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ORIGINAL ARTICLE

Innate immune responses to *Stenotrophomonas maltophilia* in immunocompromised pediatric patients and the effect of taurolidine

Christoph Härtel*, Tasja Scholz, Marie Kuhn, Meike Bendiks, Wolfgang Göpel, Melchior Lauten, Egbert Herting

Department of Paediatrics, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany

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Background: *Stenotrophomonas maltophilia* is an emerging pathogen causing invasive infections in immunocompromised pediatric patients, including neonates and pediatric oncology patients. Information on innate immune responses to *S. maltophilia* and its potential modulation are scarce.
Methods: We established an *in vitro S. maltophilia* whole blood sepsis model and studied the proinflammatory cytokine production of CD14-positive cells by flow cytometry. We compared the cytokine expression of term newborns ($n = 13$) and healthy adults ($n = 10$) and investigated *in vitro* responses of pediatric oncology patients after recovery from neutropenia ($n = 10$) with healthy adults ($n = 10$). We further evaluated the immunomodulatory role of the amino-acid derivative taurolidine in our *in vitro* sepsis model.
Results: Proinflammatory cytokine responses to *S. maltophilia* were largely diminished in the neonatal population. No remarkable differences were noted for cytokine responses between pediatric oncology patients and healthy controls. Taurolidine inhibited immunoglobulin (IL)-6, IL-8 and tumor necrosis factor-alpha expression in a dose dependent-fashion in both, pediatric oncology patients and healthy controls.
Conclusion: Deficient immune responses to *S. maltophilia* require optimized prevention strategies against infection in immunocompromised patients, including neonates. Taurolidine may be an effective immunomodulatory agent in a clinical setting.

* Corresponding author. Department of Paediatrics, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.
E-mail address: haertel@paedia.ukl.mu-luebeck.de (C. Härtel).

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Introduction

Stenotrophomonas maltophilia is an emerging gram-negative pathogen with significance for susceptible individuals, including patients with malignant diseases undergoing myeloablative therapy and cystic fibrosis patients. *S. maltophilia* is frequently found in patient respiratory secretions and may therefore cause infectious exacerbations or ventilator-associated pneumonia. Other reservoirs include the gastrointestinal tract fluids that are used in the hospital environment (e.g., parenteral nutrition). Although the virulence factors of *S. maltophilia* are limited, patients with indwelling central venous catheters are at particular risk for subsequent infection.[1] In recent years, *S. maltophilia* infections were also reported in neonatal intensive care units, in particular in infants with need for mechanical ventilation, total parenteral nutrition, or long-term stay in hospital.[2] A better understanding of host risk factors would help to improve prevention and treatment strategies of *S. maltophilia* exposure. It was the aim of this study to establish an *in vitro S. maltophilia* sepsis model and to investigate cytokine responses in whole blood monocytes of immunocompromised individuals such as neonates and pediatric oncology patients. Furthermore, the management of *S. maltophilia* infections is complicated by the inherent resistance to multiple antibiotics. In fact, current national guidelines are reluctant to recommend a standardized treatment regimen for *S. maltophilia* infection, or employ *in vitro* testing for co-trimoxazole. However, there is a need for therapeutic options beyond co-trimoxazole that is myelosuppressive and may cause bilirubin encephalopathy in susceptible neonates. Alternative treatment strategies include combinations of ciprofloxacin, ticarcillin-clavulanate or ceftazidime/ceftriaxone.[3] In order to face the issue of antibiotic resistance, the development of new therapeutic agents remains evident. One potential antimicrobial candidate is taurolidine [bis-(1,1-dioxoperhydro-1,2, 4-thiadiazinyl-4)methane], a derivative of the amino-acid taurine with broad-range antibacterial and antifungal activity.[4–6] Its mechanism of action is presumably mediated by methylol derivatives that irreversibly destroy bacterial cell walls[7] or hamper biofilm production.[8] In addition, taurolidine may have an immunomodulatory potential including antiinflammatory properties in lipopolysaccharide-stimulated monocytes and inhibitory effects on bacterial adherence to epithelial cells.[9–11] In this study, we evaluated the immunomodulatory role of taurolidine in our *in vitro S. maltophilia* sepsis model.

