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REGULAR ARTICLE

Staphylococcus epidermidis and *Staphylococcus aureus* trigger different interleukin-8 and intercellular adhesion molecule-1 in lung cells: implications for inflammatory complications following neonatal sepsis

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ABSTRACT

Aim: Staphylococci are a major contribution for neonatal sepsis, which is the main risk factor for bronchopulmonary dysplasia. This study investigated the expression of pro-inflammatory mediators in endothelial and respiratory cells from newborns exposed to staphylococci.

Methods: Human vascular endothelial cells and small airway epithelial cells were incubated with neonatal blood isolates of *Staphylococcus epidermidis* (n = 14) and *Staphylococcus aureus* (n = 14). The extracellular release of IL-8, IL-10, sICAM-1, ICAM-1 mRNA and the expression of membrane bound ICAM-1 were assessed by ELISA, RT-PCR and immunofluorescence microscopy.

Results: *Staphylococcus epidermidis* induced higher levels of IL-8 (mean 38.5 ng/mL) and ICAM-1 mRNA (mean ratio 1.037) in the small airway epithelial cells than *S. aureus* (IL-8 mean 22.2 ng/mL, $p < 0.01$ and ICAM-1 mRNA mean ratio 0.715, $p < 0.01$). In the endothelial cells, ICAM-1 remained more integrated in the cell membranes after exposure to *S. epidermidis* compared with *S. aureus,* which induced disintegration and release of soluble ICAM-1 into the supernatants.

Conclusion: *Staphylococcus epidermidis* induced a higher chemoattractive response than *S. aureus*. A persistent transmigration of granulocytes into the lung tissue in neonatal *S. epidermidis* sepsis might contribute to the development of bronchopulmonary dysplasia.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease in newborn children defined as prolonged need of extra O_2 supply ≥4 weeks of postnatal age or ≥36 weeks of gestational age corrected for prematurity (1–4). Originally, BPD was described in moderate preterm newborns following severe respiratory distress syndrome (RDS), high-pressure mechanical ventilation and toxic oxygen concentrations (1). However, the clinical spectrum of BPD has changed during the last decades as a result of improvements in neonatal intensive care and increased survival of preterm infants. Nowadays, BPD is almost exclusively seen in extreme preterm (EPT, born <30 weeks gestational age) newborns (1,3,4) and in these patients also without a preceding history of RDS or other respiratory complications (2,4–6). In contrast to the BPD of the presurfactant era, this 'new' BPD is regarded as primarily an endothelial disease, in which neonatal sepsis constitutes a main risk factor in addition to low gestational age and pulmonary immaturity (3,4,7). The immune response of EPT infants is unbalanced and characterized by an excessive up regulation in the case of inflammatory stimulation, but insufficient regulatory control (8–10). For instance, EPT newborns show increased release of IL-6, IL-8 and other proinflammatory mediators after bacteria exposure, but decreased release of IL-10 and other down regulators of the inflammatory cascade (8,9). In the development of BPD, the systemic endothelial

Key notes

- Neonatal sepsis is common and is most often caused by staphylococci.
- Neonatal sepsis is a risk factor for developing bronchopulmonary dysplasia.
- *Staphylococcus epidermidis* induces a higher chemotactic response compared with *Staphylococcus aureus* when challenging endothelial and respiratory cells. This provides condition for a persistent transmission of granulocytes into lung tissue in sepsis caused by *S. epidermidis*, which can be of major importance in the development of bronchopulmonary dysplasia.

inflammatory response interacts with the respiratory epithelium, resulting in impaired vascularization of the pulmonary capillary bed, arrest of the alveolar development and, at the end stage, lung fibrosis (3). The recruitment of circulating neutrophil granulocytes is a key factor in this process (3,11) and increased tracheal aspirate fluid levels of granulocytes, leucocyte adhesion molecules such as ICAM-1, IL-8 and other chemotactic mediators are regularly reported in preterm newborns that subsequently develop BPD (3,11,12). IL-8 is a strong neutrophil chemoattractant and is expressed by a variety of cells, whereas ICAM-1 is expressed on the surface of endothelial and epithelial cells, and mediates the transmigration of enrolled leucocytes into the surrounding tissue. As the inflammatory pathway proceeds towards down regulation, the outer part of ICAM-1 is split by enzymatic cleavage and released into the extracellular compartment as soluble ICAM-1 (sICAM-1) (13).

