Cytokine 123 (2019) 154761

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

An underestimated pathogen: *Staphylococcus epidermidis* induces pro-inflammatory responses in human alveolar epithelial cells

Ying Dong[a,*], Kirsten Glaser[a], Nicolas Schlegel[b], Heike Claus[c], Christian P. Speer[a]

[a] University Children's Hospital, University of Wuerzburg, Wuerzburg, Germany
[b] Department of Surgery I, University of Wuerzburg, Wuerzburg, Germany
[c] Institute for Hygiene and Microbiology, University of Wuerzburg, Wuerzburg, Germany

ARTICLE INFO

Keywords:
Staphylococcus epidermidis
Bronchopulmonary dysplasia
Human alveolar epithelial cells
Pro-inflammation
Toll-like receptor

ABSTRACT

Objectives: Conventionally regarded as a harmless skin commensal, *Staphylococcus epidermidis* accounts for the majority of neonatal late-onset sepsis and is shown to be associated with neonatal inflammatory morbidities, especially bronchopulmonary dysplasia. This study addressed the pro-inflammatory capacity of different *S. epidermidis* strains on human alveolar epithelial cells.

Methods: A549 cell monolayers were stimulated by live bacteria of *S. epidermidis* RP62A strain (biofilm-positive) and ATCC 12228 strain (biofilm-negative) at a multiplicity of infection ratio of 10 for 24 h. LPS (100 ng/ml) and Pam3CSK4 (1 μg/ml) were used for comparisons. Cell viability was measured by MTT method. The mRNA and protein expression of inflammatory mediators and toll-like receptor (TLR)-2 were assessed using RT-PCR, immunoassays and immunofluorescence.

Results: Both *S. epidermidis* strains induced expression of tumor necrosis factor (TNF)-α, IL-1β, interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, interferon γ-induced protein 10 (IP-10) and intercellular adhesion molecule (ICAM)-1, but not IL-10. The stimulatory effect of RP62A exceeded that of LPS ($p < 0.05$). RP62A strain showed a trend towards higher induction of pro-inflammatory mediators than ATCC 12228 strain. The co-stimulation with RP62A strain decreased cell viability compared to control and TLR agonists ($p < 0.05$). RP62A but not ATCC 12228 stimulated mRNA and protein expression of TLR2.

Conclusions: S. epidermidis drives pro-inflammatory responses in lung epithelial cells *in vitro*. The pro-inflammatory capacity of *S. epidermidis* may differ between strains. Biofilm-positive *S. epidermidis* strain seems to induce more potent pulmonary pro-inflammation than biofilm-negative *S. epidermidis* strain.

1. Introduction

Due to its ubiquitous colonization on human body surface, *Staphylococcus epidermidis* is conventionally regarded as a harmless skin commensal [1,2]. In the past two decades, *S. epidermidis* has emerged as the leading pathogen of neonatal late-onset sepsis (LOS), accounting for 50%-80% of neonatal LOS cases in many developed and developing countries [3]. Furthermore, increasing clinical studies suggest a strong link between *S. epidermidis* sepsis and other neonatal short-term and long-term morbidities, especially bronchopulmonary dysplasia (BPD) [4–7]. Neonates with *S. epidermidis* sepsis were 3–9 times more likely to develop BPD than uninfected ones [4–6]. Additionally, several lines of evidence indicated the presence of *Staphylococcus* spp., including *S. epidermidis*, in the airway microbiome of preterm neonates with evolving BPD [8,9]. Despite multiple etiological factors, inflammation is considered as one principal pathomechanism of BPD [10–12]. Pre- and postnatal injurious events, such as chorioamnionitis, oxygen exposure, mechanical ventilation, and neonatal infection, are likely to trigger and sustain adverse pulmonary inflammation [10–12]. As a consequence, normal alveolarization and angiogenesis may be impaired, with lifelong consequences [12]. So far, clinical and experimental data on *S. epidermidis*-driven pulmonary inflammation are scarce [13,14].

Advances in molecular microbiology during the last two decades have shed some light on the pathogenicity factors of *S. epidermidis*. Epidemiologic studies have demonstrated that *S. epidermidis* strains disseminating in NICUs across different regions are characterized by biofilm-forming capacity [2,15–18]. Biofilm represents a community of bacteria encased in self-produced extracellular matrix [19]. Furthermore, methicillin resistance and toxin production contribute to *S. epidermidis* pathogenicity [17,18,20]. Until now, it remains controversial

* Corresponding author at: University Children's Hospital, University of Wuerzburg, Josef-Schneider Str. 2, 97080 Wuerzburg, Germany.
E-mail address: doy1122@126.com (Y. Dong).

https://doi.org/10.1016/j.cyto.2019.154761
Received 3 March 2019; Received in revised form 6 June 2019; Accepted 11 June 2019
Available online 18 June 2019

whether *S. epidermidis* is able to induce host inflammatory responses [21–25]. In addition, several *in vitro* experimental and clinical studies have indicated that the serum level of pro-inflammatory mediators upon *S. epidermidis* stimulation may vary among *S. epidermidis* strains with different genotypes [16,26–29]. Strain-related differences in *S. epidermidis*-induced host immune responses have not been elucidated, so far.

In this study, we utilized an *in vitro* model to investigate *S. epidermidis*-driven inflammation, in particular, in human alveolar epithelial cells. Alveolar epithelial cells align more than 99% of the inner surface of the lung and play a crucial role in initiating and shaping pulmonary inflammatory responses [30]. Type II alveolar epithelial cells are an important source of cytokines and chemoattractants, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, monocyte chemoattractant protein (MCP)-1, interferon γ-induced protein (IP)-10, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 [30–32]. With phenotypic features characterizing human type II alveolar epithelial cells, A549 cell line has been widely used in *in vitro* experiments to investigate pulmonary inflammation [33–35]. Host immune responses against pathogens are largely mediated by Toll-like receptors (TLRs), with TLR2 and TLR4 being able to recognize a variety of pathogen-associated molecule patterns from Gram-positive and Gram-negative bacteria [36,37]. Here, we evaluated the expression of the abovementioned inflammatory mediators as well as TLR2 and TLR4 in A549 cells stimulated by two different *S. epidermidis* strains. In contrast to the chronic disease course of device-associated infections [38,39], neonatal sepsis is acute by nature [38]. In order to better simulate the clinical scenario of pulmonary inflammation following sepsis, *S. epidermidis* strains in planktonic growth were used for this study.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. epidermidis RP62A (ATCC 35984) and ATCC 12228 are two *S. epidermidis* strains with genome sequencing data, and were kindly provided by Dr. Wilma Ziebuhr from Institute for Molecular Infection Biology (University of Wuerzburg, Wuerzburg, Germany). S. epidermidis RP62A was isolated during an outbreak of intravascular catheter-associated sepsis, showing a strong capacity of biofilm formation [40]. In contrast, ATCC 12228 is a biofilm-negative strain [41]. This has been confirmed by our preliminary experiments (Supplemental Fig. 1). Both *S. epidermidis* strains were streaked out on tryptic soy agar (Becton Dickinson, Sparks, NV, USA) from frozen stocks, followed by inoculation into 5 ml of tryptic soy broth (Becton Dickinson) and overnight growth at 37 °C with agitation (180 rpm).

2.2. Cell cultures

A549 cells were purchased from ATCC (LGC Standards, Teddington, UK) [42]. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) with additional 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere at 37 °C with 5% CO_2. Cell monolayers reaching > 90% confluence were washed with sterile phosphate buffered saline (PBS) (Sigma-Aldrich), digested with trypsin (Sigma-Aldrich), and expanded into new culture flasks. After 6 passages, cells were collected and resuspended in fetal bovine serum with 10% dimethyl sulfoxide (Serva Electrophoresis, Heidelberg, Germany), followed by storage at −80 °C. Experiments were conducted with freshly thawed cells further expanded to passage 8.

