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Virulence factor-dependent basolateral invasion of choroid plexus epithelial cells by pathogenic *Escherichia coli* in vitro

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One sentence summary: Invasion of choroid plexus epithelial cells, which constitute an in vitro model of the blood–cerebrospinal fluid barrier, by *E. coli* K1 is dependent on the presence of virulence factors.

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ABSTRACT

Escherichia coli is the most common Gram-negative causative agent of neonatal meningitis and *E. coli* meningitis is associated with high morbidity and mortality. Previous research has been carried out with regard to the blood–brain barrier and thereby unveiled an assortment of virulence factors involved in *E. coli* meningitis. Little, however, is known about the role of the blood–cerebrospinal fluid (CSF) barrier (BCSFB), in spite of several studies suggesting that the choroid plexus (CP) is a possible entry point for *E. coli* into the CSF spaces. Here, we used a human CP papilloma (HIBCPP) cell line that was previously established as valid model for the study of the BCSFB. We show that *E. coli* invades HIBCPP cells in a polar fashion preferentially from the physiologically relevant basolateral side. Moreover, we demonstrate that deletion of outer membrane protein A, *ibeA* or *neuDB* genes results in decreased cell infection, while absence of *fimH* enhances invasion, although causing reduced adhesion to the apical side of HIBCPP cells. Our findings suggest that the BCSFB might constitute an entry point for *E. coli* into the central nervous system, and HIBCPP cells are a valuable tool for investigating *E. coli* entry of the BCSFB.

Keywords: Blood–cerebrospinal fluid barrier; *Escherichia coli* K1; host–pathogen interaction; meningitis; polar invasion; virulence factor

INTRODUCTION

Escherichia coli, a Gram-negative bacterium and member of the family of *Enterobacteriaceae*, is one of the most prevalent commensal bacterium within the human intestinal flora. Despite being apathogenic and commensal for the most part, *E. coli* is also one of the most common pathogens in a broad spectrum of infectious diseases, both intestinal and extraintestinal (Kaper, Nataro and Mobley 2004). In terms of extraintestinal infections, clinical manifestations associated with a high rate of severe complications are sepsis and meningitis. Meningitis is a condition that, when caused by *E. coli*, occurs almost exclusively in neonates.

Currently, *E. coli* is the second most common cause of neonatal bacterial meningitis after group B *Streptococcus* and is responsible for 18% of early-onset and 33% of late-onset neonatal meningitis (Gaschignard et al. 2011). Unfortunately, though incidence and mortality of overall neonatal meningitis has decreased, the fatality rate is still reported to be as high as 15% and morbidity remains virtually unchanged (Baud and Aujard 2013). A high rate of meningitis survivors has remaining neurological deficits such as vision impairment, cognitive and behavioral impairment, and overall disability (Barichello et al. 2014). A main reason for the failure to improve the outcome is the lack of understanding in the mechanisms of pathogenesis.

In order for *E. coli* to cause meningitis, the pathogen must interact with and overcome the mucosa of the gastrointestinal tract and enter circulation, consequentially leading to dissemination (Witcomb et al. 2015). This is followed by penetration of either the blood–brain barrier (BBB) or the blood–cerebrospinal fluid (CSF) barrier (BCSFB) and colonisation within the CSF spaces, causing the ultimate leptomeningeal inflammation (Kim 2008).

So far, invasion of the central nervous system (CNS) by *E. coli* has mainly been attributed to the crossing of the BBB; therefore, a lot of research has been carried out regarding this anatomical location, whereas relatively little is known about a role of the BCSFB. However, a number of both early and recent studies have suspected the BCSFB to play a role during the invasion of *E. coli* into the CSF (Parkkinen et al. 1988; Zelmer et al. 2008; Dalgakiran et al. 2014; Witcomb et al. 2015).

Differing from the BBB, the vascular endothelium at the choroid plexus (CP) is fenestrated. The barrier function is held up by a monolayer of unique CP epithelial cells, comprised of apical tight junctions, low paracellular permeability and a finely regulated set of transport mechanisms that enable the active intake of required nutrients and removal of toxic substances from the CSF (Engelhardt and Sorokin 2009).

