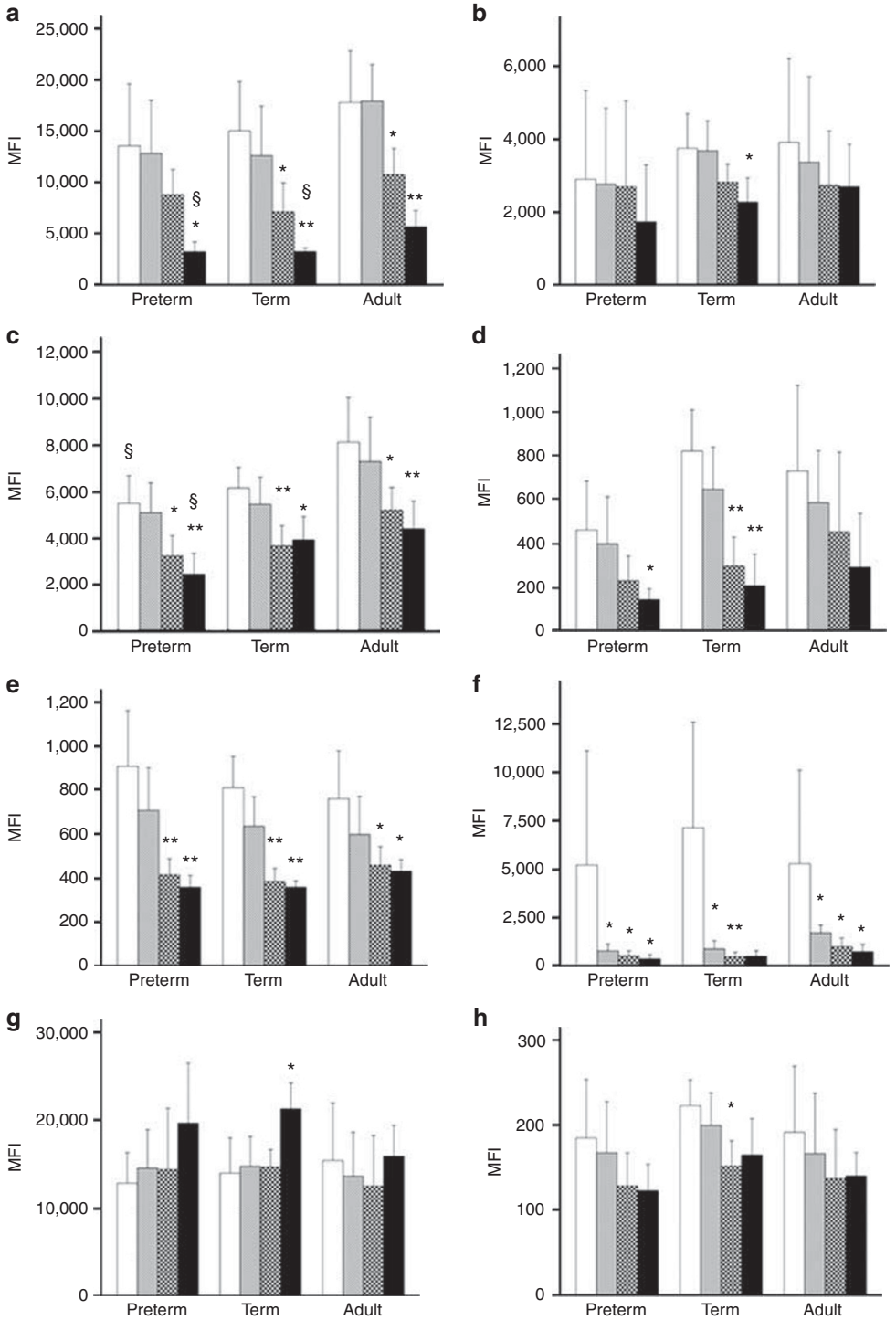

Figure 1. Pentoxifylline (PTX) differentially alters surface expression of activation and maturation markers in monocytes of term and preterm neonates compared with that in adults. To determine the effect of PTX on monocyte phenotypic maturation, whole blood was incubated with LPS (10 ng/ml) for 24 h with or without addition of different concentrations of PTX. White-filled columns, LPS only; black- and white-striped columns, LPS +PTX 20 µg/ml; black- and white-chequered columns, LPS+PTX 200 µg/ml; and black-filled columns LPS+PTX 2000 µg/ml. Cell surface molecules CD14, HLA-DR, CD11b, CD71, CD64, CD80, CD86, and CD40 were examined on CD14-positive monocytes by flow cytometry. Surface molecule expression is presented as mean fluorescence intensities (MFIs). Mean data (± SD) from adults, term, and preterm neonates ($n = 5$ for each group) are shown. A statistical analysis was performed by analysis for variance. Tukey honest significant difference *post hoc* test analysis showed a significant dose-dependent downregulation for (**a**) CD14, (**b**) HLA-DR, (**c**) CD11b, (**d**) CD71, (**e**) CD64, (**f**) CD80, and (**h**) CD40 ($^{*}P < 0.05$, $^{**}P < 0.005$); only for (**g**) CD86, an upregulation was found ($^{*}P < 0.05$). Compared with adults, preterm neonates had significantly lower CD14 and CD11b expression levels when PTX (2,000 µg/ml) was added ($^{\S}P < 0.05$).