2.3. Cell stimulation assays

A549 cells were seeded at a density of 3.5×10^5 cells/well in 6-well plates (Greiner, Frickenhausen, Germany), followed by overnight incubation to achieve > 90% confluence at the bottom of wells [43]. According to previous *in vitro* studies [13,14,21,22,26], various multiplicity of infection ratios (MOI 0.1, 1.0 and 10) of *S. epidermidis* were tested in our preliminary experiments (Supplemental Fig. 2). With regard to kinetics of inflammatory mediator expression at mRNA and protein levels, different co-incubation periods (4 h, 8 h, 24 h, 30 h, and 48 h) have been evaluated (Supplemental Fig. 2). MOI 10 was chosen for the final experiments, and A549 cell monolayers were exposed to bacteria diluted to 3.5×10^6 colony forming units (CFUs)/ml in 1 ml of antibiotic-free cell growth medium for 24 h. The count of bacteria was confirmed by serial dilution and plate counting on tryptic soy agar. Our preliminary experiments showed no effect of antibiotic-free cell culture medium on the growth of both *S. epidermidis* strains. Unstimulated cells served as the negative control. Besides live bacteria, *E. coli* lipopolysaccharide (LPS) (TLR4 agonist, Sigma-Aldrich) and Pam3CSK4 (TLR2/1 agonist, InvivoGen, San Diego, CA, USA), representing TLR agonists of Gram-negative and Gram-positive bacteria respectively, were also used for cell stimulation assays. The dose of LPS (100 ng/ml) and Pam3CSK4 (1 µg/ml) applied in this study were chosen based on previous *in vitro* approaches using the same cell line [33,35].

2.4. Cell viability assay

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed to assess cell viability [44]. Living cells with active metabolism convert the yellow MTT dye into purple-colored formazan through redox reaction [44]. After 24 h of stimulation, A549 cells were washed with PBS, and 1 ml of antibiotic-free medium containing 0.5 mg MTT was applied to each well. After incubation at 37 °C for 45 min, the medium was removed. Then, 500 ml isopropanol was added per well, followed by gentle vibration for 30 s at room temperature to dissolve purple formazan. Optical density was measured at 550 nm in triplicates in 96-well plates (Greiner) with an MR 5000 microplate reader (Dynatech, Santa Monica, CA).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

After 24 h of stimulation, total RNA was extracted from cells using NucleoSpin RNA Kit (Macherey-Nagel, Dueren, Germany). Extracted RNA was eluted in 60 µl nuclease-free H_2O (Sigma-Aldrich), quantified using a Qubit® 2.0 Fluorometer (Thermo Fisher), and immediately stored at −80 °C until further analysis. Using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher, Carlsbad, CA, USA), 0.1–0.5 µg of total RNA was reverse transcribed in each independent experiment. The first-strand cDNA was diluted 1:10 with deionized, nuclease-free H_2O (Sigma-Aldrich) and stored at −20 °C until qRT-PCR assessment. For quantitative detection of TNF-α, IL-1β, IL-6, IL-8, IL-10, MCP-1, IP-10, ICAM-1, VCAM-1, TLR2, and TLR4 mRNA, a 25 µl reaction mix containing 10 µl cDNA template, 12.5 µl iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 2 µl forward and reverse primers (10 µM), and 0.5 µl deionized H_2O was prepared. Table 1 demonstrates the primer sequences used for qRT-PCR [45,46,43]. A 7, 500 Real-Time PCR System (Applied Biosystems) was used to perform qRT-PCR with a two-step protocol: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analyses at the end of every run confirmed single PCR products. The mRNA expression of each target gene was normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), with mean fold changes calculated using the △△CT method [47].

Table 1
Primer sequences used in real-time polymerase chain reaction.

Gene name	Sequence accession	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNF-α	NM_000594.3	CAGCCTCTTCTCCTTCCT	GGTTTGCTACAACATGG
IL-1β	NM_000576.2	TTCATTGCTCAAGTGTCTG	GCACTTCATCTGTTTAGGG
IL-6	NM_000600.4	ACATCGTCGACAAAATCTCTGCA A	GCCAGTGTCTCCTTGCTGTTT
IL-8	NM_000584.3	CAGTGCATAAAGACATACTCC	TTTATGAATTCTCAGCCCTC
IL-10	NM_000572.3	GCTGTCATCGATTTCTTCC	GTCAAACTCACTCATGGCT
MCP-1	NM_002982.3	GCTGTGATCTTCAAGACC	AAGTCTTCGGAGTTTGGG
IP-10	NM_001565.3	AGCACCATGAATCAAACTG	TGTAGCAATGATCTCAACAC
ICAM-1	NM_000201.2	CAGACCTTTGTCCTGCCA	AAGGAGTCGTTGCCATAGGT
VCAM-1	NM_001078.3	GCAAGTCTACATATCACCCA	AGTTGCATTTCCAGAAAGGT
TLR2	NM_001318793.1	CCAAAGGAGACCTATAGTGAC	GCTTCAACCCACAACTACC
TLR4	NM_003266.3	TTATCCAGGTGTGAAATCCA	GATTTGTCTCCACAGCCA
GAPDH	NM_002046.5	CAAAGTTGTCATGGATGACC	CCATGGAGAAGGCTGGGG

TNF-α: tumor necrosis factor; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; IP-10: interferon γ-induced protein 10; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule; TLR: toll-like receptor. GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

2.6. Multi-analyte immunoassays

The supernatants of cells were harvested after 24 h of stimulation and stored at −80 °C. Concentrations of human TNF-α, IL-1β, IL-6, IL-8, IL-10, MCP-1, IP-10, ICAM-1, and VCAM-1 were assessed in duplicate by means of multi-analyte immunoassays using Luminex® bead technology and reagent kits (Merck Millipore, Darmstadt, Germany). For each inflammatory mediator, a standard curve was developed by xPonent® Software (Luminex Cooperation, Texas, USA). The thresholds of detection were set at 0.98 pg/ml (TNF-α), 1.54 pg/ml (IL-1β), 3.17 pg/ml (IL-6), 3.17 pg/ml (IL-8), 0.71 pg/ml (IL-10), 4.07 pg/ml (MCP-1), 3.6 pg/ml (IP-10), 1.23 pg/ml (ICAM-1), and 4.47 pg/ml (VCAM-1). Protein expression was calibrated by MTT-measured cell viability as previously described [13].

2.7. Immunofluorescence microscopy

After 24 h of stimulation as described above, cells were washed three times with PBS and fixed for 15 min in 4% Formaldehyde (Sigma-Aldrich). Non-specific binding was blocked for 30 min at room temperature with 1% bovine serum albumin (Sigma-Aldrich) in PBS. Cells were then incubated overnight with a PE anti-human TLR2 antibody (1:20, Biolegend, San Diego, CA, USA) at 4 °C in a humidified chamber, followed by 2 h at room temperature. Subsequently, nuclei were stained with Hoechst 33342 (1:5000, Invitrogen) for 15 min at room temperature. Samples were washed in PBS, mounted onto glass slides using VECTASHIELD anti-fading medium (Biozol Endogen, Eching, Germany), and examined under Leica DMI6000 inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were taken using a Leica DFC360FX digital camera with fixed exposure times. Experiments were repeated in triplicate.

2.8. Statistical analysis

All experiments were performed five times until otherwise indicated. Results from independent experiments are expressed as means ± standard deviation (SD). Differences among groups were assessed by non-parametric Kruskall-Wallis test and Dunn's *post-hoc* test for multiple comparisons. Statistical analyses were performed using Prism® 6 software (GraphPad Software, San Diego, CA, USA), with statistical significance defined as p-value < 0.05.

3. Results

3.1. Viability of A549 cells upon stimulation with S. epidermidis

Compared to control, LPS and Pam3CSK4 did not exert a significant cytotoxic effect on A549 cells following 24 h of stimulation (Fig. 1).

