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Short communication

Ureaplasma species modulate cell adhesion molecules and growth factors in human brain microvascular endothelial cells

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ABSTRACT

Ureaplasma species (spp.) are considered commensals of the adult urogenital tract, but may cause chorioamnionitis and preterm birth as well as sepsis and meningitis in neonates. Pathomechanisms in *Ureaplasma*-driven neuroinflammation are largely unknown. This study addressed mRNA and protein expression of intercellular and vascular cell adhesion molecules (ICAM-1, VCAM-1), granulocyte-colony stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF) in native or lipopolysaccharide (LPS) co-stimulated human brain microvascular endothelial cells (HBMEC) exposed to *Ureaplasma (U.) urealyticum* or *U. parvum*. *Ureaplasma* spp. reduced G-CSF mRNA ($p < 0.05$) and protein expression ($p < 0.01$) and increased VEGF mRNA levels ($p < 0.01$) in native HBMEC. Upon co-stimulation, *Ureaplasma* isolates enhanced LPS-evoked VEGF and ICAM-1 mRNA expression ($p < 0.05$), but mitigated G-CSF and VCAM-1 mRNA responses ($p < 0.05$). In line with previous findings, our results indicate an ability of *Ureaplasma* spp. to compromise blood-brain barrier integrity, mitigate immune defense, and subdue neuroprotective mechanisms. This may facilitate intracerebral inflammation, allow chronic infections, and promote brain injury. More pronounced effects in co-stimulated cells may indicate an immunomodulatory capacity of *Ureaplasma* spp.

1. Introduction

Although often considered low-virulent commensals of the adult urogenital tract, *Ureaplasma* species (spp.) are associated with chorioamnionitis and preterm birth, and may cause neonatal sepsis and meningitis [1,2]. Given reported detection rates of about 20% in cord blood and/or cerebrospinal fluid of preterm infants ≤1500 g birth weight, *Ureaplasma* spp. have to be regarded as relevant pathogens particularly in immature neonates [3,4]. Underlying pathomechanisms of *Ureaplasma*-driven inflammation in general and neuroinflammation in particular are still largely unknown. Inflammation is a carefully balanced process regulated by cytokines, chemokines, growth factors, and cell adhesion molecules. A particular relevance in neuroinflammation has to be ascribed to the blood-brain barrier (BBB). It usually maintains the homeostasis within the central nervous system (CNS) and anatomically as well as immunologically protects the brain from injurious impacts [5,6]. Having assessed the interactions between *Ureaplasma* spp. and human brain microvascular endothelial cells (HBMEC), main BBB constituents, we could recently demonstrate *Ureaplasma*-driven apoptosis as well as chemokine receptor responses. Both presumably contribute to BBB breakdown as a potential key feature in *Ureaplasma*-induced neuroinflammation [7,8]. The current study focuses on the impact of *Ureaplasma* spp. on cell adhesion molecules and growth factors in HBMEC. Intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1 facilitate endothelial adhesion of inflammatory cells, thus promoting tissue invasion and consecutive

Abbreviations: **BBB**, blood-brain barrier; **CCU**, color-changing units; **CD**, cluster of differentiation; **CNS**, central nervous system; **G-CSF**, granulocyte-colony stimulating factor; **HBMEC**, human brain microvascular endothelial cells; **ICAM-1**, intercellular adhesion molecule 1; **LPS**, lipopolysaccharide; **qRT-PCR**, quantitative real time reverse transcriptase polymerase chain reaction; **RPKM**, reads per kilo base per million mapped reads; **SD**, standard deviation; **spp.**, species; **U.**, *Ureaplasma*; **Up3**, *Ureaplasma parvum* serovar 3; **Uu8**, *Ureaplasma urealyticum* serovar 8; **VCAM-1**, vascular cell adhesion molecule 1; **VEGF**, Vascular endothelial growth factor

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inflammation [9]. Vascular endothelial growth factor (VEGF) is an angiogenic mediator, induces vascular permeability, and is involved in regulation of BBB integrity [9]. Granulocyte-colony stimulating factor (G-CSF) promotes neutrophil production and differentiation, but is also considered beneficial in neuroinflammation [10,11]. We correlated mRNA and protein expression of these mediators upon exposure of native or lipopolysaccharide (LPS) primed HBMEC to *Ureaplasma (U.) urealyticum* and *U. parvum*, using quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR), RNA sequencing, multi-analyte immunoassay, and flow cytometry.