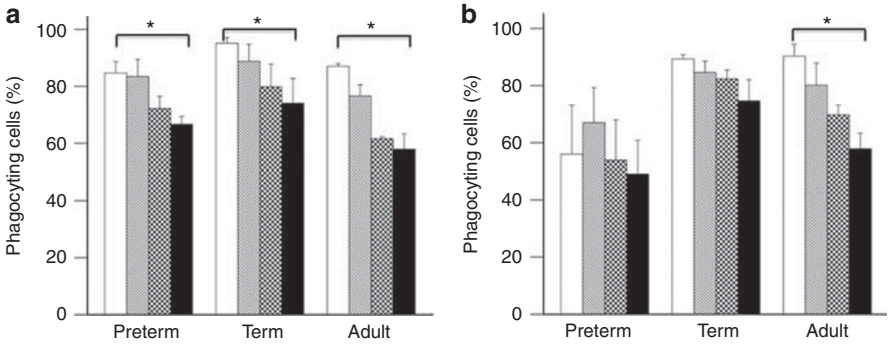

Figure 2. *E. coli* phagocytosis of monocytes and granulocytes from cord blood of term and preterm neonates and peripheral blood of adults were investigated by flow cytometry. The effect of pentoxifylline (PTX; mean values ±1 SEM) on fluorescein isothiocyanate-labeled non-opsonized *E. coli* phagocytosis in preterm and term neonates compared with that in adults was determined. White-filled columns, LPS only; black- and white-striped columns, LPS+PTX 20 µg/ml; black- and white-chequered columns, LPS+PTX 200 µg/ml; and black-filled columns LPS+PTX 2000 µg/ml. Results are given as percentage of phagocyting cells. Monocytes were characterized by CD14 and granulocytes were identified by side scatter. The analysis for phagocytosis in monocytes (**a**) showed a dose-dependent decrease of phagocytosis under PTX treatment without age-dependent differences. In granulocytes (**b**), the suppressive effect was less pronounced in neonates, but reached significance in adults (*$P < 0.05$).

manufacturer's protocol of Phagotest (Glycotope Biotechnology, Heidelberg, Germany). Monocytes were stained with APC anti-CD14 mAb (clone M5E2 by BD), and red cells were lysed with VersaLyse (Beckman Coulter). Cells were washed three times in Hank's Balanced Salt Solution. The MFI of ingested *E. coli* bacteria from 5×10^3 $CD14^+$ monocytes and granulocytes that had been gated by side scatter was determined by flow cytometry, and MFI of ingested FITC-labeled *E. coli* was analyzed.

Cell Viability Assay

A PE Annexin V Apoptosis Detection Kit I by BD Pharmingen assessed cell viability according to the manufacturer's protocols. In brief, 300 µl of whole blood (peripheral blood in adults and cord blood in neonates) was incubated with PTX (2,000 µg/ml) for 24 h. Thereafter, cells were washed with phosphate-buffered saline, and they were resuspended in binding buffer. Staining was performed with CD14 FITC, 7-amino-actinomycin, and PE-labeled Annexin V for 20 min at room temperature. Red cells were lysed with VersaLyse. Cells were washed again with phosphate-buffered saline and fixed with 4% formaldehyde, and fluorescence-activated cell sorting analysis was conducted within 1 h.

Isolation of Monocytes, RNA Extraction, and cDNA Synthesis

For TLR4-mRNA quantification, monocytes were separated by density gradient centrifugation and by means of magnetic cell sorting (Monocyte isolation kit II; Milteny Biotec, Bergisch Gladbach, Germany). Monocyte purity of negatively selected cells was usually ~95%, as determined by flow cytometry. Cells (1×10^6/ml) were seeded in culture plates and cultured in RPMI 1640 supplemented with 10% fetal calf serum in the presence or absence of LPS (10 ng/ml) or LPS+PTX (200 µg/ml), for 6 h. Thereafter, total RNA was prepared using the RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instruction. RNA purity and quantity were determined using Nanodrop (Thermo Fisher, Waltham, MA).

The ABI PRISM 7500HT sequence detection system (Applied Biosystems, Life Technologies, Grand Island, NY) was used for quantitative reverse-transcriptase PCR analysis. Primer–probe sets for TLR4 FAM and 18S rRNA VIC were obtained, pre-designed from Applied Biosystems, and tested for primer efficacy (Gene Expression Assays Hs99999901_s1 18S VIC, Hs00152939_m1 TLR4 FAM). Multiplex amplification was carried out in a total volume of 20 µl for 45 cycles of 3 s at 95 °C and 30 s at 60 °C. Initial denaturation was performed for 3 min at 95 °C. Target gene expression was normalized to 18s rRNA housekeeping gene expression. Normalized target gene expression was analyzed by the ΔΔ comparative threshold method and calculated as *x* fold expression TLR4.

Statistical Methods

Infants were enrolled into the study over a 12-month period. Statistical analysis was performed with SPSS software (SPSS 15.0 for Windows, Armonk, NY). Data were analyzed using one-way analysis for variance in accordance to Tukey *B* or the two-tailed *t*-test with Bonferroni correction. A probability of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of LPS and PTX on the Expression of Activation and Maturation Markers in Monocytes of Preterm and Term Neonates Compared with Adults

Monocytes were identified by CD14. LPS led to an upregulation of most surface markers after 24 h compared with medium-only-treated monocytes. For reasons of clarity, the LPS-only group was set as a control in **Figure 1**.