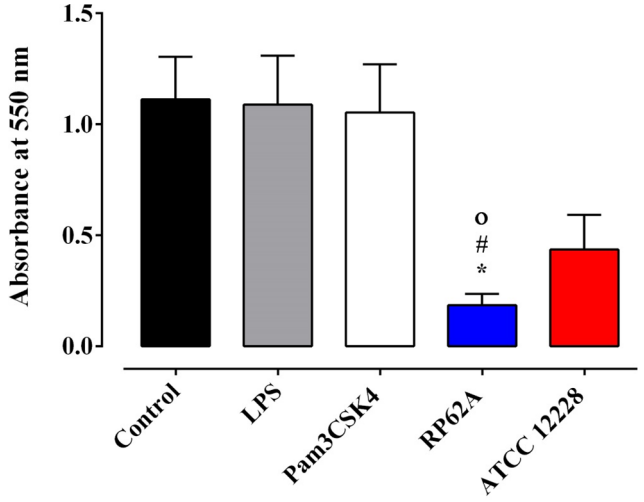

Fig. 1. The viability of A549 cells stimulated by LPS, Pam3CSK4, and *S. epidermidis* strains RP62A and ATCC 12228. Viable cells were measured by the absorbance of Methylthiazolyldiphe-nyltetrazolium bromide (MTT) at 550 nm following 24 h of stimulation. Unstimulated cells served as negative control. Data are presented as mean ± SD of four independent experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. LPS-stimulated cells; ○$p < 0.05$ vs. Pam3CSK4-stimulated cells.

Cells exposed to *S. epidermidis* RP62A strain had significantly lower viability than both control and TLR agonist-stimulated cells ($p < 0.05$). In contrast, ATCC 12228 strain did not significantly reduce A549 cell viability when compared to control, TLR agonists or RP62A strain.

3.2. Inflammatory cytokine responses in S. epidermidis-stimulated A549 cells

As shown by kinetic experiments in Supplemental Fig. 2 (data were given on TNF-α and IL-8), mRNA and protein expression of inflammatory mediators in *S. epidermidis*-stimulated A549 cells was dependent on bacterial concentration and incubation period. At MOI 10, *S. epidermidis* exerted the most pronounced stimulatory effect on A549 cells. Both *S. epidermidis* strains elicit maximal expression of inflammatory mediators at 24 h of stimulation. In the final experiment, we evaluated mRNA and protein expression of inflammatory mediators in A549 cells stimulated with *S. epidermidis* strains at a MOI of 10 for 24 h.

Without any stimuli, A549 cells exhibited very low basal synthesis of inflammatory cytokines. Compared to *S. epidermidis*, TLR agonists exerted limited stimulatory effects on A549 cells. At the level of mRNA,

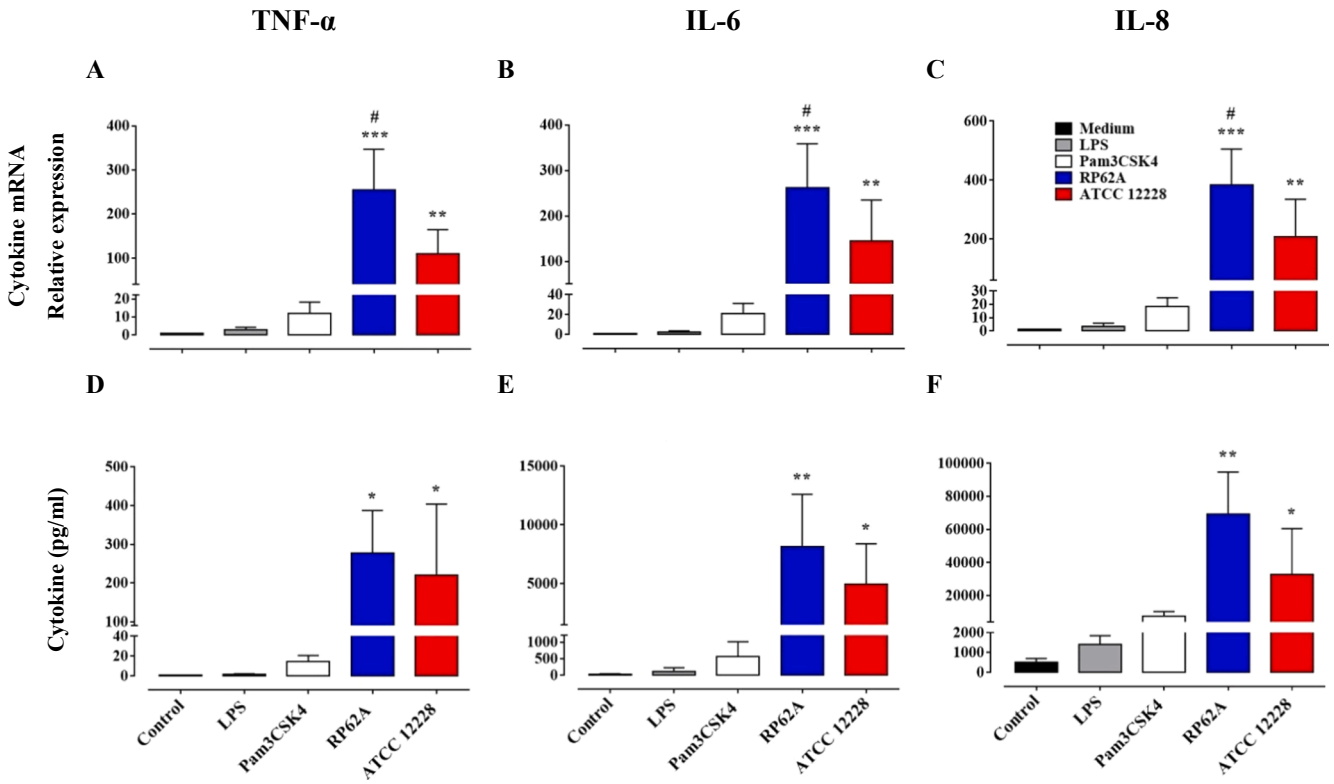

Fig. 2. Pro-inflammatory TNF-α, IL-6 and IL-8 responses in A549 cells incubated with LPS, Pam3CKS4, and *S. epidermidis* strains RP62A and ATCC 12228 for 24 h. The mRNA expression of cytokines was assessed by qRT-PCR and normalized to the reference gene GAPDH (A-C). Protein concentrations in supernatants were determined by multi-analyte immunoassays and corrected by cell viability (D-F). Unstimulated cells served as negative control. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$ vs. LPS-stimulated cells.

both *S. epidermidis* RP62A and ATCC 12228 significantly induced TNF-α (RP62A: $p = 0.0002$, ATCC 12228: $p = 0.009$), IL-1β (RP62A: $p = 0.0006$, ATCC 12228: $p = 0.019$), IL-6 (RP62A: $p = 0.0004$, ATCC 12228: $p = 0.006$) and IL-8 (RP62A: $p = 0.0003$, ATCC 12228: $p = 0.007$) compared to control (Fig. 2 A–C). The same trend applied to extracellular protein release of TNF-α, IL-6 and IL-8 (Fig. 2 D–F). IL-1β displayed a similar mRNA expression pattern as TNF-α, but was undetectable in cell supernatants (data not shown). Although *S. epidermidis* RP62A strain appeared to induce more pro-inflammatory cytokines in A549 cells than ATCC 12228 strain, this difference did not reach statistical significance. Induction of TNF-α, IL-1β, IL-6 and IL-8 mRNA expression by RP62A was significantly more pronounced than the effect of LPS (IL-1β: $p = 0.007$; other mediators: $p < 0.05$) (Fig. 2 A-C). The expression of anti-inflammatory IL-10 was negligible in both the control and stimulated cells, either at mRNA or protein level (data not shown).