The predominating aetiologic agents of sepsis in EPT newborns are coagulase negative staphylococci (CoNS), with *Staphylococcus epidermidis* as the most prevalent species, followed by *Staphylococcus aureus* (14), that is, typical pathogens in patients with granulocyte defects. Preterm neutrophils are characterized by an impaired ability of phagocytosis and insufficient bactericidal capacity, but paradoxically an increased oxidative activity compared with adults following bacterial exposure (10,15). The leak of highly oxidative products from activated neutrophils that are captured in the pulmonary capillary bed most probably contributes to exceeding tissue damage and the subsequent development of BPD (3,16). In an earlier study, we found higher incidence of BPD in preterm newborns after *S. epidermidis* sepsis than after neonatal sepsis caused by more virulent bacteria (17). Based on this clinical observation, we hypothesize that CoNS might induce a chronic low-grade pulmonary inflammation associated with a sustained recruitment of circulating granulocytes towards the respiratory epithelium. The aim of this study was to gain more insight into possible host–bacterial interactions in the pathogenesis of BPD by investigating the effect of neonatal blood isolates of *S. epidermidis* and *S. aureus* on the expression of IL-8, ICAM-1 and IL-10 in small airway epithelial and vascular endothelial cells *in vitro*.

METHODS

Bacteria

Blood isolates of *S. epidermidis* from preterm newborns (≤30 w GA) were genetically characterized by the use of pulsed-field gel electrophoresis in an earlier study, carried out at the neonatal intensive care unit of Örebro University Hospital between 1994 and 2004 (10). From that collection, 14 strains of *S. epidermidis* representing different genotypes were selected, together with 14 neonatal blood isolates of *S. aureus* collected during the same period. All bacterial isolates represented clinical sepsis (≥3 clinical signs and a peak C-reactive protein ≥20 mg/L). The bacterial isolates were suspended in preservation medium (yeast extract; DIFCO Laboratories, Detroit, MI, USA) and stored at −70°C pending further analysis. Before each experiment, the bacteria were cultured over night on blood agar medium (4.25% Columbia II Agar [BBL, Becton Dickinson, Baltimore, MD, USA], 0.3% Agar No. 2 [LAB M Ltd., Bury, UK] and 5% bovine blood) at 37°C and checked for purity. One isolate from each plate was suspended in broth, and the bacteria were cultured for 24 h at 37°C to steady state growth phase.

Cell cultures and exposure to bacteria

Early passage human vascular endothelial cells (HMVEC-L) and small airway epithelial cells (SAEC) were obtained cryopreserved from Clonetics®, Lonza Copenhagen ApS, Copenhagen, Denmark. The cells were subcultured according to instructions in EGM-2 MV Bullet Kit Medium (HMVEC-L) or SAGM Bullet Kit Medium (SAEC) obtained from Lonza.

The cells were used for experiments at passage 4–6 with 1:3 expansion of the cultures at each passage. For the gene expression analysis (RT-PCR) and the cytokine analysis in the supernatants, 30 000 cells/well were seeded in 24-well plates, and for the analysis of cell surface, ICAM-1 60 000 cells/well were seeded in 4-well chamber-slides (Nunc/VWR, Stockholm, Sweden) in the appropriate medium containing antibiotics. The cells were cultured for 3 days before the addition of bacteria, with a change to fresh medium 1 day before bacteria addition. Cells were washed three times in Medium 199 with Earle's Salt and GlutaMAX containing 15% heat-inactivated foetal bovine serum but no antibiotics. For each experiment, the bacteria were suspended in PBS, counted in a Bürker chamber and resuspended in the antibiotic-free cell culture medium at a final concentration of 10^4 CFU/mL. This is at the lower end of what could be anticipated in blood during sepsis (18). Individual bacterial strains were administrated into the wells (0.5 mL/well) and incubated at 37°C, 5% CO_2 for different times as indicated. The cellular viability was checked by visual control of the cellular attachment to the wells.