CD14 was downregulated in a dose-dependent manner by PTX in all age groups (**Figure 1a**). In the highest PTX concentration tested (2,000 µg/ml), monocytes of term and preterm infants ended up with a significantly lower CD14 expression compared with that in monocytes of adults ($P < 0.05$).

HLA-DR expression was only moderately influenced by PTX. Compared with controls, a decrease in HLA-DR level was observed in all age groups, which only reached statistical significance in the group of term infants at the highest PTX concentration ($P < 0.05$). Similar results were found for CD40 (**Figure 1h**) and CD54 expressions, but with the latter decrease not reaching statistical significance (results not shown).

CD11b, a polypeptide α-chain linked to the β2-subunit of CD18, was significantly downregulated by PTX. This effect was most pronounced in preterm neonates reaching a statistically significant difference compared with adult CD11b expression ($P < 0.05$, **Figure 1c**).

CD71, also known as the transferrin receptor, is a marker for the activation of monocytes. CD71 was statistically significantly downregulated on monocytes from term and preterm neonates and to a lesser extent in adults (**Figure 1d**).

The expression of CD64 on monocytes was downregulated in a dose-dependent manner in all age groups without any significant age-dependent differences (**Figure 1e**).

The co-stimulatory molecules, CD80 and CD86, showed a divergent pattern upon PTX stimulation. CD80 showed a clear dose-dependent downregulation (**Figure 1f**) in all age groups, whereas CD86 was upregulated in the highest dose of PTX tested, which reached significance only in term neonates because of high SD in preterm infants. Adult levels of CD86 expression were not significantly influenced (**Figure 1g**).

Analysis of the Effects of PTX on Phagocytosis Activity

PTX reduced phagocytosis activity in monocytes in a dose-dependent manner, which was most pronounced in adults (**Figure 2a**). Similarly, we found a statistically significant reduction of phagocytic activity of PTX-incubated granulocytes in adults, but there was only a tendency for it in term and preterm neonates. However, granulocytes of preterm neonates had lower phagocytic activity at baseline and showed only a moderate decline in phagocytic activity after PTX incubation (**Figure 2b**).

Effect of PTX on TNF-α Production

For intracellular TNF-α quantification, peripheral blood mononuclear cells (PBMCs) were left untreated or stimulated with LPS (10 ng/ml) ± PTX (200 μg/ml) in the presence of an inhibitor of intracellular protein transport (Brefeldin A) for 5 h. Thereafter, intracellular TNF-α was measured in CD14-positive monocytes by flow cytometry (**Figure 3a**). PTX reduced intracellular TNF-α production (measured by median fluorescence intensity (MFI) fold change) in a consistent way as shown in **Figure 3c** (mean reduction of MFI index for adult: 39.0 ± 9.3%, term: 44.4 ± 19.3%, preterm: 37.4 ± 16.3%, $P < 0.05$ for all age groups). The reduction of TNF-α-producing cells was statistically significant in all age groups (adult: 26.8 ± 16.4%, term: 43.8 ± 21.4%, preterm: 32.2 ± 14.9%; ($P < 0.05$, **Figure 3b**). No age-dependent difference in the ability of PTX to reduce TNF was found.

In a different experiment, TNF-α was measured in the supernatant of a whole-blood culture stimulated with LPS (10 ng/ml) in the presence and absence of PTX (200 μg/ml) for 4, 8, and 24 h. TNF-α levels of controls (unstimulated) were low or not detectable at all (**Table 2**). As shown before, upon LPS stimulation, monocytes of adults produced significantly more TNF-α than those of neonates at all time intervals studied. PTX led to a highly significant reduction of TNF-α levels in the supernatant in all age groups in absolute (**Table 2**) and relative numbers (**Figure 3d**), but again no age-dependent difference in the ability of PTX to reduce TNF-α was found.

Effect of PTX on the Production of IL-10, IL-1-β, IL-6, and IL-8

Activation by LPS clearly induced an increase in all cytokines investigated (**Table 2**) except in IL-12 production, for which reason we excluded IL-12 from further investigations. As demonstrated in **Figure 4a**, PTX showed an age-dependent difference for IL-10 production. We found a highly significant reduction of IL-10 in term (28.2 ± 16.1%) and preterm infants (11.2 ± 8.4%) after 4 h of incubation, whereas adult IL-10 levels were not influenced (119.0 ± 28.2%) compared with those in LPS-stimulated controls. IL-10 levels stayed significantly reduced after 8 h in term (9.2 ± 4.8%) and preterm (13.0 ± 8.8%) samples, whereas adult levels were moderately decreased (48.7 ± 19.6%). These age-dependent differences disappeared after 24 h.

LPS-induced secretion of IL-1-β and IL-6 levels was significantly reduced under the influence of PTX after 4 and 8 h in adults and neonates without any differences between the groups (**Figure 4b,c**). This suppressive effect of PTX disappeared after 24 h. A significant IL-8 suppression was only observed in preterm neonates after 4 h and in term neonates after 8 h (**Figure 4d**). We found no significant difference in IL-1-β, IL-6, and IL-8 reduction levels between the age groups investigated, although there was a trend toward a stronger suppression of IL-1-β in preterm infants compared with that in adults ($P = 0.058$) after 8 h (**Figure 4b**).