The ability of *E. coli* to cross the BBB and cause meningitis has been established in a number of studies, primarily regarding human brain microvascular endothelial cells (HBMEC) (Kim 2016). Here, transversal of the cell layer occurs by transcytosis via membrane-bound vacuoles, without intracellular replication and without altering barrier integrity (Prasadarao et al. 1999; Kim 2008). A number of virulence factors have been shown to contribute to this process, including cytotoxic necrotizing factor 1 (*cnf1*), *ibeA* protein, *fimH*, outer membrane protein A (*ompA*) and the *neuDB* gene products. *OmpA* and *fimH* were primarily associated with adhesion processes, *cnf1* and *ibeA* with the ability to enter the cell and *neuDB* as a requisite for survival upon cell entry (Prasadarao et al. 1996; Huang et al. 2001b; Kim et al. 2003; Teng et al. 2005; Wang and Kim 2013; Kim 2016).

Cnf1 is an AB type toxin, which has been shown to induce bacterial uptake into non-professional phagocytes, such as epithelial and endothelial cells (Fabbri et al. 2002). *IbeA* protein is a virulence factor that has particularly been described regarding NMEC (neonatal meningitis causing *E. coli*) and uses Caspr1 as host receptor to facilitate BBB penetration (Zhao et al. 2018). Both *cnf1* and *ibeA* facilitate bacterial invasion into HBMEC, where they promote actin reorganisation (Huang et al. 2001a; Khan et al. 2002; Maruvada and Kim 2012; Wang and Kim 2013; Kim 2016).

Important roles during adhesion to and invasion into host cells are played by adhesive bacterial structures including type 1 fimbriae of Gram-negative bacteria, which are assembled from multiple subunits (Kline et al. 2009). Located at the very tip of type 1 fimbriae in *E. coli*, *FimH* is well-characterised for its involvement in adhesion processes. This role has especially been established in the context of urinary tract epithelial cells, and could also be demonstrated for HBMECs (Teng et al. 2005, 2006; Feenstra et al. 2017). Importantly, the expression of type 1 fimbriae is phase variable (Eisenstein 1981),

OmpA is one of the major structural components within the outer membrane of *E. coli*, contributing to cell surface integrity and is involved in the pathogenicity of *E. coli* in multiple organ systems (Prasadarao et al. 1996; Confer and Ayalew 2013). Apart from its main adhesive role, upon uptake into HBMECs it leads to a reduced glucose uptake and disturbed barrier integrity (Krishnan et al. 2016).

The *neuDB* genes are gene clusters necessary for production of the cytoplasmatic precursors of n-acetylneuraminic acid polymers that are the basis for the K1 capsule, which is considered to be the only K-serotype capable of entering the brain as live bacterium and causing meningitis. In HBMEC, survival upon cell entry was shown to be dependent on the ability to avoid lysosomal fusion and subsequent degradation, and this was greatly reduced in *neuDB*-deficient mutants (Kim et al. 1992, 2003).

The aim of the following paper is to shed light on characteristics of *E. coli* pathogenesis with regard to the BCSFB. Moreover, we investigate the role of a set of virulence factors that have been shown in earlier studies to participate in BBB transversal.

MATERIALS AND METHODS

Bacterial strains and cultivation

Escherichia coli RS218 is a strain isolated from the CSF of a neonate with meningitis that has been described as the prototypic wild type causing NMEC (Huang et al. 1995; Wijetunge et al. 2015). It was used in this study along with its derivatives lacking certain virulence factors as previously described. Additionally, E44Δ*neuDB*, a strain derived from a spontaneous rifampicin resistant mutant of RS218 served as a capsule-deficient strain. The pathogenic RS218 strain and all derivative strains used for this paper are listed in Table 1. *Escherichia coli* K12 laboratory strain HB101 was used as non-pathogenic reference strain and was kindly provided by Dr Ulrich Dobrindt (Institute of Hygiene, University of Münster, Münster, Germany).

The genetic characteristics of the various strains were confirmed by growth on selective agar plates (Table 1) and PCR analysis of genomic DNA with appropriate primers (Table 2). For each experiment, bacterial viability was verified with parallel growth curves.

All strains were stored at –20°C in brain heart infusion (BHI) containing 10% glycerol. Prior to each experimental procedure, 3 ml of BHI supplemented with appropriate antibiotics according

Table 1. RS218-derived bacterial strains used in this study.

Bacterial strain	Characteristics	Antibiotics added to culture medium	References
RS218	K1 O18 H7	strep	Silver et al. (1980), Huang et al. (1995)
RS218Δcnf1	K1, cnf1 gene deletion	strep, chl	Yu and Kim (2010)
RS218ΔompA	K1, ompA gene deletion	strep, chl	Teng et al. (2006)
nRS218ΔfimH	K1, fimH gene deletion	strep, chl	Teng et al. (2005)
RS218ΔibeA	K1, ibeA gene deletion	strep, kan	Maruvada and Kim (2012)
E44ΔneuDB	K1, neuDB gene deletion	rif, chl	Kim et al. (2003)

strep, streptomycin; chl, chloramphenicol; kan, kanamycin; rif, rifampicin.