Cell cytotoxicity

Endothelial and respiratory epithelial cells were exposed for one strain of the included strains of *S. epidermidis* (94B080) and one strain of *S. aureus* (90B083) for 1, 4, 6, 12 and 18 h. The cells were washed twice in PBS after removal of medium, fixed in methanol for 20 min and incubated in crystal-violet solution (Sigma-Aldrich Sweden AB, Stockholm, Sweden) for 30 min. Cells were destained thoroughly in tap water and air-dried. Cell viability was estimated by visual inspection of stained cells in bacteria-treated versus control cultures. In addition, each bacteria-exposed cell culture was similarly stained and analysed upon termination of incubation in the case of IL-8 and sICAM-1 experiments.

Extracellular release of IL-8 and sICAM-1

For analysis of the extracellular release of cytokines, the endothelial cells were exposed for the bacteria for 12 h and

the respiratory epithelial cells for 18 h (based on differences in viability after bacterial exposure). The concentrations of IL-8 and soluble ICAM-1 (sICAM-1) in the supernatants of the cell cultures were determined by enzyme-linked immunosorbent assay (ELISA). Quantikine® Human IL-8 Immunoassay (nr D8000C R&D Systems, Minneapolis, MN, USA) were used for analysis of IL-8, and Parameter Human sICAM-1 Immunoassay (nr BBE 1B, R&D Systems) for sICAM-1. The assays were performed according to the manufacturer's instructions using a Multiskan Ascent® microplate reader (Thermo Labsystems, Stockholm, Sweden).

RNA extraction, cDNA synthesis and RT- PCR

Medium was removed from cells in 24-well plates after 18 h (SAEC) or 12 h (HMVEC-L) bacterial exposure and RNA extracted using column separation technique (RNeasy Plus Micro Kit, Qiagen AB, Solna, Sweden). 200 ng of total RNA per sample was used as template for synthesis of cDNA using a commercial kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Semiquantitative RT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) using commercially available fluorescent probes from Applied Biosystems (TaqMan Gene Expression Assays). Probes were intercellular adhesion molecule-1 (ICAM-1, public reference sequence NM_000201.1, item number Hs00164932_m1) and the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, public reference sequence NM_0020 46, item number Hs99999905_m1). PCR reagents were obtained from Applied Biosystems (TaqMan Gene Expression Master Mix). Crossing threshold (Ct) values were calculated by the 7500 Fast Real-Time PCR System software using the second derivative maximum method.

Relative level of gene expression was calculated by first subtracting Ct(ICAM1) from Ct(GAPDH) giving the dCt values, followed by subtraction of each Ct value from an arbitrary chosen Ct value serving as a reference, giving ddCt. Finally, the formula 2^-ddCt was used to calculated relative levels of ICAM-1 gene expression. Expression of GAPDH was stable throughout the experiments.

Immunofluorescence of cell surface ICAM-1

After 10 h of bacterial (*S. epidermidis and S. aureus*) exposure, the cells were washed briefly twice with PBS and incubated for 30 min at 4°C with FITC-conjugated monoclonal anti-ICAM-1 (CD54) antibodies (Beckman Coulter/ Immunotech, Stockholm, Sweden) or IgG1-FITC isotypic control antibodies from the same supplier. Cells were washed three times with PBS and fixed in 0.5% formaldehyde for 15 min at room temperature. Cells were washed twice with PBS and chambers removed. Slides were mounted in antifading medium (Vector Laboratories/Immunkemi, Stockholm, Sweden) and staining intensity evaluated by fluorescence microscopy and visual examination. Positive result was classified as +, ++ and +++. The investigator was blinded for bacterial isolate identification and species.

Statistics

Results are presented as mean ± SD in the text. Box-plots in figures show medians, 75th percentiles (boxes), 90th percentiles bars and outliers (dots). To evaluate significant changes, Student's unpaired t-test was used for ELISA data and mRNA levels. For differences in ICAM-1 staining intensities, significance was determined by use of Fisher's exact test for these categorical data. Significance levels are indicated in texts and figures.