Effects of LPS and PTX on TLR4 Surface Expression and mRNA Level

TLR4 expression was analyzed on monocytes characterized by CD14 after incubation of whole blood for 24 h with LPS (10 ng/ml) and PTX as indicated and measured by flow cytometry (**Figure 5a**). TLR4 expression was reduced in a dose-dependent manner in adults, reaching statistical significance in monocytes treated with PTX (200 μg/ml) and PTX (2,000 μg/ml). This effect was less pronounced in preterm neonates (**Figure 5b**).

We also tested whether TLR4 expression was downregulated in a PBMC culture. As shown in **Figure 5c**, TLR4 downregulation started after 12 h, although the effect was less pronounced in the PBMC culture than in whole blood and did not reach statistical significance.

Finally, we investigated TLR4 on the transcriptional level. As shown in **Figure 5d**, upregulation of messenger RNA (mRNA) level of TLR4 by LPS stimulation was significantly reduced if PTX was co-administrated.

Analysis of NF-κB Phosphorylation

This experiment was only performed in adults and preterm neonates, as we did not expect differences between term and preterm neonates based on previous results (3). Stimulation of whole blood with PTX for 24 h resulted in a significantly reduced upregulation of phospho-NF-κB (**Figure 6a,c**) and phospho-ERK1/2 (**Figure 6b,d**) upon LPS stimulation (10 ng/ml) in monocytes of preterm neonates and adults. We observed no age-dependent differences in this PTX effect. In monocytes that had not been stimulated with LPS, PTX did

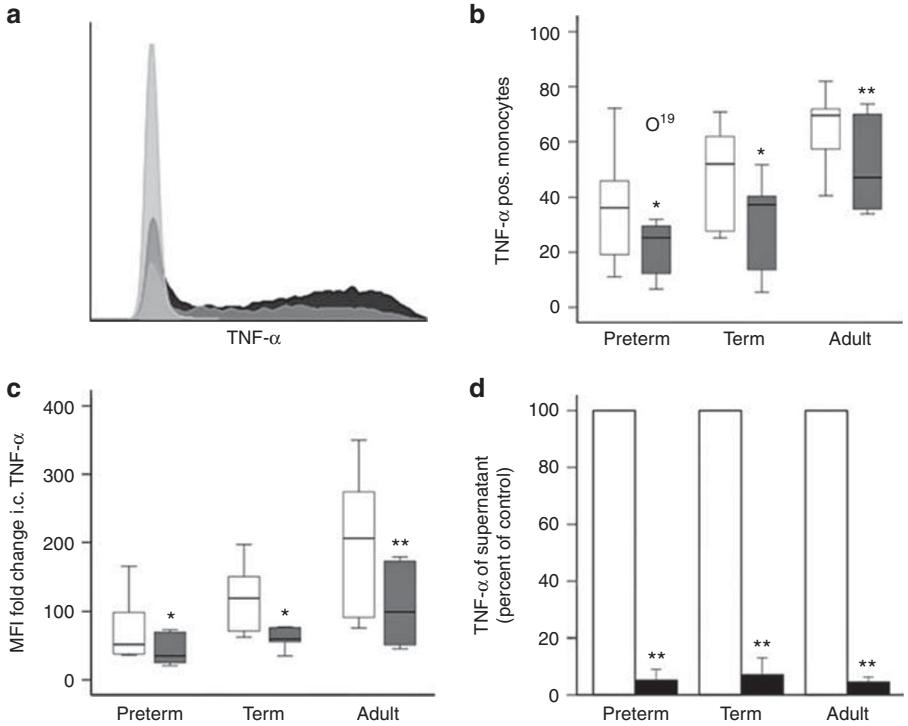

Figure 3. Effect of pentoxifylline (PTX) on tumor necrosis factor-α (TNF-α) production in monocytes of term and preterm neonates compared with that in adults. For intracellular TNF-α cytokine staining, peripheral blood mononuclear cells were incubated in the presence of LPS ± PTX (200 µg/ml) for 5 h, collected thereafter, and stained for CD14 and TNF-α. Upon LPS challenge, TNF-α was found exclusively in monocytes. Stimulation with LPS induced a robust TNF-α production in all age groups. (**a**) Respective histogram. Light gray indicates medium only, medium gray indicates LPS+PTX, dark gray indicates isotype control, and black indicates LPS only. PTX led to a significant decrease of TNF-α fold change compared with LPS-stimulated control (**c**) without differences seen in the potency of this PTX effect. The percentage of intracellular TNF-α positive monocytes was also affected by PTX in a significant way without any age-specific differences (**b**). Blank boxes indicate treatment with LPS only, and filled boxes indicate LPS+PTX. The experiment with monocytes was derived from seven adults, five term infants, and seven preterm infants (*$P<0.05$, **$P<0.005$). For the measurement of TNF-α in the supernatant, cord blood (neonates) and peripheral blood (adults) were cultured with LPS (10ng/ml) in the absence or presence of PTX (200 µg/ml). After incubation, cell-free supernatants were collected and analyzed for TNF-α levels by a capture bead array. Blank boxes indicate treatment with LPS only, and filled boxes indicate LPS+PTX. TNF-α production was significantly suppressed in all age groups (**$P<0.005$) after (**d**) 8 h.

not significantly influence expression levels of phospho-NF-κB and phopho-ERK1/2.

No Apoptotic Effect of PTX

We found no apoptotic effect of PTX on monocytes treated with the highest PTX dose tested (2,000 µg/ml) for up to 24 h using Annexin-V as the detection method (data not shown).