Table 2. PCR primers used in this study.

Target gene	Forward primer	Reverse primer
ompA	GAGTACGCGATCACTCCTGA	GCAACTACTGGAGCTGCTTC
cnf1 (1)	CGCAGTTTCAGTGATGGTGA	TCGCCAAACCAGGTATAGCA
cnf2 (2)	ATGAAGCCCCGGTTGAAGTA	TCCCCGTCTTTAAGCACAT
ibeA	TTCGGTTGTTTTGGCGGAAT	TGCCTGTTCAAGCATTGCAT
fimH	TGCCATAACGATTACCCGGA	CAGCTTTAATCGCCACTCCC
neuDB	TGGTTCCTGGGTCGGTTGTAA	GCATTAACACCGGCCTCTTT
Chloramphenicol resistance cassette	AGGGATTGGCTGAGACGAAA	GGTGAGCTGGTGATATGGGA
Kanamycin resistance cassette	GTGACGACTGAATCCGGTGA	ACCGATCGCGTATTTCGTCT

to strain (Table 1) were inoculated with the appropriate bacterial strains from cryo-stocks using sterile inoculating loops and cultivated under static conditions at 37°C for 14–18 h. In preparation for further use, they were washed twice with serum-free medium and diluted to the optical density at 600 nm (OD₆₀₀) of 0.6 for HB101 and 0.3 for all other strains.

PCR-based restriction length di-morphism assay

The human CP papilloma (HIBCPP) inverted cell culture system was used and maintained as mentioned above. For infection of HIBCPP cells, all *E. coli* strains used were added to the cells with a multiplicity of infection (MOI) of 10 for 1 h at 37°C. Following the bacterial incubation, 50 μl of the supernatant was taken from the filter compartment and boiled for 15 min at 99°C to extract the DNA from the bacteria. DNA was measured with the help of a Nanodrop (VWR, Radnor, USA). Extracted DNA (150 ng) was used for conventional Taq PCR (Qiagen, Hilden, Germany) with the OL4 and OL20 primers described previously (Smith and Dorman 1999). PCR was performed with the following cycles: initial denaturation 94°C for 3 min followed by 35 cycles, 97°C for 1 min, 58°C for 1 min, 72°C for 1 min and final extension 72°C for 8 min. After samples were cooled down, 10 μl of PCR product was challenged with FastDigest BstUI (Life Technologies, Karlsbad, USA) according to the manufacturers' instructions and loaded onto a 1.5% Agarose gel. The PCR fragment shows a length of 726 bp, whereas the restriction enzyme digested samples show products of 433 and 293 bp (phase ON populations of bacteria) and 539 and 187 bp (phase OFF populations). Bacteria containing both phase ON and phase OFF populations show all of the mentioned products.

Cell culture

The use of HIBCPP as model of the human BCSFB has been previously established (Schwerk et al. 2012). In brief, HIBCPP cells were cultivated in DMEM/F12 (Ham) medium with 4 mM

L-glutamine and 15 mM HEPES, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 5 μg/ml insulin and containing 15% FCS. For standard cell cultures, 1×10^5 cells were seeded onto filter inserts (3.0-μm pore diameter, 2.0×10^6 pores/cm², 0.33 cm² surface; Greiner Bio One, Germany) in a 24-well plate with culture medium containing 15% FCS. For inverted cell cultures, 0.7×10^5 cells were seeded on the same filter inserts that had been flipped over and placed in 12-well plates containing medium, which were then flipped back and placed in a 24-well plate the following day. The transepithelial electrical resistance (TEER) was measured daily with an epithelial tissue voltohmmeter (Millipore, Germany) and when it exceeded $70 \Omega \times \text{cm}^2$ the medium was changed to 1% FCS containing culture medium without antibiotics. Cells that had reached a TEER of $250\text{--}800 \Omega \times \text{cm}^2$ 1 or 2 days later were used in the experiments.

Evaluation of cytotoxicity and barrier function

To evaluate the effect of *E. coli* infection on the barrier function of the HIBCPP cell layer in standard and inverted cell cultures, TEER measurements were carried out every 2 h with an epithelial tissue voltohmmeter (Millipore, Germany). This was done over a period of 8 h employing MOI of 1 and 10. All assays were carried out three times in triplicates ($n = 9$).

