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Original Article

Modulation of *S. epidermidis*-induced innate immune responses in neonatal whole blood

Birte Tröger [a,*], Mathias Heidemann [a], Ines Osthues [a], Dennis Knaack [b], Wolfgang Göpel [a], Egbert Herting [a], Johannes K.-M. Knobloch [c], Christoph Härtel [a]

[a] *Department of Pediatrics at the University of Lübeck, Lübeck, Germany*
[b] *Institute of Medical Microbiology, University Hospital Münster, Münster, Germany*
[c] *Institute of Medical Microbiology, Virology and Hygiene, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany*

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Abstract *Background:* Coagulase-negative staphylococci (CoNS) such as *Staphylococcus epidermidis* are highly prevalent pathogens for sepsis in neonates. The interaction between host, environment and pathogenic factors of *S. epidermidis* are still poorly understood. Our objective was to address the role of several pathogenic factors of *S. epidermidis* on neonatal cytokine responses and to characterize the influence of three immunomodulatory drugs.
Methods: We performed an ex-vivo model of *S. epidermidis* sepsis by assessment of blood cytokine production in neonatal whole blood stimulation assays (ELISA). *S. epidermidis* strains with different characteristics were added as full pathogen to umbilical cord blood cultures and the influence of indomethacin, ibuprofen and furosemide on neonatal immune response to *S. epidermidis* was evaluated (Flow cytometry).
Results: Stimulation with *S. epidermidis* sepsis strains induced higher IL-6 and IL-10 expression than stimulation with colonization strains. Biofilm formation in clinical isolates was associated with increased IL-10 but not IL-6 levels. In contrast, stimulation with mutant strains for biofilm formation and extracellular virulence factors had no major effect on cytokine expression. Notably, addition of ibuprofen or indomethacin to *S. epidermidis* inoculated whole blood resulted in mildly increased expression of TNF-α but not IL-6, while frusemide decreased the production of pro-inflammatory cytokines, i.e. IL-6 and IL-8.

* Corresponding author. Department of Pediatrics at the University of Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. Fax: +49 451 500 42804.
E-mail address: birte.troeger@uksh.de (B. Tröger).

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Conclusions: The virulence of sepsis strains is coherent with increased cytokine production in our whole-blood *in-vitro* sepsis model. Biofilm formation and expression of extracellular virulence factors had no major influence on readouts in our setting. It is important to acknowledge that several drugs used in neonatal care have immunomodulatory potential.

Introduction

Neonatal sepsis is a systemic bacterial infection of the newborn and continues to be a major cause of mortality and long-term morbidity in term and preterm infants. The individual risk profile is mainly influenced by gestational age, immaturity of host defences, requirement of invasive treatment modalities and prolonged hospitalisation.[1,2] Coagulase-negative staphylococci (CoNS) are the predominant pathogen of late-onset sepsis (LOS) in preterm infants in developed countries.[3–5] The majority of CoNS infections are caused by *Staphylococcus epidermidis*, which colonize the skin immediately after birth and rapidly dominate the microflora of various body sites within the first month of life.[6] Although *S. epidermidis* infections are rarely fatal (1–2%), they may cause long-term morbidity among preterm infants such as chronic lung disease (bronchopulmonary dysplasia (BPD)).[1,4,7–10] In contrast to BPD in the pre-surfactant area, the new BPD is regarded as primarily an endothelial disease, in which neonatal sepsis contributes a main risk factor in addition to low gestational age and prematurity. In a previous observational study we noted that the immune response of preterm infants to *S. epidermidis* is unbalanced and characterized by up regulated and gestational age dependent proinflammatory responses but insufficient regulatory control.[11] In the development of BPD, the systemic endothelial inflammatory response interacts with the respiratory epithelium, resulting in impaired vascularization of the pulmonary capillary bed, arrest of alveolar development and lung fibrosis.[12] Ivarsson et al. investigated inflammatory responses in endothelial and respiratory cells after exposure to neonatal blood isolates of *S. epidermidis* and *Staphylococcus aureus*. *S. epidermidis* induced higher chemotactic response compared with *S. aureus* providing condition for a persistent transmission of inflammatory cells into lung tissue in sepsis caused by *S. epidermidis* potentially contributing to the development of BPD.[13]

Besides immunological factors of the host, pathogenic factors of *S. epidermidis* may account for the high vulnerability of newborns to infection. Virulence of *S. epidermidis* relies on different mechanisms, such as biofilm development, expression of extracellular virulence factors and genetic flexibility to evade hosts' immune responses.[14,15] Yet, the host-CoNS interaction in neonates is less understood which makes development of specific preventive strategies a difficult task. In addition, several immunomodulatory influences e.g. drugs frequently used in the early postnatal period of infants requiring intensive care, may impact on the delicate interplay between host immunity and exposure to CoNS.

It was the aim of our study to address the role of several pathogenic factors of *S. epidermidis* on cytokine responses in neonatal whole blood stimulation assays and to characterize the influence of immunomodulatory drugs, in particular indomethacin, ibuprofen and frusemide.

Materials and methods

Work flow – characterization of the host inflammatory response to *S. epidermidis* in neonatal whole blood

1. Characterization of *S. epidermidis* strains from clinical sepsis and colonisation isolates (biofilm production, presence of the *icaA* gene, presence of the *mecA* gene, oxacillin resistance, MLTST-ST)
2. Stimulation studies
   a. Stimulation of neonatal umbilical cord blood with clinical sepsis and colonisation isolates of *S. epidermidis*
      - Are there differences in cytokine responses to clinical sepsis and colonisation isolates?
   b. Stimulation of neonatal umbilical cord blood with *S. epidermidis* wild type 1457 and mutants with different degrees of biofilm formation and extracellular virulence factors
      - Do cytokine responses in neonatal whole blood depend on certain pathogenic factors of *S. epidermidis*?
   c. Stimulation of neonatal of neonatal umbilical cord blood with *S. epidermidis ATCC 12228* (commercially available product for research use) after incubation with indomethacin, ibuprofen and furosemide
      - Influence of immunomodulatory drugs on neonatal cytokine responses after stimulation with *S. epidermidis*?