Methods

Study population

Cord blood samples were taken after parental informed consent from healthy term newborns ($n = 13$, gestational age 37–41 weeks). Peripheral venous blood samples from healthy adult blood donors ($n = 10$) served as controls. Blood samples of pediatric oncology patients were withdrawn after recovery from neutropenia before the next cycle of chemotherapy was commenced (inclusion criteria: white blood cell count $\geq 2 \times 10^6$/mL; surgically inserted central venous line). The cohort of pediatric oncology patients consisted of three patients with acute lymphoblastic leukemia, three with neuroblastoma, one soft tissue sarcoma, one ependymoma, one nephroblastoma, one Hodgkin disease (age in years; mean/median/95% CI: 10.7/11.7/6.2–15.2), and their data were compared with healthy blood donors. Informed consent was given for all subjects, and the study was approved by the ethics committee of the University of Lübeck.

Whole blood assay

Heparinized whole blood was suspended in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 1% penicillin/streptomycin, 2 mM glutamine, 1 mM pyruvate, and nonessential amino acids (Seromed Biochrome, Berlin, Germany) at a concentration of 5×10^6 leukocytes/ml. A total of 5×10^6 [one colony forming unit (CFU)/white blood cell(WBC)] or 5×10^7 (10 CFU/WBC) vital bacteria (*S. maltophilia,* derived from a preterm infant born at 28 weeks of gestation who developed *S. maltophilia* sepsis) were added and whole blood cultures were incubated for 4 hours. Stimulation experiments with 30 ng/ml lipopolysaccharide [(LPS) Sigma/Deisenhofen, Germany] and 5×10^6 vital *Staphylococcus epidermidis* served as positive controls. As a negative control, only phosphate buffered saline (PBS) buffer was added.

The cells were washed in PBS, incubated with a buffer containing 4% paraformaldehyde (Riedel de Haen, Seelze, Germany) for another 10 minutes and washed again in PBS. They were resuspended in milk 5% and stored at 4°C overnight.

Intracellular staining of cytokines

Cells were washed in Hank's buffered salt solution (HBSS) and resuspended in a buffer consisting HBSS, 0.1% saponin (Riedel de Haen) and 0.01 M Hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer (Seromed Biochrome). A total of 200 μl aliquots of cells were added to tubes containing 0.5 μg/10 μl of monoclonal antibodies [(Mabs) BD Pharmingen Heidelberg, Germany] against CD14 (M5E2, Phycoerythrin-cyanine (PC)5 conjugated), immunoglobulin (IL)-6 (MQ2-13A5, Fluorescein Isothiocyanate (FITC)-conjugated), IL-8 (G265-8 Phycoerythrin (PE)-conjugated) and tumor necrosis factor (TNF)-alpha (Mab11, 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 cytometric analysis was performed on BD FACS Canto (Heidelberg, Germany). Data were expressed as percentage of stimulated whole blood cells ($CD14^+$ monocytes) positive for individual cytokine production.[12]

Statistical analysis

Statistical differences were tested for nonpaired data with the Mann-Whitney U-test, for paired data using Wilcoxon-

rank sum test. Statistical analyses were performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Whole blood *in vitro* sepsis model with *S. maltophilia*

In all different age groups we were able to demonstrate that *S. maltophilia* induces proinflammatory cytokine production in whole blood cultures comparable to stimulation with LPS. For example, pediatric oncology patients ($n = 10$); median number of cytokine expressing CD14+ cells, 95% confidence interval (CI), unstimulated versus stimulated with 1 CFU/WBC *S. maltophilia*; IL-6: 0.25%, 0.03—1.2% versus 28.7%, 13.6—42.9%, $p = 0.005$; IL-8: 4.0%, 2.3—8.8% vs. 64.3%, 54.8—79.1%, $p = 0.005$; TNF-alpha: 0.05%, 0.03—0.3% vs. 13.6%, 10.7—21.6%, $p = 0.005$. These data are shown in Figs. 1 and 2.

In addition to that, an inoculation-dose dependent stimulation of IL-6 and TNF-alpha responses was observed in both, cord blood samples and peripheral blood samples from adults (Fig. 1A—C).