Live/dead assays based on intracellular esterase activity (causing green fluorescence) and membrane permeability (damaged membranes allow red dye to enter the cell and bind to DNA and thus fluoresce) were performed according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

Double immunofluorescence analysis for determination of adhesion and invasion

Invasion and adhesion assays were done as previously described (Schwerk et al. 2012) with modifications. Bacteria were added to

the prepared cells at an MOI of 10 and bacterial exposure occurred for 1 h at 37°C after which the cells were stained for immunofluorescence. All assays were performed in triplicates and repeated at least five times ($n = 15$). Cell viability and barrier function during the experiment were assured with use of live/dead assays and TEER measurements.

For determination of invasion and association of *E. coli* in HIBCPP cells, intra- and extracellular bacteria were fluorescently labeled as described previously (Schwerk et al. 2012). The primary antibody used to label all *E. coli* was goat anti-*E. coli*, Serotypes O + K (Invitrogen, Rockford, USA). Extracellular bacteria were stained with antibody Alexa Fluor 594 (red) donkey anti-goat (Molecular Probes, Oregon, USA) before cell permeabilisation with 0.5% Triton X-100. After the cells had been rendered permeable, all bacteria, intra- and extracellular, were stained with Alexa Fluor 488 (green) donkey anti-goat (Molecular Probes, Oregon, USA), cell nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride diluted 1:50 000 (blue) and Phalloidin Alexa Fluor 660 (Molecular Probes, Oregon, USA) was used to stain actin filaments.

Image acquisition occurred with Zeiss Apotome® and Zen blue software (Carl Zeiss, Jena, Germany) using a 63×/1.4 objective lens. The amount of invaded or adhered bacteria per filter was extrapolated by counting green (intracellular) or both green and red (extracellular) bacteria, respectively, within 20 (1388 × 1040 pixel) fields of vision and multiplying the mean value by an area coefficient. Together with the number of bacteria present at the end point of cell incubation, which was derived from growth curves carried out in parallel to each experiment, we hereby derived the percentage of invaded and adhered bacteria.

Transmission electron microscopy of invasion assays

Invasion assays were carried out as previously described. Parallel to the samples for electron microscopy, some samples were stained for double immunofluorescence to ensure bacterial invasion analogue to previous experiments. Cell viability was assured with TEER measurements and live/dead assays. After incubation, the cells were washed with PBS, fixed with 2% glutaraldehyde for 2 h and finally washed and kept in cacodylate buffer before osmification with 1% aqueous osmium tetroxide for 1 h at room temperature. After washing with buffer, samples were embedded into 2% water agar, cut into cubes and dehydrated with a series of 10%, 30%, 50% ethanol each step for 15 min. Samples were left overnight in 70% ethanol containing 4% uranyl acetate. The following day samples were further dehydrated with 90% and 100% ethanol. Infiltration was done with LV resin applying the hard formula of the resin. Samples were flat embedded and after resin polymerisation small areas were cut out and glued onto resin to enable an 'enface cutting'. Ultrathin sections were cut with a diamond knife and counterstained with 4% uranyl acetate. Samples were examined in a Zeiss EM910 with an acceleration voltage of 80 kV. Images were recorded digitally at calibrated magnifications with a Slow-Scan CCD-Camera (ProScan, 1024 × 1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

For statistical analysis SAS software, release 9.4. (SAS Institute Inc., Cary, North Carolina, USA) has been used. In order to compare two groups regarding relative frequencies of a binary factor

(i.e. adhesion yes/no) Fisher's exact test has been used. For quantitative variables, Mann-Whitney U-test has been performed as a non-parametric test for location. Because of the rather small sizes, exact P values have been requested. The result of a statistical test has been considered as significant if the P value was less than 0.05.

RESULTS

TEER breakdown after HIBCPP cell exposure to pathogenic *E. coli* is more pronounced when exposure occurs from the basolateral side

Pathogenic *E. coli* strains lead to barrier dysfunction of epithelial and endothelial cell layers; however, this has primarily been examined following exposure from the apical side only (Khan, Iqbal and Siddiqui 2012; Wood et al. 2012). To investigate the impact of *E. coli* on barrier function of CP epithelial cells over time, we exposed HIBCPP cells to different *E. coli* strains from either the apical cell side in standard cell cultures or the basolateral cell side in inverted cell cultures, respectively, and measured TEER values in 2 h intervals. As can be seen in Fig. 1, an increasing drop in TEER was visible after infection with the pathogenic strain RS218. Noteworthy, TEER values dropped more rapidly in the inverted cell cultures compared to the standard cell cultures both after infection with an MOI of 1 and an MOI of 10. In contrast, TEER values remained stable after infection with the non-pathogenic strain HB101 under all conditions investigated (Fig. 1).