DISCUSSION

Preterm neonates are highly susceptible to bacterial infections, ultimately leading to sepsis. In sepsis, the initial pro-inflammatory response can be overwhelming, suggesting that the innate immune response determines, next to the pathogen itself, the outcome of a septic neonate. The mechanistic effects of immunomodulatory agents in neonates and preterm infants are yet to be established.

This is the first study to investigate the immunomodulatory effects of PTX on monocytes from preterm neonates *in vitro*. The results demonstrate that PTX exerts a dose-dependent downregulation of many surface markers and TLR4 expression. Our results support the anti-inflammatory effects of PTX found *in vivo* in septic preterm neonates studied by Lauterbach *et al.* (18) and described for adult PBMCs *in vitro* (19). Using a whole-blood model with final concentrations of PTX also measured in preterm neonates *in vivo* (17), we tried to resemble a most physiological condition.

The concentration of PTX measured *in vivo* in preterm neonates after infusion of PTX (5 mg/kg/h) varied from 213 to 23,859 ng/ml ((ref. 17)). This suggests that PTX clearance may be age-related, although other variables may have an effect including the method of administration. Regarding dosage, no adverse effects of PTX were observed in clinical trials in premature infants (17,18). This is consistent with the *in vitro* results of our study, demonstrating no toxic effect of PTX on monocytes treated with up to 2,000 µg/ml.

PTX significantly diminished plasma TNF-α and IL-6 levels. We found no age-dependent differences in the ability of PTX to reduce TNF-α production. In contrast to Lauterbach, but in line with the adult *in vitro* data (14), our results demonstrate a suppressive effect for IL-1β and IL-8 (**Figure 4b,d**).

Table 2. Production of cytokines by cord blood (preterm and term infants) and adult blood samples after incubation for 4, 8, and 24 h

		Unstimulated			LPS stimulated			LPS+PTX stimulated		
		Preterm	Term	Adult	Preterm	Term	Adult	Preterm	Term	Adult
Incubation period	Cytokine									
4 h										
	TNF (pg/ml)	5 ± 4	ND	ND	206 ± 183	216 ± 266	4,802 ± 6776	7 ± 7	6 ± 5	200 ± 289
	IL-10 (pg/ml)	ND	ND	ND	60 ± 13	25 ± 19	14 ± 14	7 ± 6	9 ± 9	15 ± 12
	IL-6 (pg/ml)	1,345 ± 1,542	36 ± 56	ND	17,986 ± 10,117	39,496 ± 25,865	37,196 ± 25,600	3,901 ± 1,344	17,968 ± 23,522	16,955 ± 14,459
	IL-1 β (pg/ml)	12 ± 13	ND	ND	350 ± 186	368 ± 176	570 ± 394	43 ± 26	81 ± 82	181 ± 66
	IL-8 (pg/ml)	1,856 ± 847	1,242 ± 1967	73 ± 41	20,898 ± 11,237	28,448 ± 5646	9,357 ± 2016	7,573 ± 3,763	16,803 ± 17,300	7,432 ± 2,307
8 h										
	TNF (pg/ml)	7 ± 5	ND	ND	277 ± 303	354 ± 275	3,812 ± 2,190	27 ± 44	ND	191 ± 165
	IL-10 (pg/ml)	ND	ND	ND	141 ± 92	321 ± 267	149 ± 92	141 ± 92	23 ± 8	66 ± 41
	IL-6 (pg/ml)	1,011 ± 1,694	36 ± 56	7 ± 1	28,195 ± 13,275	70,289 ± 18,754	52,129 ± 16,629	28,195 ± 13,275	27,117 ± 14,158	26,883 ± 18,119
	IL-1 β (pg/mL)	11 ± 18	ND	ND	403 ± 285	1,525 ± 492	3,188 ± 1,543	403 ± 285	208 ± 82	790 ± 450
	IL-8 (pg/ml)	5,579 ± 7,121	19,03 ± 3,008	89 ± 53	31,407 ± 19,884	78,057 ± 28,250	9,379 ± 3,750	11,966 ± 9,352	32,650 ± 17,216	9,299 ± 4,273
24 h										
	TNF (pg/ml)	5 ± 7	ND	ND	86 ± 144	4 ± 2	1,969 ± 1,550	21 ± 34	ND	6 ± 8
	IL-10 (pg/ml)	ND	ND	ND	337 ± 171	1,362 ± 1,719	988 ± 270	337 ± 134	36 ± 21	132 ± 75
	IL-6 (pg/ml)	863 ± 1,491	78 ± 100	ND	20,344 ± 8,873	65,626 ± 22,951	57,310 ± 10,469	20,344 ± 8,874	42,099 ± 28,715	43,095 ± 22,148
	IL-1β (pg/ml)	8 ± 13	ND	ND	393 ± 329	1,958 ± 269	3,865 ± 1,617	393 ± 329	224 ± 112	1,095 ± 647
	IL-8 (pg/ml)	1,623 ± 1,799	828 ± 821	618 ± 825	55,059 ± 35,192	84,050 ± 20,620	25,637 ± 4043	52,987 ± 46502	37,257 ± 18,308	37,402 ± 10,113

IL, interleukin; ND, not determined; PTX, pentoxifylline; TNF, tumor necrosis factor.
Data are represented as mean values ± SD.