Study population

In a single centre study, heparinized cord blood samples of 68 newborn infants (32 + 2 weeks–41 + 4 weeks) born in the perinatal unit of the University of Lübeck, Germany, were obtained immediately after birth. Blood was taken from the umbilical cord to avoid additional blood sampling in study infants and to acquire a sufficient amount of blood. We performed an *ex vivo* model of *S. epidermidis* sepsis, as described earlier.[11] *S. epidermidis* strains with different characteristics were added as full pathogen to whole blood cultures under defined conditions. In addition,

Lipopolysaccharide (LPS) and Pam_3Cys-Ser-$(Lys)_4$ trihydrochloride (Pam3Cys; derived from N-terminus of bacterial lipoproteins) were used as control stimulants for the initiation of immune responses. LPS, a component of gram-negative bacteria activates Toll-like receptor 4 (TLR4) whereas Pam3Cys has been shown to be a potent agonist of TLR1/TLR2 (Toll-like receptor 1 and 2 complex). Staphylococcal lipoproteins have been identified as potent TLR2 ligands. Blood from healthy adult blood donors served as controls. Cytokines were analysed directly at the single cell level by flow cytometry or assayed for their secretion in culture supernatants by enzyme-linked immunosorbent assay (ELISA).

Inclusion criteria

Inborn preterm and healthy term infants with unremarkable perinatal history for infection were included.

Exclusion criteria

Infants with lethal abnormalities, amniotic infection, histological chorioamnionitis as cause of preterm delivery and early onset sepsis (EOS, sepsis within the first 72 h of life) were not considered for this study.

Whole blood culture

All blood samples were collected in lithium—heparin tubes (Sarstedt, Nümbrecht, Germany) and were stored at room temperature for no longer than 24 h before processing. For assessment of cytokine secretion into culture supernatants, whole blood specimens were suspended in RPMI (Rosewell Park Memorial Institute; cell culture media) 1640 supplemented with 1% penicillin/streptomycin, 2 mM glutamine, 1 mM pyruvate and non-essential amino acids (Seromed Biochrome) at a concentration of 5×10^6 white blood cells/ml and stimulated with 30 ng/ml LPS or *S. epidermidis* (as indicated for each study, whole bacteria, not heat inactivated) for 24 h. Unstimulated controls were added to each experiment. After incubation, 2 ml of the supernatant was withdrawn for ELISA and frozen at $-80\ ^\circ C$ for analysis of cytokine production. ELISA analysis was performed for human IL-6, IL-10 and tumour necrosis factor α (TNF α).

Assessment of intracellular cytokine production in CD14+ cells

Heparinised whole blood was suspended in RPMI 1640 supplemented with 1% penicillin/streptomycin, 2 mM glutamine, 1 mM pyruvate and non-essential amino acids at a concentration of 5×10^6 white blood cells/ml (peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN)). To induce intracytoplasmic production of proinflammatory cytokines in monocytes, whole blood cultures were stimulated with 30 ng/ml LPS or *S. epidermidis* (strains as indicated for each study) for 4 h. Cells were exposed to 3 μM monensin (Sigma, Deisenhofen, Germany) during the whole stimulation period, followed by fixation with 4% paraformaldehyde (Riedel de Haen, Seelze, Germany). An unstimulated control was added in each experiment. For intracellular staining, cells were washed in Hanks's buffered salt solution (HBSS) and resuspended in a buffer consisting of HBSS, 0.1% saponin (Riedel de Haen, Selze, Germany) and 0.01 M HEPES buffer (Seromed Biochrome, Berlin, Germany). Cells (200 μl aliquots) were added to tubes containing 0.5 μg/10 μl of monoclonal antibodies (mAbs; BD Pharmingen, Heidelberg, Germany) against CD14 (M5E2, phycoerythrin-conjugated), IL-6 (MQ2-13A5, FITC-conjugated), tumour necrosis factor α (TNFα) (MAb11, FITC-conjugated) and IL-8 (G265-8 FITC-conjugated). Preincubation with a surplus of unconjugated anticytokine mAbs (5 μg/10 μl; Pharmingen) served as a negative control for intracellular staining to each sample. Isotype-specific antibodies were used to detect irrelevant specificity for surface molecule staining.

Flow cytometry

Flow cytometric analysis was performed on BD FACS Canto (Pharmingen). A total of at least 2000 CD14+ cells were acquired from each sample. Dead cells were excluded by forward and side scatter gating. Thresholds were set according to the unconjugated anticytokine mAb control. Positive cells $<2\%$ were allowed beyond the statistical marker. Data were expressed as a percentage of CD14+ cells.