3.3. S. epidermidis-driven chemoattractant responses in A549 cells

In the absence of any stimuli, A549 cells exhibited very low basal synthesis of chemoattractants. Regarding MCP-1 expression, only *S. epidermidis* RP62A strain exerted a significant stimulatory effect on A549 cells at both mRNA and protein levels when compared to control ($p < 0.05$) (Fig. 3 A,C). RP62A strain-induced MCP-1 mRNA expression was significantly higher than that induced by LPS ($p = 0.019$). IP-10 mRNA expression was barely detected in unstimulated cells, as well as cells stimulated with TLR agonists and *S. epidermidis* strains. However, extracellular concentration of IP-10 was significantly enhanced in A549 cells stimulated by RP62A ($p = 0.002$ vs. control, $p = 0.049$ vs. LPS) and ATCC 12228 ($p = 0.034$ vs. control). Similar as pro-inflammatory cytokine responses, ICAM-1 mRNA expression was significantly increased in A549 cells upon *S. epidermidis* infection ($p < 0.01$) (Fig. 3 B). Compared to LPS, RP62A but not ATCC 12228 provoked more ICAM-1 at the level of mRNA and protein ($p < 0.05$) (Fig. 3 B,D). ATCC 12228 strain did not significantly induce MCP-1 and ICAM-1 protein expression. Again, both mRNA and protein data showed a non-significant trend towards higher expression of chemoattractants in RP62A than in ATCC 12228. The expression of VCAM-1 was negligible in both control and stimulated cells, either at the mRNA or protein level (data not shown).

3.4. TLR2 expression of S. epidermidis-stimulated A549 cells

Via qRT-PCR, the expression of TLR4 was below the detection limit in unstimulated as well as stimulated cells (data not shown). The basal mRNA expression level of TLR2 was low in control and treated A549 cells, as indicated by cycle threshold values (data not shown). The mRNA expression of TLR2 in *S. epidermidis* RP62A infection group was higher than in control and LPS group ($p < 0.05$) (Fig. 4). Immunofluorescence was performed to qualitatively assess TLR2 expression at the protein level. Similar as control, LPS-treated cells exhibited minimal fluorescent signals for TLR2 (Fig. 5). Incubation with TLR2 agonist Pam3CSK4 enhanced TLR2 staining in the cytoplasm. *S. epidermidis* ATCC 12228 strain elicited detectable but weak fluorescence for TLR2, whereas RP62A strain considerably amplified TLR2 protein expression.

4. Discussion

This *in vitro* study demonstrated that *S. epidermidis* is able to exert pro-inflammatory effects on human alveolar epithelial cells, by stimulating the expression of pro-inflammatory mediators while hardly inducing anti-inflammatory IL-10. Cell death may occur as a result of pro-inflammatory responses upon stimulation with *S. epidermidis*. Biofilm-positive RP62A strain seems to induce more potent pulmonary pro-

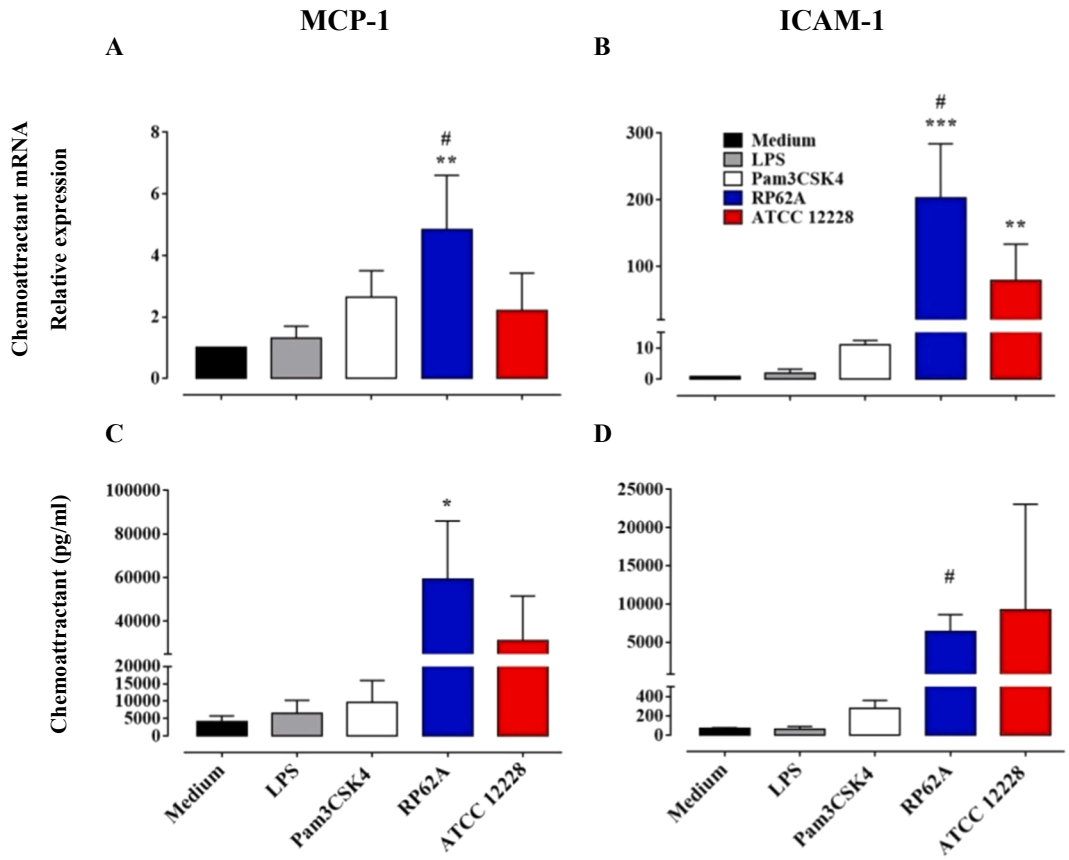

Fig. 3. Expression of chemoattractants MCP-1 and ICAM-1 in A549 cells stimulated by LPS, Pam3CKS4, and *S. epidermidis* strains RP62A and ATCC 12228 for 24 h. Levels of mRNA expression relative to gene GAPDH (A, B) and extracellular protein concentrations calibrated by cell viability (C, D) are presented as mean ± SD. Unstimulated cells served as negative control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$ vs. LPS-stimulated cells.

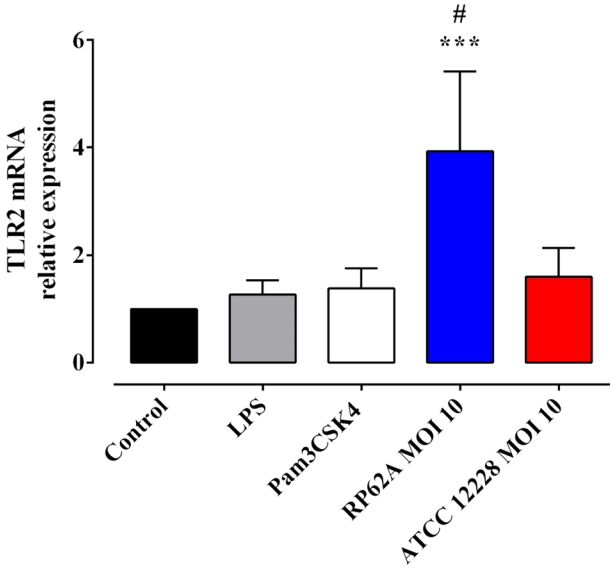

Fig. 4. The relative mRNA expression of TLR2 in A549 cells stimulated by LPS, Pam3CKS4, and *S. epidermidis* strains RP62A and ATCC 12228 for 24 h. The mRNA expression of TLR2 was assessed by qRT-PCR and normalized to the reference gene GAPDH. Unstimulated cells served as negative control. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$ vs. LPS-stimulated cells.

inflammation than biofilm-negative ATCC 12228 strain.