RESULTS

Kinetics of cytokine and sICAM-1 release of bacteria-stimulated cells

To estimate cytokine and sICAM-1 release from the cells after bacteria-stimulation, ELISA measurements were performed over a time period of 18 h of bacteria incubation. For this purpose, one strain of *S. epidermidis* (94B080) and one of *S. aureus* (93B083) were selected. The release of IL-8 increased during the entire incubations regardless of cell type or the two bacterial species tested (Fig. 1A and B). *Staphylococcus aureus* induced successive increasing levels of sICAM-1 in the supernatants, whereas stimulation with *S. epidermidis* resulted in very low, or not detectable levels of sICAM-1 at all measurement time points (Fig. 1C and D). IL-10 was generally below the detection limit for the ELISA kit (<4 pg/mL). Thus, a minimum of 12 h incubation time was required to detect IL-8 and sICAM-1 in our system.

Cytotoxicity

Using the same bacteria strains as above for the kinetic analysis, we found that the respiratory epithelial cells could be incubated with bacteria for up to 18 h without cytotoxic effects, whereas the endothelial cells tolerated a maximum of 12 h incubation. The validity of this for each bacteria strain was confirmed in the IL-8 and sICAM-1 experiments (data not shown).

Cellular release of IL-8

In the respiratory epithelial cells, incubation with *S. epidermidis* resulted in significantly higher release of IL-8 (38.5 ± 9, 11 ng/mL) than stimulation by *S. aureus* (22.8 ± 12.2 ng/mL) ($p < 0.01$; Fig. 2A). For the endothelial cells, IL-8 release was similar for the two types of bacteria; after stimulation with *S. epidermidis,* the release was 668 pg/mL ± 83.2 pg/mL, and with compared to *S. aureus* the release was 642 ± 130 pg/mL (Fig. 2B).

Cellular release of sICAM-1

In the respiratory epithelial cells, all bacterial strains induced release of sICAM-1 into the supernatants, and the levels were similar regardless of bacterial species (*S. aureus* 3.2 ± 1.9 ng/mL; *S. epidermidis* 2.8 ± 0.42 ng/mL, $p = 0.39$). In the endothelial cells, however, none of the isolates of *S. epidermidis* induced any detectable release of sICAM-1 into the supernatants in contrast to all strains of *S. aureus* (mean 3.02 ± 1.72 ng/mL; Fig. 3A).

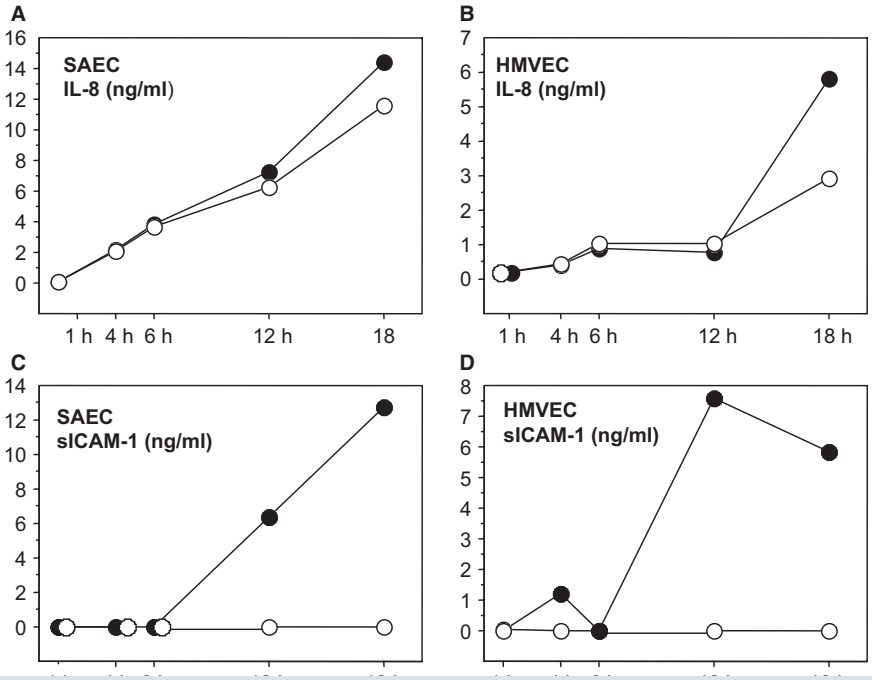

Figure 1 Kinetics in the release of IL-8 and sICAM-1 from small airway respiratory epithelial cells (SAEC) and human vascular endothelial cells (HMVEC) after exposure to *Staphylococcus aureus* 90B083 (n = 2) (●) and *Staphylococcus epidermidis* 94B080 (n = 2) (○). Both bacterial strains originate from neonatal blood cultures.