Characterization of *S. epidermidis* strains

The clinical isolates were identified by mass spectrometry using matrix-assisted laser desorption ionization — time of flight (MALDI-TOF; Bruker Daltonics, Bremen, Germany) and genotypic characterization was performed by multi locus sequence typing (MLST).[16] Oxacillin-resistance was determined phenotypically by using an oxacillin screening agar (6 mg/L oxacillin) as well as Vitek 2 (bioMérieux) and by detection of the *mecA* gene by polymerase-chain reaction (PCR). *S. epidermidis mecA* primers were used in accordance with Rohde et al.[17] Biofilm formation was carried out according to the method described by Christensen.[18,19] Therefore, the phenotypic biofilm-forming capacity was investigated by a well plate assay. Biofilm formation was analysed with trypticase soy broth (TSB) growth medium and quantified in a microtiter plate reader after staining with gentian violet with subsequent ethanol elution. A biofilm positive phenotype was defined as a value of ≥ 0.1 in the optical density measurement in at least one of the used growth media. Additional, the presence of *icaA* gene as a surrogate marker for the possibility of the formation of a polysaccharide intracellular adhesin (PIA) - dependent biofilm was verified by PCR.

Preparation of *S. epidermidis* strains

The extinction at 1×10^8 CFU (Colony Forming Units) per millilitre was determined in previous experiments for each strain. Bacteria were grown on a sheep's blood agar, picked and dissolved by vortexing in an isotonic sodium chloride solution. In each case, 5×10^6 leukocytes were used, and were placed in sterile 6-well plates with RPMI medium, which was previously treated with 10,000 μg/ml Penicillin/

streptomycin, 200 mM L-alanyl-L-glutamine, 100 mM sodiumpyruvate and non-essential amino acids. Subsequently, the blood was stimulated with 1 or 10 CFU/WBC (Colony Forming Unit/White Blood Cell) and incubated for 24 h at 37 °C in a 5% CO_2-enriched atmosphere. An unstimulated sample served as a negative control. The cell-free supernatants were transferred into small tubes and frozen at −20 °C before in preparation for ELISA.

***In vitro* study I: evaluation of cytokine responses to *S. epidermidis* strains with different pathogenic factors**

Cytokine response was tested for 13 *S. epidermidis* strains with different pathogenic factors using ELISA Origin and properties of the bacterial strains are shown in Table 1. *S. epidermidis* strains were characterized using MALDI-TOF, genotypic characterization, phenotypically by using an oxacillin screening agar as well as Vitek 2 and by detection of the *mecA* gene by PCR. Biofilm formation was carried out according to the method described by Christensen.[18,19] In total, cord blood was available from 29 patients. Cord blood of n = 5 patients was stimulated with the sepsis strains. Cord blood of n = 19 patients was used for stimulation with colonizing strains. Each colonization isolate was used to stimulate blood of n = 5 patients. Due to low umbilical cord blood volume it was not possible to perform stimulation of every patient's blood with each colonization isolate. Additionally, cord blood samples from n = 5 patients were stimulated with *S. epidermidis* 1457 and mutants. Assessment of intracellular cytokine production was performed as described before.

In vitro* study II: influence of immunomodulatory drugs on neonatal immune response to *S. epidermidis

Cord blood of 11 newborns was incubated with three different concentrations of indomethacin (0.1 μg/ml, 1 μg/ml, 10 μg/ml) and cord blood of 22 newborns was pre-incubated with two different concentrations of ibuprofen (1 μg/ml, 10 μg/ml) (1 h incubation time of indomethacin and ibuprofen). The usual therapeutic dose *in vivo* is 1—4 μg/ml for indomethacin and 10—100 μg/ml for ibuprofen. Assessment of intracellular cytokine production in CD14+ cells was performed as described before and cytokine concentration was measured by flow cytometry after stimulation by *S. epidermidis* ATCC 12228 and LPS. Cord blood of 6 newborns was incubated with three different concentrations of frusemide for 1 h (1 μg/ml, 10 μg/ml, 100 μg/ml). The therapeutic frusemide dose *in vivo* is 5—50 μg/ml. Cytokine concentration was measured by flow cytometry after stimulation by 10 CFU/WBC *S. epidermidis* ATCC 12228, Pam3Cys and LPS.

Ethical approval

Ethical approval was given by the University of Lübeck Ethical Committee (AZ 11-187). Informed written consent was given by parents (as legal representatives) on behalf of their infants.

Statistical analysis

Data analysis was performed using the SPSS 22.0 data analysis package (Munich, Germany). The chi-square test,

Table 1 *S. epidermidis* sepsis-, colonizing, wild type- and deletion mutant-strains and characteristics.

Strain name	Origin	Biofilm production	Presence of *icaA* gene	Presence of *mecA* gene	Oxacillin resistant/ sensitive	MLST-ST
S1 −r	Preterm infant with sepsis	negative	negative	positive	resistant	59
S2 −r	Preterm infant with sepsis	negative	negative	positive	resistant	59
C1 +r	Routine swabs	positive	positive	positive	resistant	2
C2 +r	Routine swabs	positive	negative	positive	resistant	498
C3 −r	Routine swabs	negative	negative	positive	resistant	5
C4 −r	Routine swabs	negative	negative	positive	resistant	new
C5 +r	Routine swabs	positive	positive	positive	resistant	2
C6 −s	Routine swabs	negative	negative	negative	sensitive	495
C7 −s	Routine swabs	negative	negative	negative	sensitive	110
M1 +s[a]	*S. epidermidis* 1457	positive	positive	negative	sensitive	86
M2 +s[b]	*S. epidermidis* 1457 M10	negative	positive	negative	sensitive	86
M3 +s[c]	*S. epidermidis* 1457sigB	positive	positive	negative	sensitive	86
M4 +s[d]	*S. epidermidis* 1457sigBagr	positive	positive	negative	sensitive	86

The following code is used to characterize the strains: S = sepsis isolate, C = colonization isolate; M = *S. epidermidis* 1457 and its mutants, ability to form biofilms in TSB-medium (+ = biofilm positive; − = biofilm negative), oxacillin-resistance (s = oxacillin-sensitive; r = oxacillin-resistant). The superscript indicates the characteristics of *S. epidermidis* 1457 and his mutants.