Our results are in line with the two previous *in vitro* studies, which showed the induction of TNF-α, IL-6, IL-8 and ICAM-1 in *S. epidermidis*-stimulated bronchial epithelial cells [13,14]. TNF-α, IL-6 and IL-8 are cytokines signifying the initial stage of inflammation [12,48]. Chemoattractants such as MCP-1, IP-10 and ICAM-1 may sustain and aggravate the inflammation by recruiting neutrophils, monocytes/macrophages, and lymphocytes [49–51]. Increased expression of the abovementioned pro-inflammatory mediators in airway secretions, bronchoalveolar lavage samples, or the systemic circulation of preterm neonates, has been suggested by plentiful clinical investigations to be associated with the development of BPD [52–59]. In the present study, we did not detect IL-1β at the protein level. IL-1β has an intracellular precursor form [60], and pro–IL-1β is likely to be cleaved after cytolysis [61]. Given the cytotoxic effect of *S. epidermidis* on A549 cells, our failure to determine extracellular IL-1β concentration does not necessarily imply absent IL-1β synthesis. Due to the negligible expression of VCAM-1 at both mRNA and protein levels in our experiment, we assume that *S. epidermidis*, unlike some other pathogens [32], may have limited capacity to provoke VCAM-1 response in human alveolar epithelial cells.

Anti-inflammatory cytokine IL-10 is considered to play an important role in counterbalancing pro-inflammation [12,46,48,53,62]. IL-10 was shown to be inducible in A549 cells by bacterial and chemical stimuli [63,64]. However, both *S. epidermidis* strains failed to elicit IL-10 at mRNA and protein levels in this study. Previous experimental data demonstrated a compromised IL-10 response against pro-inflammatory cytokines in *S. epidermidis*-infected blood cells [21,23,65]. Our study is the first to report *in vitro* IL-10 response in lung cells upon *S. epidermidis* stimulation. Imbalanced inflammatory responses favoring pro-inflammation may destruct alveolar capillary units *in vivo*, causing lung injuries [12,53]. This was supported by our results showing reduced viability of human lung epithelial cells upon *S. epidermidis* stimulation.

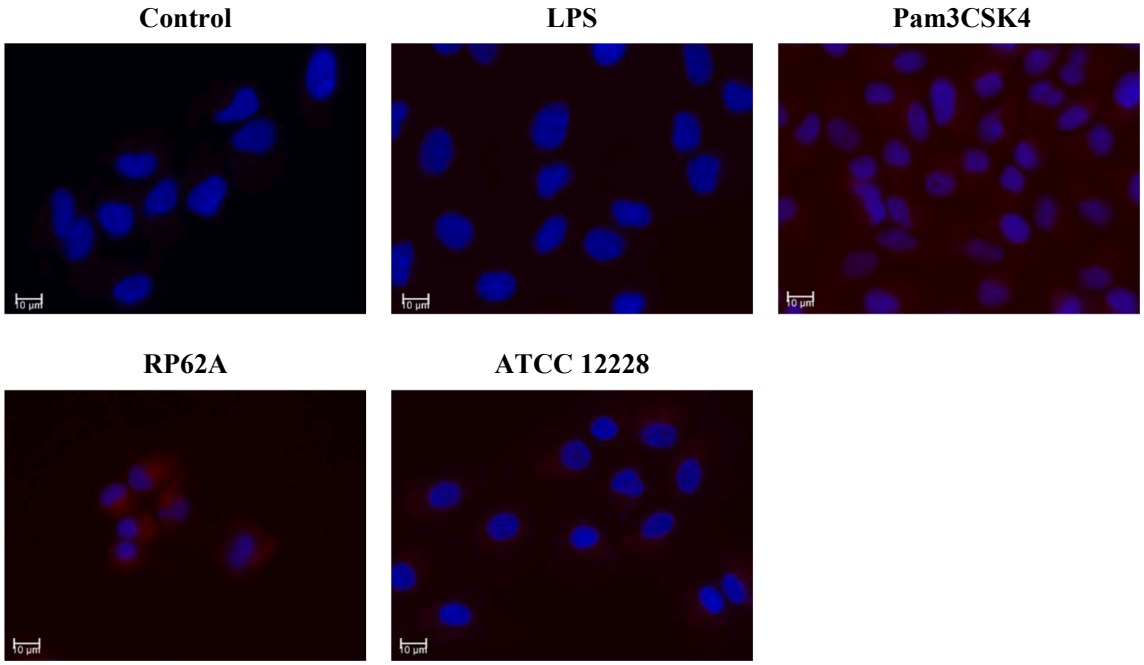

Fig. 5. Immunofluorescence of TLR2 in A549 cells induced by LPS, Pam3CKS4, and *S. epidermidis* strains RP62A and ATCC 12228. Red fluorescent signals of TLR2 stained by PE anti-human TLR2 antibody were visualized in cytoplasm. Cell nuclei were identified by Hoechst 33342 (blue). Unstimulated cells served as negative control. Scale bar represents 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In many studies, the strain characteristics of *S. epidermidis* were unknown, possibly contributing to inconsistency on host inflammatory responses induced by *S. epidermidis* [21–23]. We have chosen two *S. epidermidis* strains with genome-sequencing data [40,41], in order to minimize uncertainties on *S. epidermidis* pathogenicity factors. While biofilm-positive *S. epidermidis* strains were shown by some studies to be immune evasive [26,39,66], others demonstrated that they may induce similar or higher levels of pro-inflammatory cytokines compared to biofilm-negative strains [28,29,67]. The discrepancies among studies may be partly explained by differences in bacterial growth mode and cells used for experiments. Notably, pathogenicity factors apart from biofilm-forming capacity may also play an important role in *S. epidermidis*-driven pro-inflammation. Methicillin resistance of *S. epidermidis* is mediated by *mecA* gene located on mobile genetic elements [68]. One study investigating neonatal coagulase-negative staphylococci sepsis demonstrated that the average serum C-reactive protein level was 74% higher in infants with *mecA*-positive isolates than with *mecA*-negative isolates [16]. Moreover, α-type phenol-soluble modulin (PSM) peptide toxins of *S. epidermidis*, especially PSM-mec, have been indicated to be more potent pro-inflammatory agents than *E. coli* LPS [69–72]. Although both RP62A and ATCC 12228 strains produce α-type PSMs [69,70], PSM-mec is encoded only by RP62A due to the correlation between *psm-mec* gene locus and *mecA* [72,73]. Consistently, our study showed that lung cells treated by RP62A, but not ATCC 12228, had significantly higher pro-inflammatory mediator expression than LPS-treated cells. Taken together, *S. epidermidis*-induced inflammatory responses *per se* may be very complex, reflecting the interplay between multiple pathogenicity factors and the host immunity.

Numerous experimental and clinical data have demonstrated that *S. epidermidis* may induce the expression of TLRs at various sites of infection [24,25,37,74,75]. This study is the first to suggest an *in vitro* immunomodulatory effect of *S. epidermidis* on TLR2 expression in human lung epithelial cells. We showed induction of TLR2 in A549 cells stimulated by *S. epidermidis* RP62A but not ATCC 12228, indicating strain-related difference. Given the qualitative nature of immunofluorescence assay, our results need to be validated by further studies.

Our study belongs to the first several lines of *in vitro* evidence showing a pro-inflammatory capacity of *S. epidermidis* on human lung cells [13,14]. Strength of this study includes the application of viable bacteria. Inactivated *S. epidermidis*, as commonly prepared in *in vitro* investigations, may differentially stimulate the innate immune system and lead to biased interpretation of host inflammatory responses upon *S. epidermidis* infection [22,76,77]. There are also several limitations of this study. As host inflammatory responses are affected by genetic predisposition, immunological characteristics, and environmental conditioning factors, the net effect and temporal pattern of pro- and anti-inflammatory mediators observed *in vitro* may vary from that *in vivo* [36]. Although widely used, A549 cell line is originated from an adult and may not fully represent neonatal inflammatory responses. Freshly isolated cell lines or animal disease models of a neonatal origin provide alternatives for future experimental approaches, but may be difficult to develop.