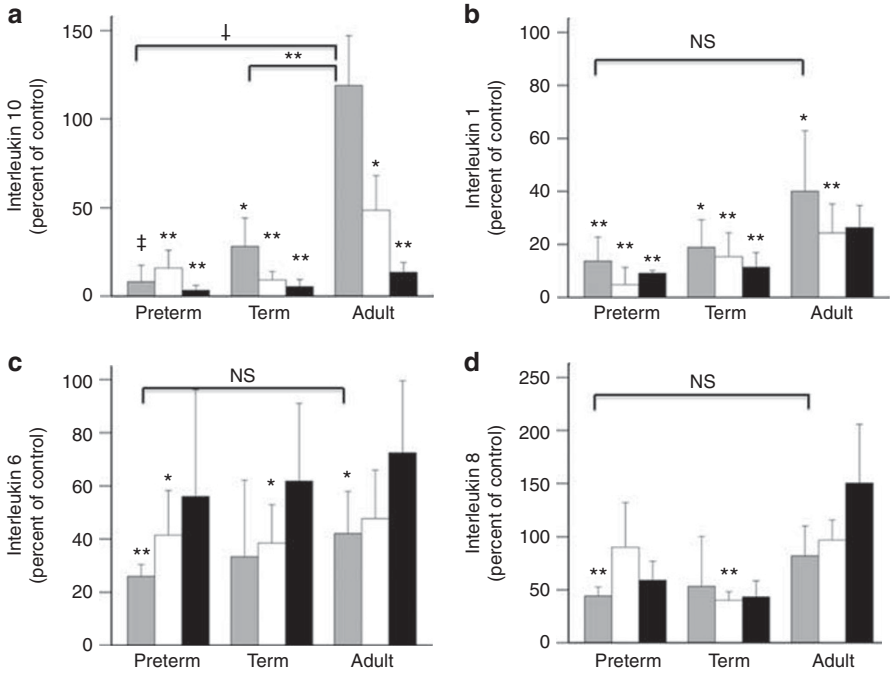

Figure 4. Effect of pentoxifylline (PTX) on interleukin (IL)-1, IL-6, IL-8, and IL-10 production in whole blood of term and preterm neonates compared with that in adults. Cord blood (neonates) and peripheral blood (adults) were cultured with LPS (10 ng/ml) in the absence or presence of PTX (200 µg/ml). After incubation for 4 (blank), 8 (gray), or 24 (black) hours, cell-free supernatants were collected and analyzed for IL-10 (**a**), IL-1β (**b**), IL-6 (**c**), and IL-8 (**d**) levels by a capture bead array. PTX led to a significant reduction of IL-10 levels after 4 h in preterm neonates ($^{‡}P<0.0001$) and to a lesser extent in term neonates ($^{*}P>0.05$). In contrast, IL-10 production in adults was not influenced after 4 h. This age-dependent difference was highly significant for preterm ($^{‡}P<0.0001$) and term neonates ($P<0.005$). After 8 h, the IL-10 production in adults decreased statistically ($^{*}P<0.05$), whereas in preterm and term neonates, the suppressive effect of PTX was still more pronounced ($^{**}P<0.005$). After 24 h, IL-10 production was significantly reduced in all groups without any age differences. PTX also reduced IL-1, IL-6, and IL-8 levels significantly in neonates and adults (except for IL-8). There was a tendency for a more pronounced effect in preterm neonates compared with that in adults ($P=0.08$ after 4 h; $P=0.058$ after 8 h). Data from adults, term, and preterm neonates are shown. Bars show mean values ± SD of the cytokine levels (%) compared with LPS-stimulated samples. $^{*}P<0.05$, $^{**}P<0.005$, $^{‡}P<0.0001$.

Notably, our results indicate an age-dependent, divergent influence of PTX on IL-10 production (Figure 4a). Adult IL-10 levels were not influenced by PTX after 4 and 8 h but showed a significant decrease after 24 h, as has been published earlier (14). In neonates, IL-10 levels were already significantly reduced by 4 h and stayed reduced at 8 and 24 h. These results suggest that PTX may not only inhibit the Th1 cytokine profile but also suppress IL-10, an anti-inflammatory cytokine in term and preterm neonates at an early stage. Previous *in vitro* studies of murine macrophages confirm our findings of the ability of PTX to reduce IL-10 production (20), whereas other *in vivo* studies showed an increase of IL-10 mRNA in human blood mononuclear cells (MNCs) (21) after PTX treatment. The role of IL-10 has been increasingly recognized in modulating the immunologic response to sepsis. IL-10 limits inflammation by inhibiting APC and effector T-cell function (22). In an experimental model, it was shown that the administration of recombinant murine IL-10 protects from lethal endotoxemia, even when IL-10 was injected 30 min after LPS administration (23). In contrast, the immunoneutralization of IL-10 led to increased levels of TNF and IL-6 in mice (24). Despite these clear protective effects, IL-10 actions might not always be beneficial, as studies found that IL-10 levels did correlate with mortality in pediatric intensive care unit patients (24).