2. Materials and methods

2.1. Bacterial strains and culture conditions

As described previously [8], *U. urealyticum* serovar 8 (Uu8) and serovar 3 of *U. parvum* (Up3) (American Tissue Culture Collection #27618, #27815) were cultured in an in-house medium ("broth") containing pleuropneumonia-like organism medium (Becton, Dickinson & Company, Franklin Lakes, NJ, USA), 10% heat-inactivated horse serum, 1% urea, and 0.002% phenol red (all: Sigma-Aldrich, St. Louis, CA, USA). Serial dilutions were incubated to obtain *Ureaplasma* titers of 1×10^9–1×10^{10} color-changing units (CCU)/ml (5×10^7–6×10^8 copies/ml, PCR: Institute of Medical Microbiology and Hospital Hygiene, Duesseldorf, Germany). Re-culture on selective agar plates (medco Diagnostika GmbH, Ottobrunn, Germany) confirmed viability.

2.2. Cell line and culture conditions

Non-immortalized adult HBMEC (ACBRI 376, Cell Systems, Kirkland, WA, USA) were propagated in gelatin (Serva Electrophoresis, Heidelberg, Germany) coated culture flasks (Greiner Bio-One, Frickenhausen, Germany) in a humid atmosphere with 5% CO_2 at 37 °C. Cells were cultivated in RPMI-1640 medium (Sigma-Aldrich), containing 10% fetal calf serum (Thermo Fisher, Waltham, MA, USA), 10% Nu-Serum (BD Biosciences, San Jose, CA, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimum essential medium non-essential amino acids (all: Thermo Fisher), 5 U/ml heparin (Biochrom, Berlin, Germany), and 0.3% endothelial cell growth supplement (Cell Systems). Cells at passage 8 were used for all experiments.

2.3. Stimulation assays

HBMEC were transferred to gelatin-coated 6-well culture plates (Greiner Bio-One) at a density of 2×10^5 cells/well and propagated for 48 h. Confluent monolayers were washed, and 1 ml fresh growth medium was added per well. Cells were inoculated with 10^9-10^{10} CCU of *Ureaplasma* isolates per well and/or 100 ng/ml *Escherichia coli* serotype 055:B5 LPS (Sigma-Aldrich) [8]. Cultures were incubated for 4 and 30 h for mRNA analysis or 24 and 48 h for protein assessment. Doses and incubation periods had been determined by previous experiments [8]. Unstimulated HBMEC served as negative controls. To account for potential broth effects, *Ureaplasma*-exposed cells were additionally compared to broth control, and results were considered relevant if both comparisons proved significant.

2.4. RNA extraction and qRT-PCR

The NucleoSpin® RNA Kit (Macherey-Nagel, Dueren, Germany) was used for extraction of total RNA, which was eluted in 60 µL RNAse-free H_2O (Macherey-Nagel) and quantified (Qubit® 2.0 Fluorometer, Thermo Fisher). 1 µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). For quantitative mRNA detection, cDNA was diluted 1:10 with nuclease-free H_2O (Sigma-Aldrich) and analyzed in duplicates of 25 µL reaction mixture containing 12.5 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µL nuclease-free H_2O, and 1 µL of a 10 µM primer solution, respectively (G-CSF: NM_000759.3, forward 5′-CTGCTTGAGCCAACTCCA-3′, reverse 5′-AGTTCTTCCATCTGCTGCC-3′; VEGF: NM_001171623.1, forward 5′-GTACATCTTCAAGCCATCC-3′, reverse 5′-GCTCTATCTTTCTTTGGT CTG-3′; ICAM-1: NM_000201.2, forward 5′-CAGACCTTTGTCCTG CCA-3′, reverse 5′-AAGGAGTCGTTGCCATAGGT-3′; VCAM-1: NM_001078.3, forward 5′-GCAAGTCTACATATCACCCA-3′, reverse 5′-AGTTGCATTTCCAGAAAGGT-3′; all: Sigma-Aldrich), using an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher). Amplification was normalized to the reference gene hypoxanthine phosphoribosyltransferase 1 (NM_000194.2, forward 5′-CTGGCGTCGT GATTAGTG-3′, reverse 5′-AGTCCTGTCCATAATTAGTCC-3′, Sigma-Aldrich). Experiments were repeated 5 times (n = 5).