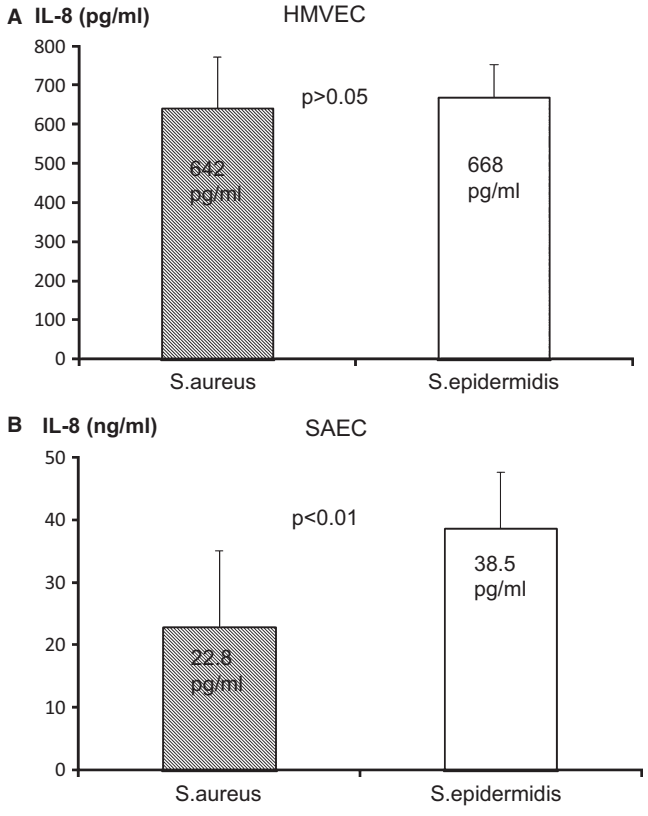

Figure 2 Release of IL-8 in human vascular endothelial cells (A) and small airway respiratory epithelial cells (B) after exposure to neonatal blood isolates of *Staphylococcus aureus* (n = 14) and *Staphylococcus epidermidis* (n = 14). *Staphylococcus epidermidis* induced significantly more IL-8 in epithelial cells than did *S. aureus* ($p < 0.01$).

ICAM-1 mRNA analysis

Staphylococcus epidermidis induced a 45% increase in ICAM-1 mRNA in the respiratory epithelial cells compared with *S. aureus* ($p < 0.001$; Fig. 3B). In the endothelial cells, incubation with the two different bacterial species resulted in similar levels of ICAM-1 mRNA (Fig. 3B).

Cellular expression of membrane bound ICAM-1

Immunofluorescence microscopy of the endothelial cells showed expression of membrane bound ICAM-1 after exposure to all isolates of *S. epidermidis* (n = 14, one isolate +++, eleven isolates ++ and two isolates +) and three isolates of *S. aureus* (one isolate +++, one isolate ++ and one isolate +), whereas most isolates of *S. aureus* (n = 11) were not associated with endothelial cellular expression of membrane bound ICAM-1 ($p < 0.001$; Fig. 4).

In the respiratory cells, *S. epidermidis* was associated with a more intense ICAM-1 staining but it was difficult to compare the strains due to that *S. aureus* also induced an increased cellular spreading that might have caused a decrease intensity per cell surface area. Thus, we could not draw any firm conclusions from the ICAM-1 surface expression with respect to the respiratory cells.

DISCUSSION

In this study, we investigated the inflammatory response in endothelial and respiratory epithelial cells after exposure to neonatal blood isolates of *S. epidermidis* and *S. aureus* and found significant differences between bacterial species on expression of pro-inflammatory chemotactic mediators. Thus, we found an increased release of IL-8 in small airway