In summary, by demonstrating a substantial *in vitro* pro-inflammatory capacity of *S. epidermidis* on human alveolar epithelial cells, our results challenge the conventional view of *S. epidermidis* being an innocuous skin inhabitant. It is necessary to reevaluate *S. epidermidis*, especially biofilm-positive strains, as relevant pathogens of inflammatory disorders. The screening of clinical *S. epidermidis* isolates for pathogenicity factors, if integrated into the routine sepsis work-up, may better guide anti-sepsis strategies and thus improve sepsis-associated neonatal outcome.

Acknowledgements

We thank Dr. Wilma Ziebuhr, Dr. Ana Eulalio, Dr. Carmen Aguilar, Dr. Markus Fehrholz for constructive advices during the study. Furthermore, we would like to thank Hilde Merkert, Silvia Seidenspinner and Mariola Dragan for excellent technical support.

Declaration of Competing Interest

None.

Funding

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154761.

References

[1] E.A. Grice, H.H. Kong, S. Conlan, C.B. Deming, J. Davis, A.C. Young, et al., Topographical and temporal diversity of the human skin microbiome, Science 324 (2009) 1190–1192, https://doi.org/10.1126/science.1171700.
[2] Y. Dong, C.P. Speer, The role of *Staphylococcus epidermidis* in neonatal sepsis: guarding angel or pathogenic devil? Int. J. Med. Microbiol. 304 (2014) 513–520, https://doi.org/10.1016/j.ijmm.2014.04.013.
[3] Y. Dong, C.P. Speer, Late-onset neonatal sepsis: recent developments, Arch. Dis. Child. Fetal. Neonatal. Ed. 100 (2015) F257–F263, https://doi.org/10.1136/archdischild-2014-306213.
[4] M. Liljedahl, L. Bodin, J. Schollin, Coagulase-negative staphylococcal sepsis as a predictor of bronchopulmonary dysplasia, Acta. Paediatr. 93 (2004) 211–215, https://doi.org/10.1080/08035250310008168.
[5] M.M. Lahra, P.J. Beeby, H.E. Jeffery, Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study, Pediatrics 123 (2009) 1314–1319, https://doi.org/10.1542/peds.2008-0656.
[6] A. Anderson-Berry, B. Brinton, E. Lyden, R.G. Faix, Risk factors associated with development of persistent coagulase-negative staphylococci bacteremia in the neonate and associated short-term and discharge morbidities, Neonatology 99 (2010) 23–31, https://doi.org/10.1159/000292567.
[7] Y. Dong, C.P. Speer, K. Glaser, Beyond sepsis: *Staphylococcus epidermidis* is an underestimated but significant contributor to neonatal morbidity, Virulence 9 (2018) 621–633, https://doi.org/10.1080/21505594.2017.1419117.
[8] P. Lohmann, R.A. Luna, E.B. Hollister, S. Devaraj, T.A. Mistretta, S.E. Welty, et al., , The airway microbiome of intubated premature infants: characteristics and changes that predict the development of bronchopulmonary dysplasia, Pediatr. Res. 76 (2014) 294–301, https://doi.org/10.1038/pr.2014.85.
[9] M. Pammi, C.V. Lal, B.D. Wagner, P.M. Mourani, P. Lohmann, R.A. Luna, et al., Airway microbiome and development of bronchopulmonary dysplasia in preterm infants: a systematic review, J. Pediatr. pii S0022–3476 (18) (2018) 31216–31222, https://doi.org/10.1016/j.jpeds.2018.08.042.
[10] P. Groneck, D. Reuss, B. Götze-Speer, C.P. Speer, Effects of dexamethasone on chemotactic activity and inflammatory mediators in tracheobronchial aspirates of preterm infants at risk for chronic lung disease, J. Pediatr. 122 (1993) 938–944, https://doi.org/10.1016/S0022-3476(09)90024-5.
[11] P. Groneck, B. Götze-Speer, M. Oppermann, H. Eiffert, C.P. Speer, Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates, Pediatrics 93 (1994) 712–718.
[12] C.P. Speer, Chorioamnionitis, postnatal factors and proinflammatory response in the pathogenetic sequence of bronchopulmonary dysplasia, Neonatology 95 (2009) 353–361, https://doi.org/10.1159/000209301.
[13] R. Hussain, I. Oliynyk, G.M. Roomans, M. Björkqvist, Modulation of ENaC, CFTR, and iNOS expression in bronchial epithelial cells after stimulation with *Staphylococcus epidermidis* (94B080) and *Staphylococcus aureus* (90B083), APMIS 121 (2013) 814–826, https://doi.org/10.1111/apm.12138.
[14] M. Ivarsson, J. Schollin, M. Björkqvist, *Staphylococcus epidermidis* and *Staphylococcus aureus* trigger different interleukin-8 and intercellular adhesion molecule-1 in lung cells: implications for inflammatory complications following neonatal sepsis, Acta. Paediatr. 102 (2013) 1010–1016, https://doi.org/10.1111/apa.12350.
[15] P. Villari, C. Sarnataro, L. Iacuzio, Molecular epidemiology of *Staphylococcus epidermidis* in a neonatal intensive care unit over a three-year period, J. Clin. Microbiol. 38 (2000) 1740–1746.
[16] C. Klingenberg, E. Aarag, A. Rønnestad, J.E. Sollid, T.G. Abrahamsen, G. Kjeldsen, et al., Coagulase-negative staphylococcal sepsis in neonates. Association between antibiotic resistance, biofilm formation and the host inflammatory response, Pediatr. Infect. Dis. J. 24 (2005) 817–822, https://doi.org/10.1097/01.inf.0000176735.20008.cd.
[17] C. Klingenberg, A. Rønnestad, A.S. Anderson, T.G. Abrahamsen, J. Zorman, A. Villaruz, et al., Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness, Clin. Microbiol. Infect. 13 (2007) 1100–1111, https://doi.org/10.1111/j.1469-0691.2007.01818.x.
[18] V. Hira, M. Sluijter, S. Estevão, D. Horst-Kreft, A. Ott, R. De Groot, et al., Clinical and molecular epidemiologic characteristics of coagulase-negative staphylococcal bloodstream infections in intensive care neonates, Pediatr. Infect. Dis. J. 26 (2007) 607–612, https://doi.org/10.1097/INF.0b013e318060cc03.
[19] J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections, Science 284 (1999) 1318–1322, https://doi.org/10.1016/j.actaastro.2004.05.028.
[20] S.M.K. Schoenfelder, C. Lange, M. Eckart, S. Hennig, S. Kozytska, W. Ziebuhr, Success through diversity - how *Staphylococcus epidermidis* establishes as a nosocomial pathogen, Int. J. Med. Microbiol. 300 (2010) 380–386, https://doi.org/10.1016/j.ijmm.2010.04.011.