Deficient proinflammatory cytokine responses in neonates

As described in Fig. 1, neonates display a deficient IL-8 and TNF-alpha response as compared to adults after LPS stimulation and *S. maltophilia* inoculation of whole blood samples. With regard to IL-6, reduced levels were only found in cord blood levels stimulated with 1 CFU/WBC *S. maltophilia*.

S. maltophilia in vitro responses in pediatric oncology patients with central venous device and effect of taurolidine

As outlined in Fig. 2, the proinflammatory cytokine expression (IL-6, IL-8, TNF-alpha) of several *in vitro* sepsis models (LPS; 1 CFU/WBC *S. epidermidis* and 1 CFU/WBC *S. maltophilia*) did not differ between pediatric oncology patients and healthy adult controls.

We also tested the effect of different concentrations of taurolidine after pre-incubation of whole blood cultures of patients and controls for 1 hour (Fig. 3). Again, no significant differences were noted between patients and controls. We were able to demonstrate that addition of 20 μg/ml taurolidine to whole blood samples of pediatric oncology patients caused a significant inhibition of IL-6 (median number of cytokine expressing CD14+ cells, 95% CI 28.7%, 13.6—42.9% vs. 2.4%, 0.05—17.4%, $p = 0.005$, $n = 10$) and TNF-alpha (13.6%, 10.7—21.6% vs. 3.9%, 2.2—10.4%, $p = 0.005$), while lower concentrations (1, 5, and 10 μg/ml) had no significant effect. A concentration of 50 μg/ml taurolidine was needed to affect patients' IL-8 expression (64.3%, 54.8—79.1% vs. 2.0%, 0.7—4.2%, $p = 0.005$). Similar results were noted for healthy adult controls (Fig. 3A—C).