[a] Isolate of an infected central venous catheter, PIA/HA positive, strongly biofilm-forming. (PIA as the main matrix component,[29] low expression of extracellular virulence factors).[36]

[b] PIA negative by insertion of transposon Tn917 in *ica*A gene *ica*ADBC; biofilm negative, depending on the environment low biofilm formation possible (in TSB and glucose medium biofilm formation DNA and protein rich),[29] other virulence factors.[37,38]

[c] Decreased biofilm formation, increased expression extracellular virulence factors (lipases and proteases).[39]

[d] Decreased biofilm forming, decreased extracellular virulence factors.

Fisher's exact test and Mann—Whitney U test were applied for statistical analysis of differences between different groups. The level of significance was defined as $p < 0.05$ in single comparisons.

Results

In vitro study I: sepsis strains induce higher IL-6 and IL-10 levels in cord blood than colonizing strains

As outlined in Fig. 1a, IL-6 expression in cord blood was higher after stimulation with isolates derived from *S. epidermidis* sepsis cases as compared to isolates derived from colonization swabs. We also noted higher IL-10 levels after stimulation with *S. epidermidis* sepsis strains at an inoculation dose of 10 CFU/WBC as compared to stimulation with colonizing isolates (Fig. 1b). Detailed data are given in Table 2a.

No differences in IL-6 and IL-10 expression were detected between sepsis- and colonizing isolates of *S. epidermidis* in adult whole blood stimulation assays (n = 5 adults) (Table 2b).

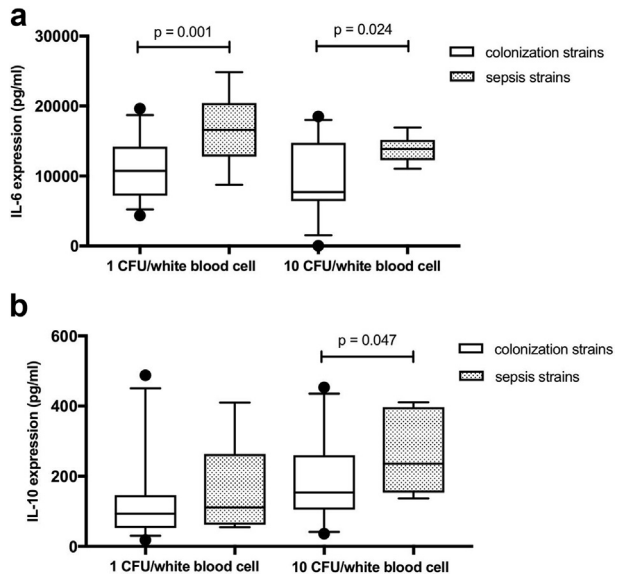

Figure 1. a: The role of different *S epidermidis* strains for the induction of proinflammatory cytokine production. Cord blood samples of n = 5 newborns were stimulated with sepsis strains, cord blood samples of n = 19 newborns were stimulated with colonizing strains. Increased IL-6 in pg/ml production after stimulation with *S. epidermidis* sepsis strains. Whole blood samples were stimulated with 1 or 10 colony-forming units (CFU)/WBC *S. epidermidis* colonization (white bars) and sepsis (grey bars) strains at the same time for 24 h as indicated. Cytokines were measured by ELISA (pg/ml). Vertical bars indicate 25/75 percentiles and 5—95% CI, horizontal lines indicate the median value of all tested subjects. Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant. **b: The role of different *S. epidermidis* strains for the induction of anti-inflammatory cytokine production**. Cord blood samples of n = 5 newborns were stimulated with sepsis strains, cord blood samples of n = 19 newborns were stimulated with colonizing strains. Increased IL-10 production in pg/ml after stimulation with 10 CFU/WBC *S. epidermidis* sepsis strains. Whole blood samples were stimulated with 1 or 10 colony-forming units (CFU)/WBC *S. epidermidis* colonization (white bars) and sepsis (grey bars) strains at the same time for 24 h as indicated. Cytokines were measured by ELISA (pg/ml). Vertical bars indicate 25/75 percentiles and 5—95% CI, horizontal lines indicate the median value of all tested subjects. Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

Pathogenic factors and cytokine responses

We investigated whether biofilm formation and sensitivity/resistance to oxacillin influenced cytokine production. Biofilm formation did not affect IL-6 expression while higher IL-10 levels were found after stimulation with 10 CFU/WBC biofilm-forming *S. epidermidis* isolates (Table 3a). Oxacillin-sensitive strains induced higher IL-6 levels after inoculation with 10 CFU/WBC as compared to resistant strains (Table 3b).

S. epidermidis mutants and cytokine responses *in vitro*

Cytokine responses were tested for *S. epidermidis 1457 wild type and mutants* (M1-M4, strain characteristics Table 1). As outlined in Fig. 2a, no differences for IL-6 were seen between stimulation of cord blood (n = 5 newborns) with *S. epidermidis* 1457 wild-type and mutants of regulators expressing different phenotypes for biofilm formation and extracellular virulence factors.

The same applies to IL-10, although biofilm forming *S. epidermidis* colonizing isolates induced slightly, but non-significantly, higher levels of IL-10 compared to that of the biofilm non-producers when stimulated with 10 CFU/WBC (Fig. 2b).

Table 2a Interleukin expression of sepsis compared to colonizing isolates of *S. epidermidis* in newborns.