2.5. RNA sequencing

Total RNA from 3 individual experiments (n = 3) was extracted by means of NucleoSpin® RNA Kit (Macherey-Nagel). RNA sequencing was performed at the Core Unit Systems Medicine, University of Wuerzburg, Germany, as described previously [8]. The Illumina TruSeq stranded mRNA Kit (Illumina, San Diego, CA, USA) was used with 700 ng of input RNA. Libraries were pooled, and reads obtained by sequencing (NextSeq 500, Illumina) were processed employing FastQC 0.11.5. Sequences were mapped to the human genome, those aligning to specific genes were quantified using bedtools subcommand intersect (version 2.15.0), and DESeq2 (version 1.16.1) helped to identify differentially expressed genes. Reads per kilo base per million mapped reads (RPKM) were calculated using DGEList of the edgeR package.

2.6. Multi-analyte immunoassay

Supernatants were collected and cytokine concentrations were determined using Luminex® multiplex kits and xPonent® software (Merck, Darmstadt, Germany). Lower detection limits were 1.44 pg/ml (G-CSF), 7.30 pg/ml (VEGF), 25.52 pg/ml (ICAM-1), and 11.48 pg/ml (VCAM-1); values underneath were set to 0. Samples were analyzed in duplicate, experiments were repeated 5 times (n = 5).

2.7. Flow cytometry

After Fc-receptor blocking with 4 mg/ml gamunex (Grifols, Frankfurt, Germany), cells were stained with a Brilliant Violet conjugated antibody to cluster of differentiation (CD) 31 (BV510, BD Biosciences), a Pacific Blue conjugated antibody to ICAM-1 (BioLegend), a PE conjugated antibody to VCAM-1 (BioLegend), and Fixable Viability Dye eFluor™ 780 (eBioScience, Thermo Fisher). Cells were separated by centrifugation and resuspended in phosphate buffered saline (Sigma-Aldrich) supplemented with 1% human serum (Biochrom). Samples were read on a FACSCanto™ II flow cytometer (BD Biosciences), acquiring a minimum of 10,000 events to be analyzed using FACSDiva v6.1.3 software (BD Biosciences). Doublets were excluded using a SSC-height versus FSC-width dot plot, and events were gated for CD31 positive, viable cells. Experiments were repeated ≥5 times (n ≥ 5).

2.8. Statistical analysis

Results were analyzed using Prism® 6 software (GraphPad, San Diego, CA, USA). A one way ANOVA was followed by Tukey's multiple comparisons test. Differences at $p < 0.05$ were considered statistically significant.