A reduced phagocytic activity in sepsis has also been shown to be associated with a poor outcome in adults and neonates (25,26). We found that the suppressive effect of PTX on phagocytosis was age- and dose-dependent. Monocytes and granulocytes from neonates were less influenced than were adult APCs. It has been demonstrated that IL-10 might enhance CD14-dependent phagocytosis (27). We therefore next explored the functional response to the ligand of TLR4/CD14 (LPS) after PTX treatment. A dose-dependent downregulation of CD14 was found in all age groups, most pronounced in preterm neonates. Moreover, PTX led to a significant dose-dependent downregulation of TLR4 in monocytes of adults, and to a lesser degree in monocytes of preterm neonates. This effect was confirmed on mRNA level. The analysis of signal transduction in monocytes that had been pre-incubated with PTX before LPS stimulation showed a downregulation of phospho-NF-κB and phospho-ERK 1/2. These data go in line with the published results on decreased IκB and NF-κB nuclear translocation in adult PBMCs treated with PTX (19). TNF-α production is dependent on the activation of NF-κB genes, and downregulation of TNF-α

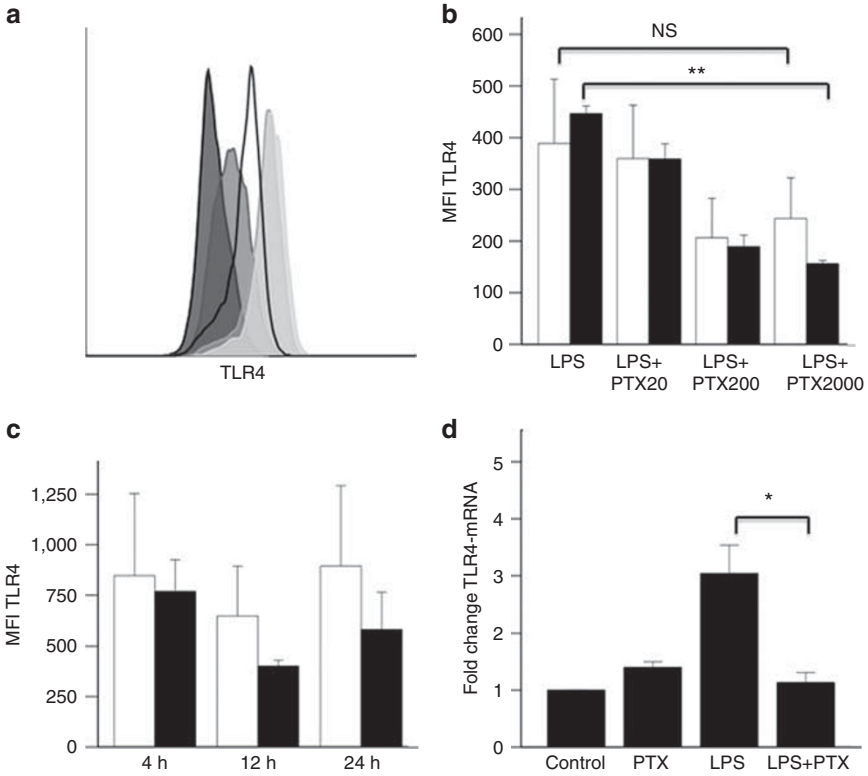

Figure 5. Effect of pentoxifylline (PTX) on Toll-like receptor 4 (TLR4) surface expression and TLR4 messenger RNA (mRNA) from monocytes of preterm neonates and adults. (**a**) Respective histograms of TLR4 expression on monocytes of a preterm neonate treated with LPS (10 ng/ml) and PTX (20–2,000 µg/ml). Black line with white filling indicates isotype, light gray indicates LPS only, medium gray indicates LPS+PTX 20 µg/ml, dark gray indicates LPS+PTX 200 µg/ml, and black fill indicates LPS+PTX 2000 µg/ml. (**b**) Downregulation of TLR4 in monocytes of a whole-blood culture (24 h). In adults (filled boxes), TLR4 expression was significantly downregulated in a dose-dependent manner (**$P < 0.005$). In preterm neonates (blank boxes), this effect was less pronounced and did not reach significance. Values are shown as mean fluorescence intensity (MFI). The experiment was conducted with three independent samples from adult and preterm infants each. Bars show mean value ± SEM. (**c**) Downregulation of TLR4 in monocytes of a peripheral blood mononuclear cell culture incubated for 4–24 h with LPS (10 ng/ml; blank boxes) and LPS+PTX (200 µg/ml; black boxes). Results indicate a downregulation of TLR4 starting at 12 h of PTX treatment, although not statistically significant. The experiment was conducted with three independent adult samples. Bars show mean value ± SEM. (**d**) For TLR4 m-RNA, monocytes were stimulated with LPS (10 ng/ml) and PTX (200 µg/ml) for 6 h. Reverse-transcriptase PCR was conducted as described in the Methods section, and m-RNA was normalized to 18s RNA. On mRNA level, TLR4 was upregulated upon LPS stimulation as expected. This effect was significantly suppressed if PTX was co-administrated (*$P < 0.05$). PTX alone did not have any influence on TLR4 mRNA. Values are shown as fold change in relation to unstimulated control. The experiment was conducted with three independent adult samples.

production by IL-10 may occur through a mechanism involving the inhibition of NF-κB activation directly or indirectly by stabilizing IκB in human monocytes and macrophages (28,29).

Age-dependent differences found in this study most likely arise from functional immunological immaturity of preterm neonates. Well-described differences between the adult and neonatal immune system concern physiological inhibitory substances in neonatal blood that might accelerate the effects of PTX. Neonatal blood plasma contains relatively high concentrations of adenosine, and neonatal cord blood MNCs demonstrate increased sensitivity to the cyclic AMP (cAMP)-mediated inhibitory effects of adenosine (30). PTX is an inhibitor of PDEs and most of its functions are thought to be mediated by enhanced cyclic AMP levels as a result of reduced degradation of cyclic AMP via PDE (31). As shown for human PMN, immunomodulatory properties of PTX are mediated via adenosine-dependent pathways. Adenosine plus PTX lead to a profound inhibitory effect on pro-inflammatory cytokine production (32). It is conceivable that high adenosine concentrations in neonates contribute to a differentiated effect of PTX in the hyperinflammatory state of neonatal sepsis.