	Sepsis strains Median, 95% CI, pg/ml	Colonization strains Median, 95% CI, pg/ml	p
IL-6 (1 CFU/WBC)	16599, 13368—20247	10822, 9832—12747	**0.001**
IL-6 (10 CFU/WBC)	13640, 12224—15093	10590, 8362—11613	**0.024**
IL-10 (1 CFU/WBC)	111, 67—260	85, 81—179	0.415
IL-10 (10 CFU/WBC)	235, 181—341	153, 140—220	**0.047**

Neonatal interleukin-expression in cord blood of newborns was assessed after stimulation with sepsis and colonizing isolates of *S. epidermidis*. Cytokines were measured by ELISA (pg/ml). Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

Table 2b Interleukin expression of sepsis compared to colonizing isolates of *S. epidermidis* in adults.

	Sepsis isolates Median, 95% CI, pg/ml	Colonizing isolates Median, 95% CI, pg/ml	p
IL-6 (1 CFU/WBC)	4602, 3310—5953	5350, 3998—6694	0.40
IL-6 (10 CFU/WBC)	9301, 7136—13982	10515, 8441—13632	0.68
IL-10 (1 CFU/WBC)	68, 43—138	94, 56—205	0.60
IL-10 (10 CFU/WBC)	163, 141—238	179, 143—252	0.60

Adult interleukin-expression was assessed after stimulation with sepsis and colonizing isolates of *S. epidermidis*. Cytokines were measured by ELISA (pg/ml). Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

Table 3a Biofilm and interleukin expression.

	Biofilm negative Median, 95% CI, pg/ml	Biofilm positive Median, 95% CI, pg/ml	p
IL-6 (1 CFU/WBC)	10839, 9765—13633	9331, 1879—12438	0.24
IL-6 (10 CFU/WBC)	10094, 8536—13054	11087, 7897—11915	0.56
IL-10 (1 CFU/WBC)	72, 56—154	124, 89—164	0.071
IL-10 (10 CFU/WBC)	138, 104—182	260, 170—313	**0.025**

Neonatal interleukin-expression in cord blood of n = 19 newborns was assessed after stimulation with biofilm positive and biofilm negative colonizing isolates of *S. epidermidis*. Cytokines were measured by ELISA (pg/ml). Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

Table 3b Oxacillin sensitivity and interleukin expression.

	Oxacillin sensitive Median, 95% CI, pg/ml	Oxacillin resistant Median, 95% CI, pg/ml	p
IL-6 (1 CFU/WBC)	14628, 9865—16088	955, 8623—11784	0.10
IL-6 (10 CFU/WBC)	15164, 10722—16909	7445, 7581—10382	**0.003**
IL-10 (1 CFU/WBC)	74, 38—228	95, 81—133	0.83
IL-10 (10 CFU/WBC)	168, 118—243	13, 135.0—241.7	0.60

Neonatal interleukin-expression in cord blood of n = 19 newborns after stimulation with oxacillin sensitive and oxacillin resistant colonizing isolates of *S. epidermidis*. Cytokines were measured by ELISA (pg/ml). Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

For adult control samples (n = 5) no differences for IL-6 and IL-10 were seen between stimulation with *S. epidermidis* 1457 wild-type and mutants (data not shown).

In vitro study II: increased TNF-α production after incubation with indomethacin and ibuprofen after stimulation with *S. epidermidis*

At the single-cell level, the intracytoplasmic TNF-α response of neonatal cord blood CD14+ cells to infection with 1 CFU *S. epidermidis*/WBC was mildly increased after incubation with indomethacin (Fig. 3a) and ibuprofen (Fig. 3b), while Il-6 levels remained unaffected. Stimulation with LPS resulted in a decreased IL-6 (mean ± SD; 63.4 ± 9.1 vs. 57.4 ± 8.8%, p = 0.003) but increased TNF-α response after incubation with 10 μg/ml indomethacin TNF-α (mean ± SD, 13.8 ± 8 vs. 18.4 ± 10%; p = 0.013). After incubation with 10 μg/ml ibuprofen, only stimulation with LPS resulted in an increased TNF-α expression (mean ± SD, TNF-α 24.8 ± 14 vs. 35.9 ± 18%; p = 0.001). IL-6 expression remained unaffected after stimulation with LPS.

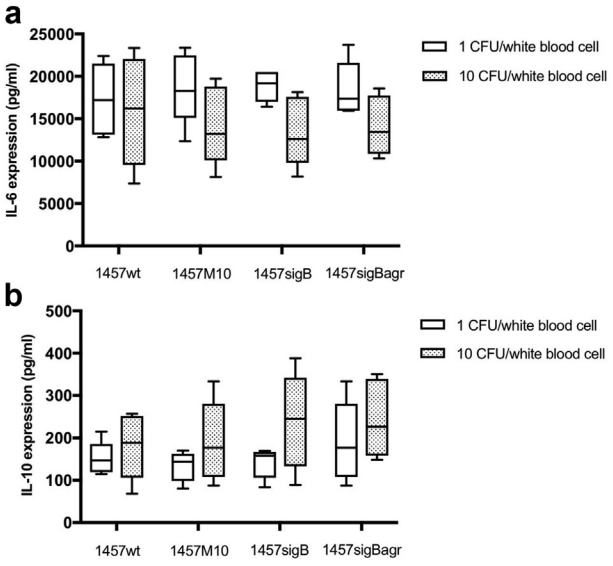

Figure 2. a: IL-6 production after stimulation of *S. epidermidis* 1457 wild type and mutants. b: IL-10 production after stimulation of *S. epidermidis* 1457 wild type and mutants. Cord blood samples of n = 5 newborns were stimulated with 1 (white bars) or 10 (grey bars) colony-forming units (CFU) of *S. epidermidis* 1457 wild type and mutants at the same time for 24 h as indicated. Cytokines were measured by ELISA in pg/ml. Vertical bars indicate 25/75 percentiles and 5—95% CI, horizontal lines indicate the median value of all tested subjects. Characteristics of *S. epidermidis* 1457 wild type and mutants *S. epidermidis* M10, *S. epidermidis* 1457sigB and *S. epidermidis* 1457sigBagr are listed in Table 1.