3. Results and discussion

This *in vitro* study provides further insights into *Ureaplasma*-driven

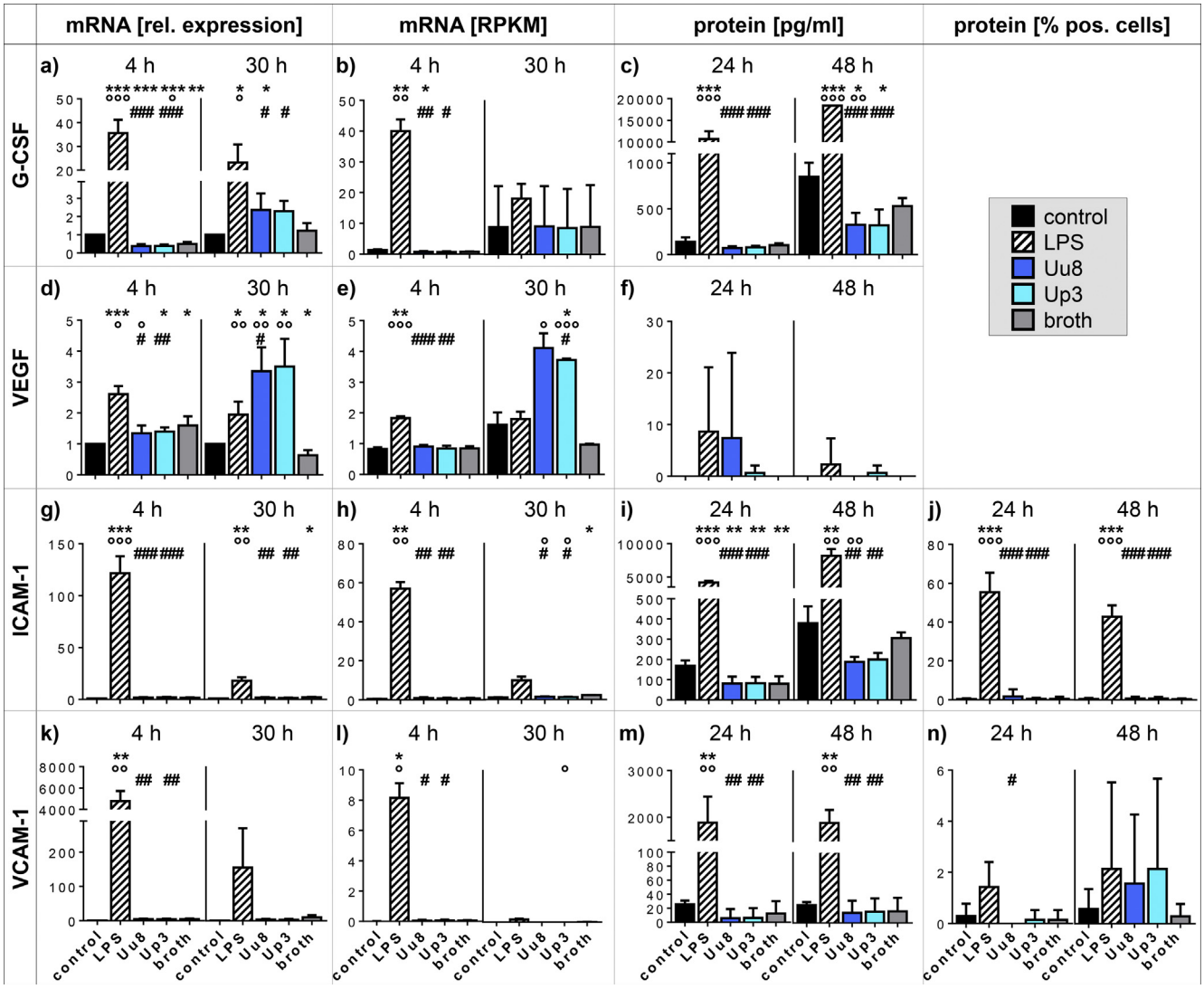

Fig. 1. Expression of G-CSF, VEGF, ICAM-1 and VCAM-1 in HBMEC exposed to *Ureaplasma* spp. or LPS. Expression of mRNA was assessed by qRT-PCR (a, d, g, k) and RNA sequencing (b, e, h, l). Multi-analyte immunoassay was employed for quantification of secreted protein (c, f, i, m), intracellular protein was determined using flow cytometry (j, n). Data are presented as means ± SD from 3 (RNA sequencing), 5 (qRT-PCR, multi-analyte immunoassay), or ≥5 (flow cytometry) individual experiments ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. unstimulated control; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs. broth; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LPS).

neuroinflammation and demonstrates an impact of *Ureaplasma* spp. on cell adhesion molecules and growth factors.

Whereas LPS induced a profound increase in G-CSF mRNA and protein abundance in HBMEC, *Ureaplasma* spp. evoked opposing effects and reduced G-CSF mRNA expression after 4 h (qRT-PCR: Up3 $p < 0.05$ vs. broth) as well as protein secretion after 48 h (Uu8 $p < 0.01$) (Fig. 1a–c). G-CSF is relevant particularly in neutrophil inflammation [11]. Our results may therefore indicate suppressed inflammatory responses in HBMEC upon *Ureaplasma* exposure. This is in line with our previous findings [7] and may ultimately impair pathogen elimination and facilitate long-term infection. Clinical implications are illustrated by descriptions of chronic *Ureaplasma*-meningitis in preterm infants [1]. Moreover, G-CSF is considered neuroprotective and anti-apoptotic and may improve the outcome after neonatal hypoxia-ischemia [10]. *Ureaplasma*-driven G-CSF depression may therefore increase the brain's vulnerability to injurious impacts and may even be an underlying cause for the potential association between *Ureaplasma* colonization and development of intraventricular hemorrhage or cerebral palsy in preterm infants [4,12]. Given an additional BBB stabilizing effect of G-CSF [10], *Ureaplasma*-associated G-CSF decrease may, moreover, promote BBB injury. Our previous studies describing *Ureaplasma*-induced apoptosis and chemokine receptor responses in HBMEC similarly indicate an ability of *Ureaplasma* spp. to impair the BBB [7,8]. A compromised BBB integrity is a key feature in neuroinflammation, since impaired barrier properties promote pathogen and inflammatory cell entry into the CNS [5,6].