[21] M.A. Mohamed, S. Cunningham-Rundles, C.R. Dean, T.A. Hammad, M. Nesin, Levels of pro-inflammatory cytokines produced from cord blood in-vitro are pathogen dependent and increased in comparison to adult controls, Cytokine 39 (2007) 171–177, https://doi.org/10.1016/j.cyto.2007.07.004.
[22] A.M.F. Tatad, M. Nesin, J. Peoples, S. Cheung, H. Lin, C. Sison, et al., Cytokine expression in response to bacterial antigens in preterm and term infant cord blood monocytes, Neonatology 94 (2008) 8–15, https://doi.org/10.1159/000112541.
[23] J.D. Peoples, S. Cheung, M. Nesin, H. Lin, A.M. Francesca Tatad, D. Hoang, et al., Neonatal cord blood subsets and cytokine response to bacterial antigens, Am. J. Perinatol. 26 (2009) 647–657, https://doi.org/10.1055/s-0029-1220788.
[24] N.T. Stevens, I. Sadovskaya, S. Jabbouri, T. Sattar, J.P. O'gara, H. Humphreys, et al., *Staphylococcus epidermidis* polysaccharide intercellular adhesin induces IL-8 expression in human astrocytes via a mechanism involving TLR2, Cell. Microbiol. 11 (2009) 421–432, https://doi.org/10.1111/j.1462-5822.2008.01264.x.
[25] D. Bi, L. Qiao, I. Bergelson, C.J. Ek, L. Duan, X. Zhang, et al., *Staphylococcus epidermidis* bacteremia induces brain injury in neonatal mice via Toll-like receptor 2-dependent and -independent pathways, J. Infect. Dis. 212 (2015) 1480–1490, https://doi.org/10.1093/infdis/jiv231.
[26] C. Härtel, I. Osthues, J. Rupp, B. Haase, K. Röder, W. Göpel, et al., Characterisation of the host inflammatory response to *Staphylococcus epidermidis* in neonatal whole blood, Arch. Dis. Child. Fetal. Neonatal. Ed. 93 (2008) F140–F145, https://doi.org/10.1136/adc.2007.124685.
[27] H.N. Granslo, C. Klingenberg, E.G. Fredheim, A. Rønnestad, T.E. Mollnes, T. Flaegstad, Arginine catabolic mobile element is associated with low antibiotic resistance and low pathogenicity in Staphylococcus epidermidis from neonates, Pediatr. Res. 68 (2010) 237–241, https://doi.org/10.1203/00006450-201011001-00463.
[28] B. Haase, K. Faust, M. Heidemann, T. Scholz, M. Demmert, B. Tröger, et al., , The modulatory effect of lipids and glucose on the neonatal immune response induced by *Staphylococcus epidermidis*, Inflamm. Res. 60 (2011) 227–232, https://doi.org/10.1007/s00011-010-0258-5.
[29] B. Tröger, M. Heidemann, I. Osthues, D. Knaack, W. Göpel, E. Herting, et al., Modulation of *S. epidermidis*-induced innate immune responses in neonatal whole blood, J. Microbiol., Immunol. Infect. (2018)https://doi.org/10.1016/j.jmii.2018.04.008.
[30] O.D. Chuquimia, D.H. Petursdottir, M.J. Rahman, K. Hartl, M. Singh, C. Fernández, The role of alveolar epithelial cells in initiating and shaping pulmonary immune responses: communication between innate and adaptive immune systems, PLos One 7 (2012) e32125, , https://doi.org/10.1371/journal.pone.0032125.
[31] H. Takizawa, Airway epithelial cells as regulators of airway inflammation (Review), Int. J. Mol. Med. 1 (1998) 367–378, https://doi.org/10.1016/S0091-6749(98)70008-9.
[32] S. Herold, W. von Wulffen, M. Steinmueller, S. Pleschka, W.A. Kuziel, M. Mack, et al., Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules, J. Immunol. 177 (2006) 1817–1824, https://doi.org/10.4049/jimmunol.177.3.1817.
[33] J.T. Leverence, M. Medhora, G.G. Konduri, V. Sampath, Lipopolysaccharide-induced cytokine expression in alveolar epithelial cells: role of PKCζ-mediated p47phox phosphorylation, Chem. Biol. Interact. 189 (2011) 72–81, https://doi.org/10.1016/j.cbi.2010.09.026 Epub 2010 Oct 30.
[34] T. Victoni, F. Gleonnec, M. Lanzetti, H. Tenor, S. Valença, L.C. Porto, et al., , Roflumilast N-oxide prevents cytokine secretion induced by cigarette smoke combined with LPS through JAK/STAT and ERK1/2 inhibition in airway epithelial cells, PLos One 9 (2014) e85243, , https://doi.org/10.1371/journal.pone.0085243.
[35] M.M. Rahman, P. Prabhala, N.N. Rumzhum, B.S. Patel, T. Wickop, P.M. Hansbro, et al., , TLR2 ligation induces corticosteroid insensitivity in A549 alveolar epithelial cells: Anti-inflammatory impact of PP2A activators, Int. J. Biochem. Cell. Biol. 78 (2016) 279–287, https://doi.org/10.1016/j.biocel.2016.07.030.
[36] K. Glaser, C.P. Speer, Toll-like receptor signaling in neonatal sepsis and inflammation: a matter of orchestration and conditioning, Expert. Rev. Clin. Immunol. 9 (2013) 1239–1252, https://doi.org/10.1586/1744666X.2013.857275.
[37] M.R. Power Coombs, K. Kronforst, O. Levy, Neonatal host defense against Staphylococcal infections, Clin. Dev. Immunol. 2013 (2013) 826303, , https://doi.org/10.1155/2013/826303.
[38] T.H. Nguyen, M.D. Park, M. Otto, Host response to *Staphylococcus epidermidis* colonization and infections, Front. Cell. Infect. Microbiol. 7 (2017) 90, https://doi.org/10.3389/fcimb.2017.00090.
[39] K.Y. Le, M.D. Park, M. Otto, Immune evasion mechanisms of *Staphylococcus epidermidis* biofilm infection, Front. Microbiol. 9 (2018) 359, https://doi.org/10.3389/fmicb.2018.00359.
[40] S.R. Gill, D.E. Fouts, G.L. Archer, E.F. Mongodin, R.T. DeBoy, J. Ravel, et al., Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain, J. Bacteriol. 187 (2005) 2426–2438, https://doi.org/10.1128/JB.187.7.2426-2438.2005.
[41] Y.Q. Zhang, S.X. Ren, H.L. Li, Y.X. Wang, G. Fu, J. Yang, et al., Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228), Mol Microbiol. 49 (2003) 1577–1593, https://doi.org/10.1046/j.1365-2958.2003.03671.x.
[42] D.J. Giard, S.A. Aaronson, G.J. Todaro, P. Arnstein, J.H. Kersey, H. Dosik, et al., *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors, Natl. Cancer. Inst. 51 (1973) 1417–1423, https://doi.org/10.1093/jnci/51.5.1417.
[43] S. Kunzmann, B. Ottensmeier, C.P. Speer, M. Fehrholz, Effect of progesterone on