The intracytoplasmic TNF-α response of adult blood CD14+ cells to infection with 1 CFU *S. epidermidis*/WBC was increased after incubation with 10 μg/ml ibuprofen (mean ± SD 6.8 ± 3.1 vs. 7.9 ± 3.2%; p = 0.004), while Il-6

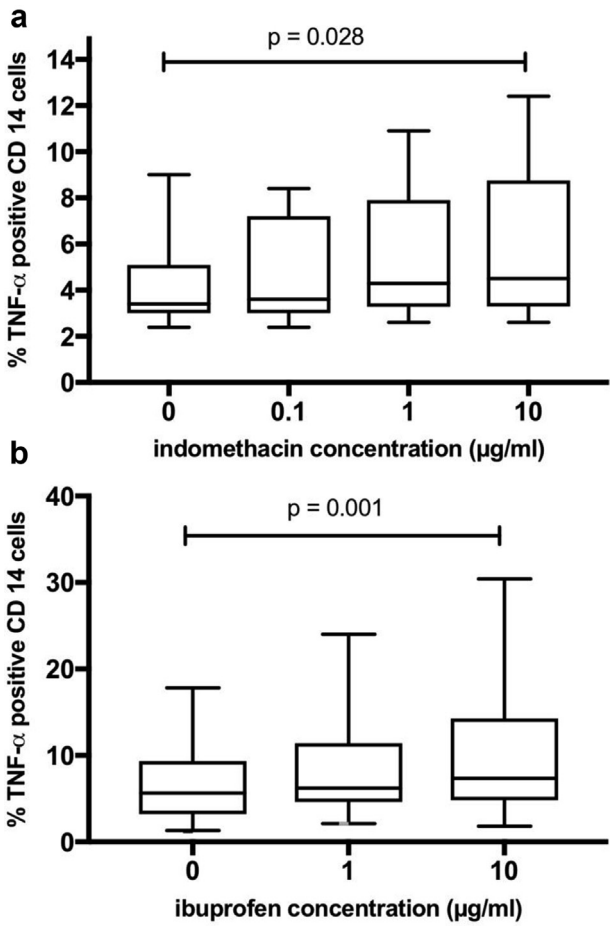

Figure 3. a: Pro-inflammatory cytokine response to *S. epidermidis* is increased after incubation with indomethacin. b: Pro-inflammatory cytokine response to *S. epidermidis* is increased after incubation with ibuprofen. Whole blood cultures were pre-incubated with indomethacin (cord blood of n = 11 newborns) and ibuprofen (cord blood of n = 22 newborns) for 1 h and stimulated for 4 h with 1 colony-forming unit/white blood cell (CFU/WBC) *S. epidermidis* to induce TNF-α response on a single-cell level in CD14+ cells (intracytoplasmic production assessed by flow cytometry). Data are expressed as percentage of cytokine-positive cells. Vertical bars indicate 25/75 percentiles and 5—95% CI, horizontal lines indicate the median value of all tested subjects. Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

levels remained unaffected. After exposure to LPS, the IL-6 and TNF-α expression of adult CD14+ cells was increased after incubation with ibuprofen, i.e. IL-6 (mean ± SD 40.3 ± 12.6 vs. 55.5 ± 10.5%; p = 0.044), TNF-α (40.3 ± 12.6 vs. 55.5 ± 10.5%, p = 0.001).

Decreased IL-6 and IL-8 production after incubation with frusemide

Cord blood of 6 newborns was pre-incubated for 4 h with 15 µg frusemide (10 µg/ml). TNF-α, IL-6 and IL-8 production was assessed on a single-cell level in CD14+ cells after stimulation by *S. epidermidis* ATCC 12228, LPS and Pam3Cys (1 µg/ml). A decreased IL-6 and IL-8 production was found after incubation with frusemide. Frusemide had an anti-inflammatory effect after stimulation with *S. epidermidis* but not after LPS and Pam3Cys (Fig. 4a and b). No effect was detected for TNF-α expression.

Discussion

The immaturity of the neonatal immune system has been assumed to be responsible for the high susceptibility to neonatal infections. Given the high prevalence of complications secondary to late onset infections including bronchopulmonary dysplasia (BPD), continuing research is required to characterize the host inflammatory responses to predominant pathogens such as *S. epidermidis*. In the first part of the present study we analysed *S. epidermidis* strains with different clinical impact (sepsis-versus colonizing strains). In this context, the role of several pathogenic factors of *S. epidermidis* on neonatal cytokine responses was evaluated in detail using *S. epidermidis* 1457