Both *Ureaplasma* isolates significantly increased VEGF mRNA expression after 30 h (qRT-PCR: $p < 0.01$ vs. broth), with effects more pronounced than those evoked by LPS (Fig. 1d–f). VEGF promotes vessel permeability. Disturbed VEGF signaling may contribute to several neonatal morbidities such as chronic lung disease and retinopathy of prematurity [13]. Enhanced VEGF levels have furthermore been associated with BBB leakage and are, for example, implicated in T-cell mediated BBB disruption [9,14]. Induction of VEGF may therefore represent yet another mechanism *Ureaplasma* spp. employ to impair BBB integrity, although we could not demonstrate an effect on protein secretion. This may be due to suboptimal incubation periods or complex post-transcriptional processes regulating VEGF mRNA degradation, translation, and secretion [15]. Of note, in a previous study, we could demonstrate increased VEGF mRNA also in *Ureaplasma*-stimulated

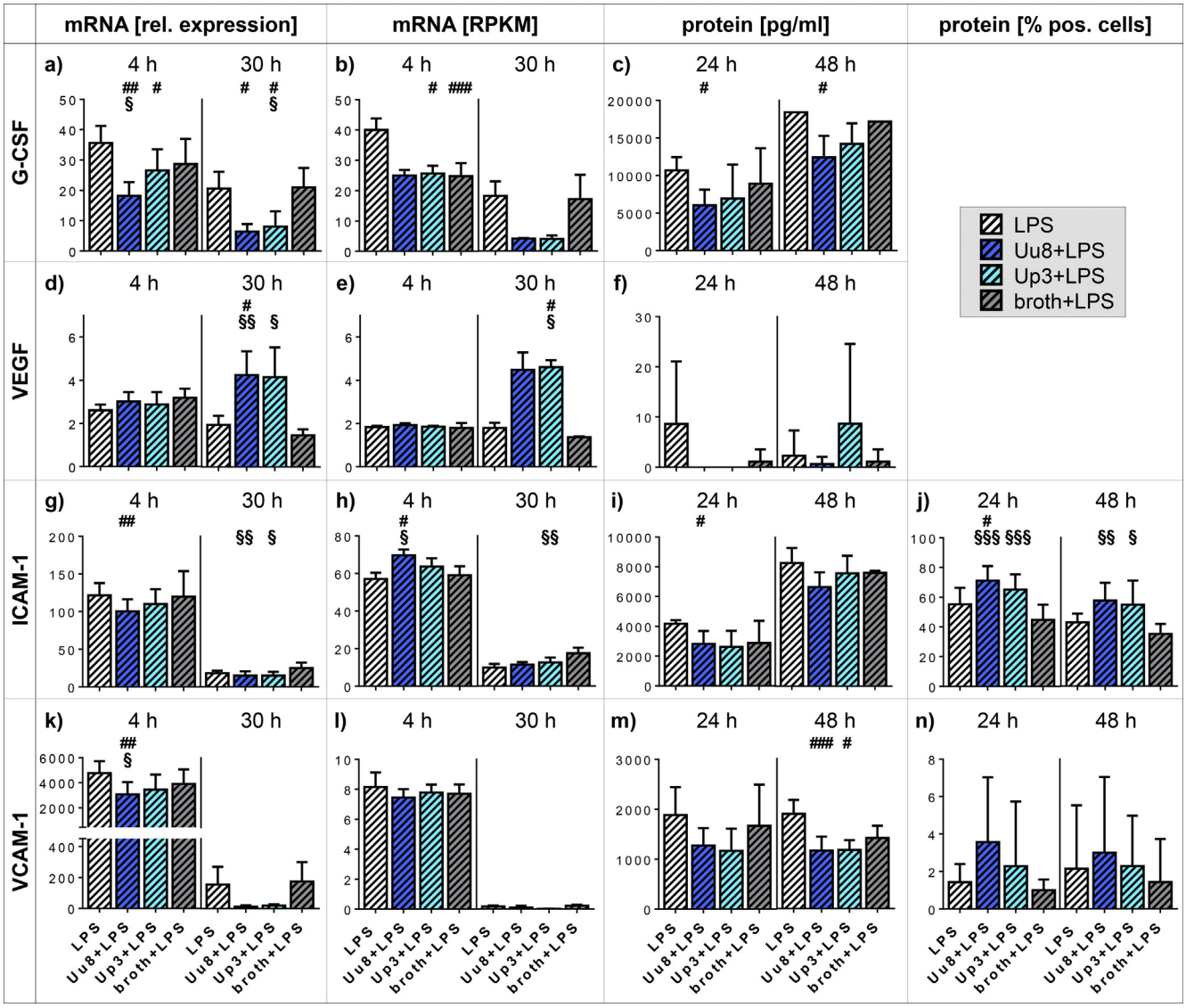

Fig. 2. Expression of G-CSF, VEGF, ICAM-1 and VCAM-1 in co-stimulated HBMEC. Using qRT-PCR (a, d, g, k) and RNA sequencing (b, e, h, l), mRNA expression was determined. Secreted protein was quantified using multi-analyte immunoassay (c, f, i, m), and intracellular protein was assessed by flow cytometry (j, n). Data are presented as means ± SD from 3 (RNA sequencing), 5 (qRT-PCR, multi-analyte immunoassay), or ≥5 (flow cytometry) individual experiments ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LPS; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ vs. broth + LPS).

human neonatal and adult monocytes [16].

Whereas LPS distinctly enhanced cell adhesion molecules, *Ureaplasma* stimulation did not relevantly influence ICAM-1 and VCAM-1 mRNA or protein synthesis in native HBMEC (Fig. 1g–n). Both molecules mediate leukocyte adhesion during inflammation, however, to which extent they contribute to leukocyte entry into the CNS is not yet fully understood [9]. *Ureaplasma* spp. alone thus appear to provoke barrier breakdown rather than classic pro-inflammation.

In co-stimulated HBMEC, we observed a modulation of LPS-induced responses by *Ureaplasma* spp. *Ureaplasma* isolates mitigated G-CSF mRNA expression upon 4 and 30 h of stimulation (qRT-PCR: $p < 0.05$ vs. LPS and vs. broth + LPS), with a non-significant extent to protein levels (Fig. 2a–c). The above described *Ureaplasma*-driven VEGF mRNA enhancement was apparent also in co-stimulated