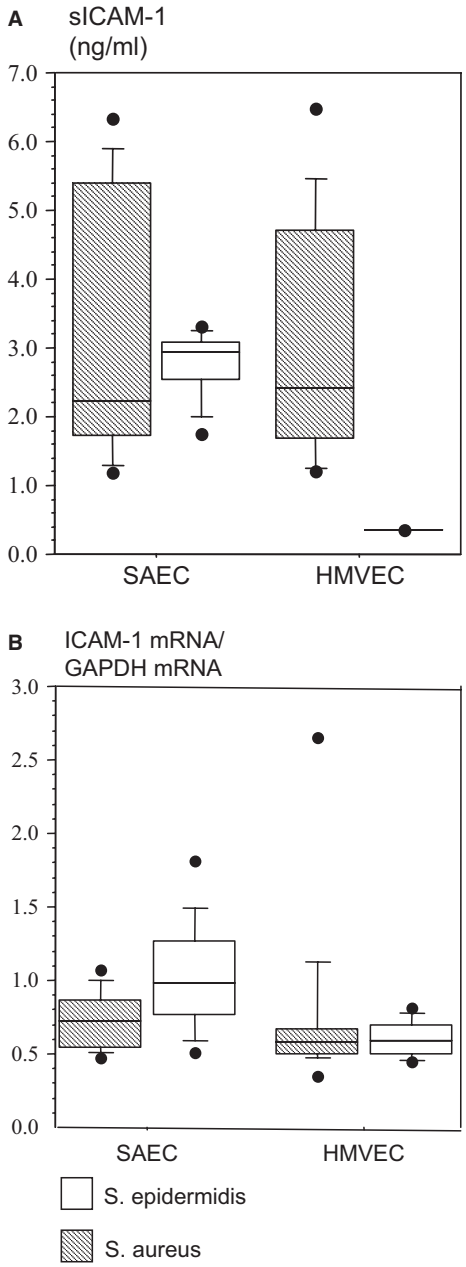

Figure 3 Release of sICAM-1 (A) and expression of ICAM-1 mRNA (B) in small airway respiratory epithelial cells and in human vascular endothelial cells after exposure to neonatal blood isolates of *Staphylococcus aureus* (n = 14) and *Staphylococcus epidermidis* (n = 14). *Staphylococcus aaureus* induced significantly more sICAM-1 in endothelial cells than did *S. epidermidis* ($p < 0.05$). *Staphylococcus epidermidis* stimulated a higher ICAM-1 mRNA expression in epithelial cells than did *S. aureus* ($p < 0.05$).

respiratory cells after stimulation with *S. epidermidis* compared with *S. aureus*. This supports the view that *S. epidermidis* trigger an increased pro-inflammatory activity and attraction of granulocytes towards the pulmonary tissue which is more pronounced compared with that of *S. aureus*. These *in vitro* results are of interest from a clinical point of view, as increased and sustained chemotactic activity in tracheal aspirate fluid is reported to predict

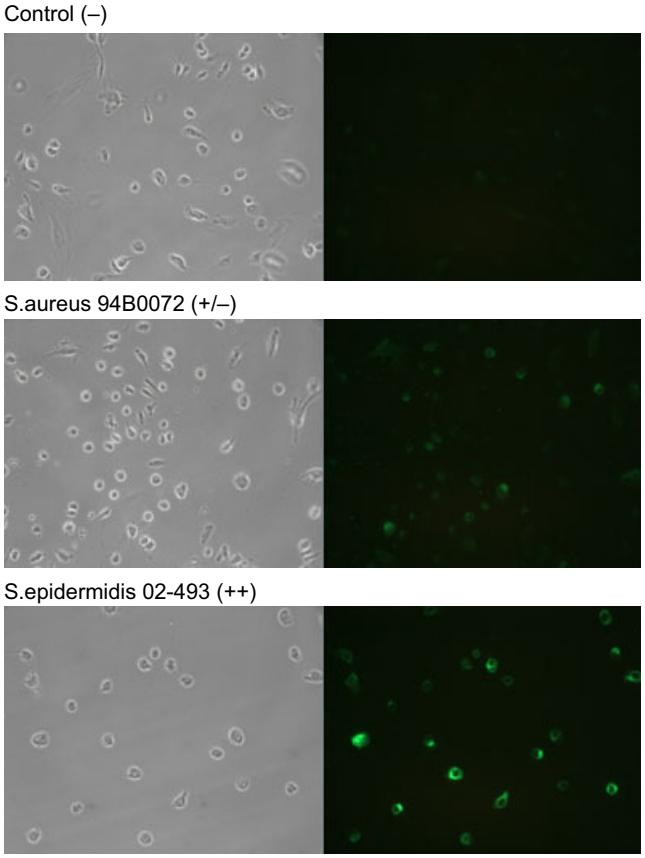

Figure 4 Immunofluorescence images of cell membrane bound expression of ICAM-1 in human vascular endothelial cells after exposure to neonatal blood isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*. The assessments of immunofluorescence intensity are shown in brackets. Phase-contrast images of the same field are shown to visualize the cells. Numbers denote strain identity.