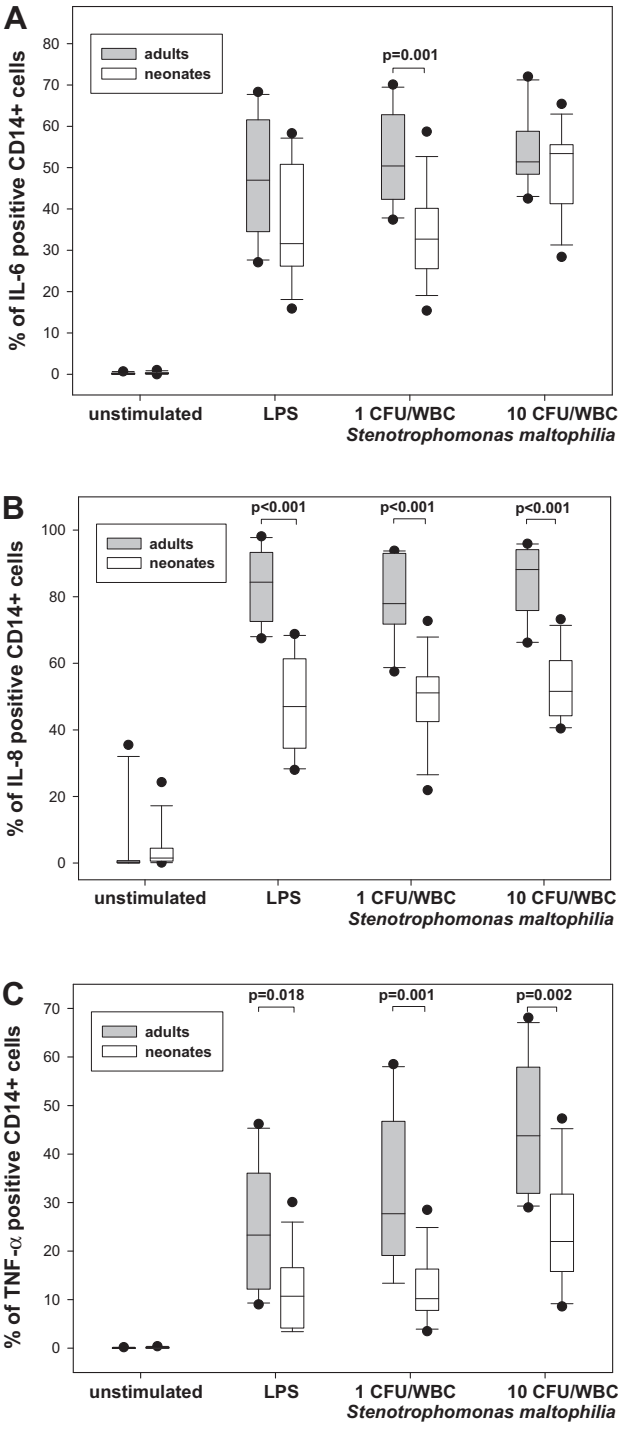

Figure 1. Deficient neonatal pro-inflammatory cytokine responses in the *S. maltophilia in vitro* sepsis model. The figure depicts the intracellular expression of (A) IL-6; (B) IL-8; and (C) TNF-alpha in CD14 + cells after incubation with PBS (control), 30 ng/ml LPS or with *S. maltophilia* 1 or 10 CFU/WBC. Neonatal (white boxes, $n = 13$) and adult data (grey boxes, $n = 10$) are presented as box plots indicating median (95% CI, 25% and 75% quartiles and outliers) percentage of cytokine expressing CD14+ cells. P-values are derived from Mann-Whitney U-test, a p value < 0.05 was regarded as significant. CI = confidence interval; CFU = colony forming unit; IL = immunoglobulin; LBS = lipopolysaccharide; PBS = phosphate buffered saline; TNF = tumor necrosis factor; WBC = white blood cell.