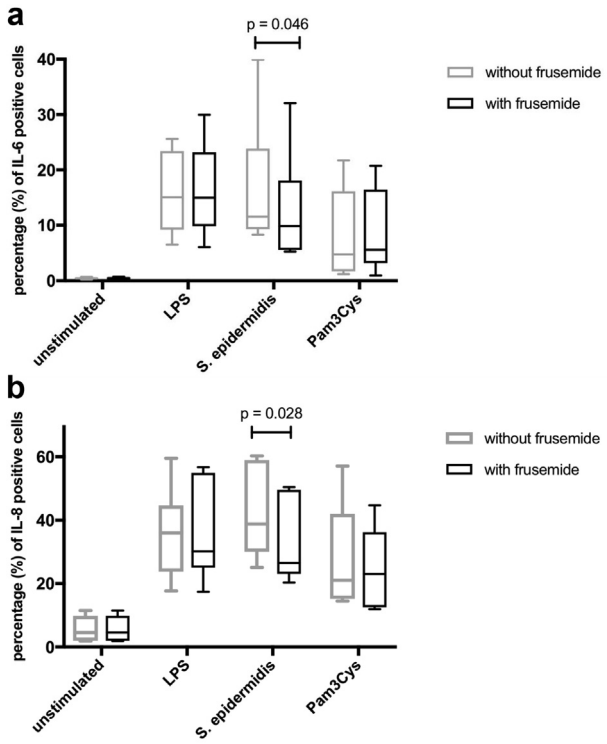

Figure 4. a: Effect of frusemide on IL-6 production in newborns. b: Effect of frusemide on IL-8 production in newborns. Whole blood cultures of term newborns (n = 6) were 4 h incubated with 15 µg frusemide and stimulated for 4 h with *S. epidermidis*, *Pam3Cys* and *LPS* to induce IL-6 and IL-8 production on a single-cell level in CD14+ cells (intracytoplasmic production assessed by flow cytometry). Data are expressed as percentage of cytokine-positive cells. Vertical bars indicate 25/75 percentiles and 5—95% CI, horizontal lines indicate the median value of all tested subjects. Statistical analysis was performed with the Wilcoxon test, a p value of <0.05 was regarded as significant.

and mutants with different phenotypes for biofilm formation and extracellular virulence factors to evaluate neonatal cytokine responses. In addition to the role of several pathogenic factors of *S. epidermidis*, we evaluated in the second part of the study the influence of frequently used immunomodulatory drugs on neonatal cytokine responses in *S. epidermidis* sepsis.

In our whole-blood *S. epidermidis* in-vitro sepsis model stimulation with clinical sepsis isolates induced higher IL-6 and IL-10 expression than stimulation with colonization strains. Biofilm formation in clinical isolates was associated with increased IL-10 expression which was not verified by stimulation settings with mutant strains for biofilm formation and extracellular virulence factors. The potential importance of several drugs used in neonatal care was proposed by the findings that ibuprofen or indomethacin may have even pro-inflammatory effects (TNF-α ↑) while frusemide may inhibit pro-inflammatory cytokine expression (IL-6, IL-8↓).

The reasons for the emergence of *S. epidermidis* as important pathogen in neonates are diverse, including need for invasive procedures and immaturity of the skin barrier.[1,3,7] On the other side pathogenic factors of CoNS like biofilm formation, expression of extracellular virulence factors and resistance to antibiotics play an important role.[20,21]

Previous studies described the strong induction of TNF-α in a whole-blood assay stimulated with cell-free supernatant of CoNS, whereas other groups used whole, heat-inactivated CoNS to induce TNF-α, IL-1ß, IL-6 and IL-12 in human mononuclear leukocytes.[22] In our study we demonstrated that pro- and anti-inflammatory cytokine responses differ between colonizing and sepsis-strains of *S. epidermidis*. After stimulation of whole blood samples with *S. epidermidis* strains isolated from preterm infants with blood-culture proven clinical sepsis (biofilm negative, oxacillin resistant) a significantly higher production of the pro-inflammatory cytokine IL-6 and IL-10 was seen compared to stimulation with *S. epidermidis* colonization-strains.

To examine the effect of pathogenic factors in detail, we compared in the second part of our study pro- and anti-inflammatory cytokine responses to *S. epidermidis* wild type 1457 (isolate from an infected central venous catheter PIA/HA positive, strongly biofilm-forming with low expression of extracellular virulence factors) and three mutants with different degrees of biofilm formation and extracellular virulence factors like lipases and proteases.

Given the limitations of our study setting, biofilm formation, virulence factors and antibiotic resistance had no major impact on neonatal pro- and anti-inflammatory cytokine responses to *S. epidermidis*. However the data set was restricted to n = 5 in a strictly in-vitro experimental setting.