cells ($p < 0.05$). Uu8 significantly augmented LPS-evoked increases in ICAM-1 mRNA (RNA sequencing: $p < 0.05$) and surface protein levels ($p < 0.05$) (Fig. 2g–j). For VCAM-1, we observed a significant Uu8-driven mitigation of LPS-induced mRNA expression (qRT-PCR: $p < 0.05$) (Fig. 2k–n). These influences of *Ureaplasma* spp. on LPS-evoked responses add to previous *in vitro* studies [7,8,16,17] and may, again, indicate an immunomodulatory capacity of *Ureaplasma* spp. in the event of co-infection *in vivo*. *Ureaplasma*-driven mitigation of LPS-induced effects, as seen for G-CSF and VCAM-1, could entail suppressed inflammatory responses and impaired pathogen elimination. Enhanced ICAM-1 and VEGF levels in co-stimulated cells may indicate an impairment of BBB integrity caused by *Ureaplasma* spp. particularly in the presence of another pathogen. Both ways are likely to undermine key CNS immune defense mechanisms, increasing the brain's susceptibility to secondary infections or non-infectious impacts, ultimately facilitating neuroinflammation and brain injury. This may be of particular clinical relevance given the common polymicrobial colonization of preterm neonates. Our *in vitro* findings are underlined by a clinical study describing a higher risk for sepsis in *Ureaplasma*-colonized preterm infants [18] and by mitigated LPS-induced immune responses in chronic *Ureaplasma*-colonized fetal sheep [19].

4. Conclusion

This *in vitro* study adds to previous findings, demonstrating an impairment of immune defense and neuroprotective mechanisms by

Ureaplasma spp. Particularly BBB breakdown seems to emerge as a key feature in *Ureaplasma*-driven neuroinflammation. Ultimate consequence may be an increased CNS vulnerability to secondary injurious hits. Effects may be enhanced in the event of co-infections, indicating immunomodulatory capacities of *Ureaplasma* spp.

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Authors' contributions

CS, CPS, KG: study conception and design. CS, AH, MF, KG: acquisition and analysis of data. CS, CPS, AH, MF, HC, NS, KG: interpretation of data. CS, CPS, AH, MF, HC, NS, KG: drafting and critical revision. All authors read and approved the final manuscript.

Declarations

Declaration of Competing Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Availability of data and materials: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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