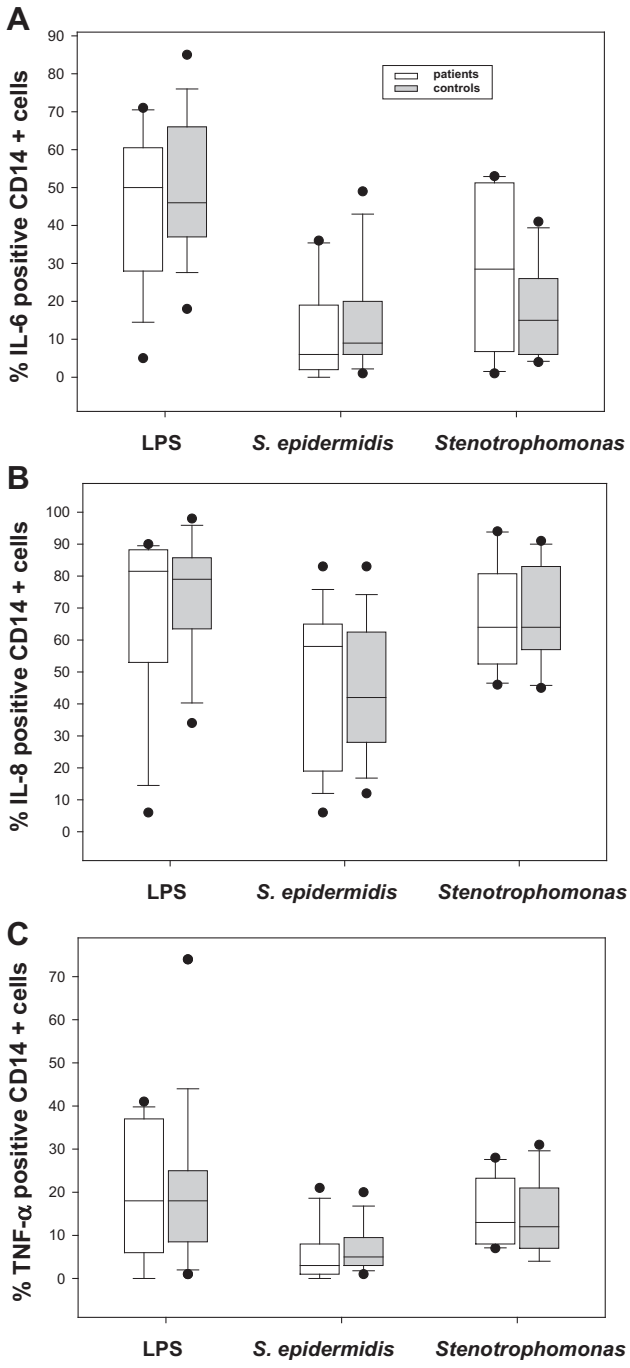

Figure 2. *S. maltophilia in vitro* responses in pediatric oncology patients with central venous device. The figure depicts the intracellular expression of (A) IL-6; (B) IL-8; and (C) TNF-alpha in CD14 + cells after incubation with 30 ng/ml LPS, l CFU/WBC *S. epidermidis* or with 1 CFU/WBC *S. maltophilia*. Data of patients (white boxes, $n = 10$) and healthy adult controls (grey boxes, $n = 10$) are presented as box plots indicating median (95% CI, 25% and 75% quartiles and outliers) percentage of cytokine expressing CD14+ cells. No statistical differences exist (Mann-Whitney U-test). CI = confidence interval; CFU = colony forming unit; IL = immunoglobulin; LBS = lipopolysaccharide; TNF = tumor necrosis factor; WBC = white blood cell.

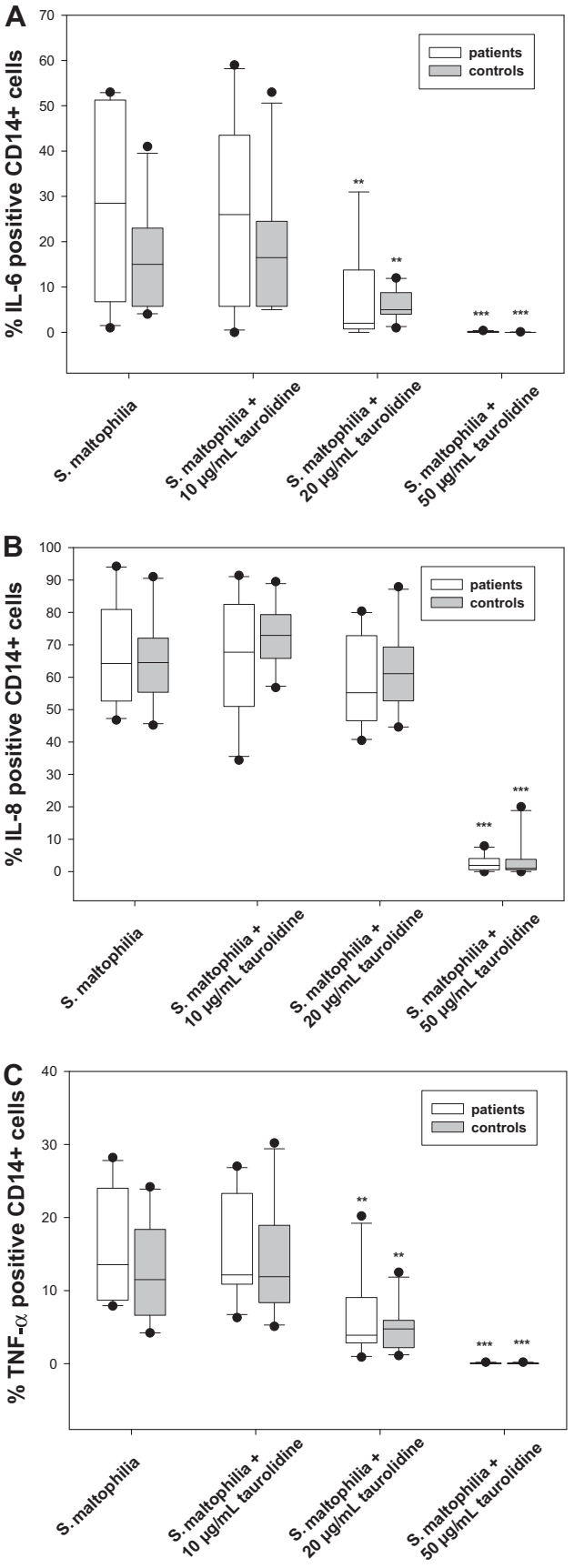

Figure 3. Effect of taurolidine in the *S. maltophilia in vitro* sepsis model. The figure depicts the intracellular expression of