In the context with current literature, we propose that not only distinct pathogenic bacterial factors seem to be responsible for cytokine responses but also their interplay with individually regulated host immune responses and environment. In line with this, Rohde et al. (2004) were able to show that an analysis of *ica*ADBC gene (biofilm) and the *mec*A gene (oxacillin-resistance) is not useful for the differentiation between invasive and colonizing isolates.[17] Vandecasteele et al. (2003) showed that also the analysis of *ica*, *aap* (biofilm) and *atlE* genes (initial adhesion) was unsuitable for the discrimination between colonizing- and sepsis strains.[23] Furthermore, Jain et al. (2009) demonstrated that biofilm formation is found more frequently in sepsis as compared to colonizing isolates.[24,25] Polymorphisms of TLR, IL-6, IL-8 and TNF- may be associated with the differentiated individual risk of developing *S. epidermidis* sepsis.[26,27] In addition, environmental factors may influence the expression of immune-related genes via DNA methylation.[28] A second explanation for our negative findings with regard to biofilm formation might be the clinical setting. Klingenberg et al. reported in the year 2005[14] an increased C-reactive-protein (CRP) response, as a marker for an increased proinflammatory response, in methicillin/aminoglycoside resistant CoNS strains compared with susceptible isolates, while we found lower pro-inflammatory cytokine levels upon stimulation with oxacillin-resistant isolates. Furthermore, Klingenberg et al. demonstrated lower CRP-levels in biofilm-positive CoNS isolates compared with biofilm-negative CoNS strains and concluded that *S. epidermidis* may evade the host's proinflammatory response by masking with a biofilm multilayer (escape-theory). In our setting, biofilm-negative sepsis isolates showed higher pro-inflammatory cytokine expression compared to colonizing isolates with mixed biofilm characteristics. Contrary to the assumption that the ability to form a PIA positive biofilm reduces interleukin production in the host, neither in neonatal nor in the adult group a significant difference in cytokine expression was detectable between the strong biofilm-forming *S. epidermidis* 1457 or its mutants M10 (PIA negative, no biofilm formation), the *sigB* deletion mutant (decreased biofilm formation, increased expression of lipases and proteases) and the *sigBagr* deletion mutant (decreased biofilm formation, decreased extracellular virulence factors). These data do not coincide with a study published by Fredheim in 2011, showing that PIA-negative *S. epidermidis* 1457 M10 was able to mount significantly higher cytokines in comparison to wild-type 1457, which formed a PIA positive biofilm. In the latter study a total of thirteen pro- and two anti-inflammatory cytokines, excluding IL-10, were measured. The mild differences in cytokine expression were significant, however, inter-individual regulation variability was pronounced.[29] A second major aspect is gestational age, as our group previously demonstrated dependency of *S. epidermidis* induced pro-inflammatory but not anti-inflammatory responses with maturity at birth.[11] In this previous study we found that stimulation by a biofilm forming, *ica*ADBC-positive *S. epidermidis* strain isolated from a preterm infant with blood-culture proven sepsis resulted in a significantly lower IL-6-expression than by a biofilm negative strain. Interestingly, the study of Granslo et al. (2013) showed that neonatal immune responses to *S. epidermidis* 1457 and the PIA-negative deletion mutant M10 provided higher IL-6 and IL-8 levels in neonates compared to adults.[15] In our study no differences were seen between neonates and adults which may be related to a different study protocol. Granslo et al. applied incubation times of 30 min representing the initial phase of immune responses, while we selected an incubation period of 24 h and standardized the number of white blood cells in our whole-blood assay.

In the second part of our study we evaluated the immunomodulatory effects of three drugs (indomethacin, ibuprofen and furosemide) on cytokine production. In our experiments we used drug concentrations which are similar to the concentrations used *in vivo*. Indomethacin and ibuprofen led to an increased TNF-α production in the *S. epidermidis* sepsis model as well as with LPS stimulation. However, the clinical relevance of the observation is unclear. Even in the adult group, after stimulation with *S. epidermidis* and LPS, an increased number of TNF-α positive macrophages could be detected with addition of ibuprofen. This result is surprising because one would have expected the anti-inflammatory drugs acting with a reduced pro-inflammatory cytokine production. Studies from recent years also came to similar conclusions. A study by Page et al. 2010 could prove that NSAIDs increase in monocytes from adult patients the production of TNF-α both *in vitro* as well as *in vivo* supporting our own previous observations.[30] It is believed that the increase in TNF-α production is due to the partial anti-inflammatory property of PGE2. By inhibiting COX II through NSAIDs the formation of PGE2 is suppressed. PGE2 has significant immunosuppressive effects by affecting cytokine production. It serves as a negative feedback in macrophages by inhibiting the TNF-α production and limits the inflammatory activity.[31] Furthermore, PGE2 promotes IL-10 production.[32] Apparently by inhibiting PGE2 synthesis with NSAIDs the lack of negative feedback mechanism results in an increased TNF-α production. By addition of PGE2, the effect of NSAIDs can be reversed on TNF-α production.[30,33] By inducing an increased TNF-α production, ibuprofen and indomethacin have a pro-inflammatory effect on the immune system. Considering the fact that pro-inflammatory cytokines play an important role in the pathogenesis of neonatal diseases, these immunomodulatory effects need to be critically considered. In contrast, frusemide was associated with reduced IL-6 and IL-8-expression when stimulated by *S. epidermidis* while no effect on cytokine responses was seen when whole blood was stimulated by LPS or Pam3Cys. The dose-dependent immunosuppressive potential of frusemide (IL-6, IL-8, TNF-α ↓) has been previously described.[34,35] However, the observation in a neonatal *S. epidermidis* sepsis model is novel and deserves and deserves further study including cell specific effect on other immune cell types such as lymphocytes.

There are some limitations to this study. First, immunological markers may be influenced by cause of antenatal exposure to antibiotics and foetal inflammatory response. The majority of study infants were born term rather than preterm. Especially preterm infants with gestational age <28 weeks are considered to be at highest risk for *S. epidermidis* infections and need for immunomodulatory drugs. In addition, individual host immune responses may influence the results due to the small specimen numbers. Finally, whole blood assays are an adequate tool to generate hypotheses *in vitro* but future *in vivo* studies are needed to draw firm conclusions.

In summary, we demonstrated that the virulence of sepsis strains is coherent with increased cytokine production in our whole-blood *in-vitro* sepsis model. Biofilm formation and expression of extracellular virulence factors had no major influence on readouts in our setting. Additionally, indomethacin, ibuprofen and furosemide have had immunomodulatory potential in our study. We conclude that not only distinct pathogenic bacterial factors seem to influence the occurrence of ConS infections, but also their interplay with individually regulated host immune responses in different patient populations (preterm vs. term infants, polymorphisms of Toll-like receptors and TNF) and the environment (invasive devices and catheter care practices, gut colonization and early enteral feeding, prevention strategies of healthcare associated infections, drugs, expression of immune-related genes via DNA methylation influenced by environmental factors) plays an important role in susceptibility to ConS.

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