Further studies on (1) the mechanisms by which neonatal adenosine concentration influences the dose-dependent efficacy of PTX (2), the signaling capacities of TLR4 under the influence of PTX, and (3) the ability of PTX to reduce phagocytic activity are required to elucidate the complex mechanisms and interactions that may occur in the scenario of neonatal sepsis.

Study Limitations

The present study has several limitations. First, for some of our experiments, we used whole-blood assays to mimic the

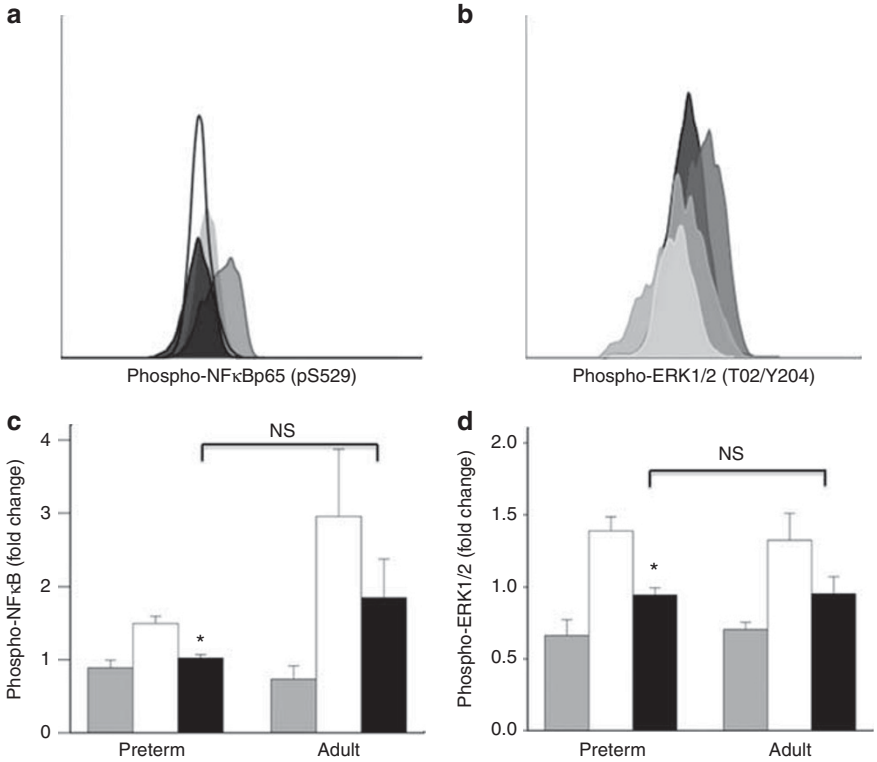

Figure 6. Effect of pentoxifylline (PTX) on phosphorylation pattern of nuclear factor-κB and ERK 1/2. Whole blood was incubated with PTX (200 µg/ml) for 24 h. Thereafter, blood was stimulated with LPS (10 ng/ml) for 10 min at 37 °C. (**a**) Respective histogram of phosphorylation of NF-κB. White filling indicates medium only, light gray indicates isotype, dark gray indicates LPS, and black indicates LPS+PTX. (**b**) Phosphorylation of ERK 1/2 in CD14-positive monocytes of a preterm neonate. Light gray indicates medium only, medium gray indicates isotype, dark gray indicates LPS, and black indicates LPS+PTX. PTX stimulation resulted in a significant reduction of phospho-nuclear factor-κB (**c**) and phospho-ER 1/2 activity (**d**) in the preterm neonate (*$P < 0.05$). There was no age-dependent difference regarding this effect of PTX. Gray boxes indicate PTX, blank boxes indicate LPS, and black boxes indicate LPS+PTX. Mean data (± SD) from adults and preterm neonates are shown.

physiological environment. However, by applying this method, our results might have been influenced by interference from other cells and mediators. For this reason, our main results (CD14, TLR4, and TNF-α) were confirmed in monocytes from a PBMC culture or shown in primarily isolated monocytes (mRNA of TLR4). Because of the technical problems, we could not measure intracellular IL-10.

Second, incubation time was up to 24 h in the whole-blood culture in some experiments. We cannot exclude any influence of sample hypoxia or glucose deprivation on our results by the length of incubation time.

Third, because of the very small sample size volumes of cord blood in preterm neonates of ⩽32 weeks of gestation, not all tests could be performed with each sample of neonatal blood, and the number of individuals tested varied for each age group depending on the experiment.

CONCLUSION

The immunomodulatory properties of PTX cause a wide range of effects on differentiation, maturation, cytokine production, as well as TLR4 expression, signaling, and phagocytic activity of neonatal and adult monocytes. Our *in vitro* model provides insight into age-dependent differences of PTX with a focus on preterm infants. We found distinct immunological responses in preterm infants leading to quantitative and qualitative differences in the levels of surface markers and cytokine production. The present data contribute to our understanding of the therapeutic mechanisms and beneficial effects of PTX for adjunctive treatment of sepsis in preterm infants.

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