Discussion

In this study, we established an *in vitro* S. *maltophilia* sepsis model and determined proinflammatory cytokine responses in whole blood monocytes of immunocompromised individuals such as neonates and pediatric oncology patients. In line with previous data regarding exposure to various antigenic or microbial stimuli,[13] neonates displayed a reduced, immature cytokine response to S. *maltophilia* that underlines the basic concept of susceptibility to infection in these vulnerable patients. Innate immune pathways represent the first defence mechanism for neonates, and maturation of immune responses is an essential part of early postnatal development.[14] On the other hand, impairment of adequate cytokine regulation in neonates (or disturbed balance in the "inflammatory situation") might be another key element for adverse outcomes associated with gram-negative bacilli infection in newborns.[15] Recent epidemiologic data indicate that S. *maltophilia* is an emerging pathogen on neonatal intensive care units,[2] and likewise for pediatric oncology patients.[1,16–19] Due to several intrinsic and acquired resistance mechanisms of S. *maltophilia*, the choice of adequate antibiotic treatment is difficult.[20] Thus preventive strategies are of high importance, particularly hygienic measures, restriction of invasive procedures including mechanical ventilation and insertion of central venous lines.[21] The taurine derivative taurolidine proved to be a promising alternative to antibiotic agents as it has antimicrobial and immunomodulatory capacities and no resistance patterns have been described yet.

In our *in vitro* setting we were able to demonstrate that pediatric oncology patients after recovery from neutropenia are able to mount an adequate proinflammatory cytokine response against whole S. *maltophilia* bacteria, which may be sufficiently reversed by addition of taurolidine to whole blood in a concentration of 20–50 μg/mL. Compared with minimal inhibitory concentrations (MIC) to kill S. *maltophilia* ranging from 500–1000 μg/ml,[6] anti-inflammatory properties are evident at much lower concentrations. While several prospective studies showed that taurolidine-lock-solutions reduce the rate of central venous access device (CVAD)-associated infections,[22–25] relevant bloodstream concentrations may, however, counter-regulate a systemic proinflammatory response. On one hand, this phenomenon might be associated with an increased risk of a deficient first-line response against S. *maltophilia*; on the other hand, the counter-regulatory potential could be beneficial to prevent systemic inflammation and organ failure. We noted that the immunomodulatory effect is concentration-dependent in both pediatric oncology patients and healthy controls. Even if concentrations of 100 μg/ml were applied, no cytotoxic effects were observed. Previous data indicate that taurolidine might be considered as antimicrobial agent for local application in lock or flushing solutions of central venous lines; in our experience, it may also be applied for counter-regulation of exaggerated proinflammatory responses in an S. *maltophilia* sepsis situation which needs to be further studied in an animal model.

There are limitations to this study. First, we used cord blood samples of healthy term neonates that do not reflect the cohort of extremely vulnerable preterm infants. Furthermore age-dependent maturation processes occur in early childhood that do not allow to superimpose our data to all pediatric oncology patients at all age groups. Second, we only investigated monocyte responses in our setting, while cytokine networks are based on additional immune cell types such as lymphocytes, dendritic cells and endothelial cells. Moreover, we described a survey of a limited selection of pro-inflammatory markers for the immune system rather than a hypothesis-driven study of individuals "at risk." In addition, our *in vitro* whole blood assay preserves physiologic concentrations of factors involved in innate immune responses but can only serve as a model for the *in vivo* situation. Third, the case numbers in all experimental groups were small, and future studies will need to evaluate whether the immunomodulatory effect of taurolidine improves clinical outcome.

In summary, deficient immune responses to S. *maltophilia* require optimized prevention strategies against infection in vulnerable patient cohorts including neonates. Taurolidine may be an effective immunomodulatory agent in a clinical setting.

(A) IL-6; (B) IL-8; and (C) TNF-alpha in CD14 + cells after incubation with 1 CFU/WBC S. *maltophilia* co-incubated with different concentrations of taurolidine. Pediatric oncology patients (white boxes, $n = 10$) and healthy adult controls (grey boxes, $n = 10$) are presented as box plots indicating median (95% CI, 25% and 75% quartiles and outliers) percentage of cytokine expressing CD14+ cells. Statistical differences (co-incubated cultures vs. controls; Wilcoxon-Rank-Sum test) are indicated as asterisks. No statistical differences exist between patients and controls (Mann-Whitney U-test). $^{**}p < 0.005$. $^{***}p < 0.0005$. CI = confidence interval; CFU = colony forming unit; IL = immunoglobulin; TNF = tumor necrosis factor; WBC = white blood cell.

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