BPD in EPT newborns with respiratory distress syndrome, whereas non-BPD preterm newborns with this syndrome showed a down regulation of chemotactic parameters during the further progression of the inflammatory process (11). The production of ICAM-1 mRNA in endothelial cells was similar regardless of staphylococci species, but on exposure to *S. epidermidis,* ICAM-1 remained integrated in the endothelial cell membrane, whereas *S. aureus* induced a release of sICAM-1 into the supernatants. Our results are compatible with that transmigration of granulocytes over the endothelial borders after exposure for *S. epidermidis* is sustained compared with *S. aureus*, whereas *S. aureus* results in a more regulated inflammatory pathway that proceeds towards its down regulation. In contrast to *S. aureus*, CoNS sepsis in EPT newborns is not associated with increased mortality, but neonatal CoNS sepsis generates a considerable morbidity in the acute phase but also regarding late sequels for instance retinopathy or intraventricular haemorrhage, (14,19–21). Interestingly, in an earlier study, we found higher incidence of BPD in preterm newborns after *S. epidermidis* sepsis than after neonatal sepsis caused by more virulent bacteria (17). That study was

based on a limited number of patients, but also other authors report at least similar risk of BPD after neonatal CoNS sepsis compared with *S. aureus* or gram negatives (5). In this study, we found support for an increased and sustained chemoattractant activity in endothelial and small airway epithelial cells after stimulation with neonatal blood isolates of *S. epidermidis*. We suggest that these findings constitute *in vitro* support for clinical observations that more virulent bacteria are not more strongly associated with inflammatory manifestations such as BPD, compared to less virulent bacteria (17).

Staphylococci are able to persist alive after incorporation into phagocytosing cells (22). Taking together the insufficient inflammatory regulatory control in EPT newborns, impaired bactericidal capacity in preterm granulocytes, increased release of neutrophil oxidative reagents, and our findings of this study, we hypothesize that *S. epidermidis* generates a chronic low-grade endothelial inflammation and persistent transmigration of activated neutrophils into the surrounding pulmonary tissue. Besides BPD, it is worth to consider whether similar host–bacterial interactions also are involved in the pathogenesis of other postinflammatory endothelial complications associated with sepsis in EPT newborns, that is, retinopathy and intraventricular haemorrhage. A high percentage of EPT newborns become colonized by nosocomial strains of *S. epidermidis* in the respiratory tract during the first week of life (10,23,24). Bacterial colonization of the respiratory tract increases the release of pro-inflammatory mediators (25), but most studies so far have focused on bacteria transferred from the maternal vaginal flora, mainly *Ureaplasma* and *Mycoplasma* spp. Clinical studies of the association between local airway colonization of staphylococci and BPD in EPT newborns are of interest. This study was not designed to investigate differences in the inflammatory response between different subpopulations of staphylococci within the two groups of *S. epidermidis* and *S. aureus* strains, but still we observed individual variations that were consistent in repeated experiments, which is in accordance with other authors (8,26). We used living bacteria for challenge of the cultured cells, as inactivation of staphylococci is known to influence the inflammatory response and extended the duration of the bacterial incubations to a maximum with regard to integrity of the cultured cells.

In summary, we found an increased expression of chemoattractant inflammatory mediators in response to neonatal blood isolates of *S. epidermidis* compared with *S. aureus*. This provides conditions for a sustained capillary transmigration of highly activated neutrophil granulocytes from the pulmonary capillary bed into the surrounding lung tissue in case of neonatal CoNS sepsis. Further studies on interactions between bacteria and endothelial and respiratory epithelial cells are warranted to develop strategies for the prevention of BPD in EPT newborns.

CONFLICTS OF INTEREST

None.

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