Smad signaling and TGF-β/Smad-regulated genes in alveolar epithelial cells, PLoS One 13 (2018) e0200661, , https://doi.org/10.1371/journal.pone.0200661.
[44] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods. 65 (1983) 55–63, https://doi.org/10.1016/0022-1759(83)90303-4.
[45] K. Glaser, C. Silwedel, M. Fehrholz, A.M. Waaga-Gasser, B. Henrich, H. Claus, et al., *Ureaplasma* species differentially modulate pro- and anti-inflammatory cytokine responses in newborn and adult human monocytes pushing the state toward pro-inflammation, Front. Cell. Infect. Microbiol. 7 (2017) 484, https://doi.org/10.3389/fcimb.2017.00484.
[46] K. Glaser, C. Silwedel, A.M. Waaga-Gasser, B. Henrich, M. Fehrholz, H. Claus, et al., *Ureaplasma* isolates differentially modulate growth factors and cell adhesion molecules in human neonatal and adult monocytes, Cytokine 105 (2018) 45–48, https://doi.org/10.1016/j.cyto.2018.01.026.
[47] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[48] G.A. Duque, A. Descoteaux, Macrophage cytokines: involvement in immunity and infectious diseases, Front. Immunol. 5 (2014) 491, https://doi.org/10.3389/fimmu.2014.00491.
[49] S. Montefort, S.T. Holgate, Adhesion molecules and their role in inflammation, Respir. Med. 85 (1991) 91–99.
[50] L.F. Neville, G. Mathiak, O. Bagasra, The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily, Cytokine. Growth. Factor. Rev. 8 (1997) 207–219, https://doi.org/10.1016/S1359-6101(97)00015-4.
[51] S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya, Monocyte chemoattractant protein-1 (MCP-1): an overview, J. Interferon. Cytokine. Res. 29 (2009) 313–326, https://doi.org/10.1089/jir.2008.0027.
[52] T. Kojima, M. Sasai, Y. Kobayashi, Increased soluble ICAM-1 in tracheal aspirates of infants with bronchopulmonary dysplasia, Lancet 342 (1993) 1023–1024, https://doi.org/10.1016/0140-6736(93)92880-3.
[53] C.A. Jones, R.G. Cayabyab, K.Y. Kwong, C. Stotts, B. Wong, H. Hamdan, et al., Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns, Pediatr. Res. 39 (1996) 966–975, https://doi.org/10.1203/00006450-199606000-00007.
[54] S. Kotecha, L. Wilson, A. Wangoo, M. Silverman, R.J. Shaw, Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity, Pediatr. Res. 40 (1996) 250–256, https://doi.org/10.1203/00006450-199608000-00010.
[55] B. Jónsson, K. Tullus, A. Brauner, Y. Lu, G. Noack, Early increase of TNF alpha and IL-6 in tracheobronchial aspirate fluid indicator of subsequent chronic lung disease in preterm infants, Arch. Dis. Child. Fetal. Neonatal. Ed. 77 (1997) F198–F201, https://doi.org/10.1136/fn.77.3.F198.
[56] P. Ballabh, J. Kumari, A.N. Krauss, J.J. Shin, A. Jain, P.A.M. Auld, et al., , Soluble E-selectin, soluble L-selectin and soluble ICAM-1 in bronchopulmonary dysplasia, and changes with dexamethasone, Pediatrics 111 (2003) 461–468, https://doi.org/10.1542/peds.111.3.461.
[57] R.J. Baier, A. Majid, H. Parupia, J. Loggins, T.E. Kruger, CC chemokine concentrations increase in respiratory distress syndrome and correlate with development of bronchopulmonary dysplasia, Pediatr. Pulmonol. 37 (2004) 137–148, https://doi.org/10.1002/ppul.10417.
[58] Z.H. Aghai, J.G. Saslow, K. Mody, R. Eydelman, V. Bhat, G. Stahl, et al., IFN-γ and IP-10 in tracheal aspirates from premature infants: relationship with bronchopulmonary dysplasia, Pediatr. Pulmonol. 48 (2013) 8–13, https://doi.org/10.1002/ppul.22540.
[59] L. Rivera, R. Siddaiah, C. Oji-Mmuo, G.R. Silveyra, P. Silveyra, Biomarkers for bronchopulmonary dysplasia in the preterm infant, Front. Pediatr. 4 (2016) 33, https://doi.org/10.3389/fped.2016.00033.
[60] D.J. Hazuda, J. Strickler, P. Simon, P.R. Young, Structure-function mapping of interleukin 1 precursors. Cleavage leads to a conformational change in the mature protein, J. Biol. Chem. 266 (1991) 7081–7086.
[61] L. Shi, D.M. Manthei, A.G. Guadarrama, L.Y. Lenertz, L.C. Denlinger, Rhinovirus-induced IL-1β release from bronchial epithelial cells is independent of functional P2X7, Am. J. Respir. Cell. Mol. Biol. 47 (2012) 363–371, https://doi.org/10.1165/rcmb.2011-0267OC.
[62] W. Ouyang, S. Rutz, N.K. Crellin, P.A. Valdez, S.G. Hymowitz, Regulation and functions of the IL-10 family of cytokines in inflammation and disease, Annu. Rev. Immunol. 29 (2011) 71–109, https://doi.org/10.1146/annurev-immunol- 031210-101312.
[63] H. Mikamo, A.K. Johri, L.C. Paoletti, L.C. Madoff, A.B. Onderdonk, Adherence to, invasion by, and cytokine production in response to serotype VIII group B Streptococci, Infect. Immun. 72 (2004) 4716–4722, https://doi.org/10.1128/IAI.72.8.4716-4722.2004.
[64] C. Odewumi, L.M. Latinwo, A. Sinclair, V.L.D. Badisa, A. Abdullah, R.B. Badisa, Effect of cadmium on the expression levels of interleukin-1α and interleukin-10 cytokines in human lung cells, Mol. Med. Rep. 12 (2015) 6422–6426, https://doi.org/10.3892/mmr.2015.4316.
[65] K.D. Kronforst, C.J. Mancuso, M. Pettengill, J. Ninkovic, M.R. Power Coombs, C. Stevens, et al., , A neonatal model of intravenous *Staphylococcus epidermidis* infection in mice < 24 h old enables characterization of early innate immune responses, PLos One 7 (2012) e43897, , https://doi.org/10.1371/journal.pone.0043897.
[66] N.N. Schommer, M. Christner, M. Hentschke, K. Ruckdeschel, M. Aepfelbacher, H. Rohde, *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1, Infect. Immun. 79 (2011) 2267–2276, https://doi.org/10.1128/IAI.01142-10.
[67] E.G. Aarag Fredheim, H.N. Granslo, T. Flægstad, Y. Figenschau, H. Rohde, I. Sadovskaya, et al., *Staphylococcus epidermidis* polysaccharide intercellular adhesin activates complement, FEMS. Immunol. Med. Microbiol. 63 (2011) 269–280, https://doi.org/10.1111/j.1574-695X.2011.00854.x.
[68] K. Hiramatsu, L. Cui, M. Kuroda, T. Ito, The emergence and evolution of methicillin-resistant *Staphylococcus aureus*, Trends. Microbiol. 9 (2001) 486–493.
[69] S.Y. Queck, B.A. Khan, R. Wang, T.H.L. Bach, D. Kretschmer, L. Chen, et al., Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA, PLoS. Pathog. 5 (2009) e1000533, , https://doi.org/10.1371/journal.ppat.1000533.
[70] G.Y.C. Cheung, K. Rigby, R. Wang, S.Y. Queck, K.R. Braughton, A.R. Whitney, et al., *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils, PLoS. Pathog. 6 (2010) e1001133, , https://doi.org/10.1371/journal.ppat.1001133.
[71] G.Y.C. Cheung, H.S. Joo, S.S. Chatterjee, M. Otto, Phenol-soluble modulins–critical determinants of staphylococcal virulence, FEMS. Microbiol. Rev. 38 (2014) 698–719, https://doi.org/10.1111/1574-6976.12057.
[72] L. Qin, F. Da, E.L. Fisher, D.C.S. Tan, T.H. Nguyen, C.L. Fu, et al., Toxin mediates sepsis caused by methicillin-resistant *Staphylococcus epidermidis*, PLos Pathog. 13 (2017) e1006153, , https://doi.org/10.1371/journal.ppat.1006153.
[73] L. Qin, J.W. McCausland, G.Y.C. Cheung, M. Otto, PSM-Mec-A Virulence determinant that connects transcriptional regulation, virulence, and antibiotic resistance in staphylococci, Front. Microbiol. 7 (2016) 1293, https://doi.org/10.3389/fmicb.2016.01293.
[74] J.P. Zhang, Y. Yang, O. Levy, C. Chen, Human neonatal peripheral blood leukocytes demonstrate pathogen-specific coordinate expression of TLR2, TLR4/MD2, and MyD88 during bacterial infection *in vivo*, Pediatr. Res. 68 (2010) 479–483, https://doi.org/10.1203/PDR.0b013e3181f90810.
[75] T. Strunk, M.R.P. Coombs, A.J. Currie, P. Richmond, D.T. Golenbock, L. Stoler-Barak, et al., TLR2 mediates recognition of live *Staphylococcus epidermidis* and clearance of bacteremia, PLos One 5 (2010) e10111, , https://doi.org/10.1371/journal.pone.0010111.
[76] T. Strunk, A. Prosser, O. Levy, V. Philbin, K. Simmer, D. Doherty, et al., Responsiveness of human monocytes to the commensal bacterium *Staphylococcus epidermidis* develops late in gestation, Pediatr. Res. 72 (2012) 10–18, https://doi.org/10.1038/pr.2012.48.
[77] T. Strunk, P. Richmond, A. Prosser, K. Simmer, O. Levy, D. Burgner, et al., Method of bacterial killing differentially affects the human innate immune response to *Staphylococcus epidermidis*, Innate. Immun. 17 (2011) 508–516, https://doi.org/10.1177/1753425910379840.