Also, we were interested in investigating the impact of described virulence factors of pathogenic *E. coli* on barrier function following the challenge of HIBCPP cells. For this purpose, we monitored the TEER of standard and inverted cell cultures in 2-h intervals over the course of 8 h after infection with the various deletion strains. We did not observe a difference in TEER development between the different RS218-derived strains (Fig. 1).

The results of the TEER measurements coincided with observations in live/dead assays, where all RS218-derived strains displayed a strong toxic effect on the cells, while no clear difference was detectable between the different strains. HB101, however, had no influence on cell viability and no difference could be seen in comparison to the control setting (Supplementary Fig. S1).

Escherichia coli transverse HIBCPP cell layer transcellularly within membrane-bound vacuoles

To investigate cell invasion and adhesion, bacteria were stained for visual analysis with Apotome immunofluorescence microscopy 1 h after infection, as described in Materials and Methods, and in parallel some filter inserts were fixated for EM images. Both in the double immunofluorescence images as well as in EM images, *E. coli* RS218 could be detected within the cells, while there was no evidence of bacteria to be found in between cells (Fig. 2). In many cases, however, extracellular bacteria could be identified attached to the apical cell surface (Fig. 2a, c and d), most likely after having successfully penetrated the cell layer. Ultrathin section images confirmed the intracellular location of the bacteria in membrane-bound compartments. Thus, we assume that *E. coli* is transported within membrane-bound vacuoles (Fig. 2e and f) through the cell. Bacteria were frequently found as groups of two and more bacteria within one vacuole (Fig. 2e).

Figure 1. *Escherichia coli* induced changes to TEER in HIBCPP standard and inverted cultures over time. Changes in TEER after infection of HIBCPP cells with *E. coli* at an (a) MOI of 10 in standard cultures, (b) MOI of 10 in inverted cultures, (c) MOI 1 in standard cultures and (d) MOI of 1 in inverted cultures. Values are given as % of the initial TEER prior to infection (0 h value); initial TEER was between 300 and $660 \Omega \times \text{cm}^2$ (SD $\pm 129 \Omega \times \text{cm}^2$). All conditions were carried out three times in triplicates ($n = 9$).

FimH is required for adhesion of pathogenic *E. coli* to the apical side of HIBCPP cells

Adhesion of *E. coli* to host cells presents an important step during the process of cell invasion and barrier transversal (Kim 2016). Therefore, we studied the adhesive properties of the different strains to the apical side of HIBCPP cells by double immunofluorescence microscopy as described in Materials and Methods. It is necessary to point out that adhesion to the basolateral cell side could not be evaluated due to the obstruction by the filter.

A major role during adhesion to host cells is played by type 1 fimbriae (Kline et al. 2009). Since the expression of type 1 fimbriae in *E. coli* is phase variable (Eisenstein 1981), we analysed the state of type 1 fimbrial expression in the *E. coli* strains by a PCR-based restriction length di-morphism assay as described in Materials and Methods. We found similar levels of ON phase for all strains, except for the *ompA*-deficient strain, where ON phase was reduced, and the *fimH*-deficient strain, where ON phase was nearly absent (Supplementary Fig. S2). Due to the deletion of *fimH*, the minimal levels of ON phase should not be relevant in the *fimH*-deficient strain.

As shown in Fig. 3a, all pathogenic bacterial strains adhered to the apical cell side in comparable amounts, except for the *fimH*-deficient strain, in which the average amount of adhered bacteria was decreased by 94% ($P < 0.0001$; U-test). Noteworthy, there was no significant difference in adhesion of RS218Δ*fimH* compared to HB101, a non-pathogenic strain in which the *fimH* gene is not expressed ($P = 0.2148$; Fisher's test).

Escherichia coli invasion into HIBCPP cells occurs in a polar fashion and is dependent on *cnf1*, *ompA*, *ibeA* and capsule proteins

Several virulence factors have been established for their role in the crossing of the BBB and the development of meningitis (Kim 2016). To identify virulence factors involved in *E. coli* invasion across the BCSFB, invasion assays were carried out with double immunofluorescence microscopy as described in Materials and Methods.

As depicted in Fig. 3b, bacterial invasion occurred from the basolateral side, whereas little to no intracellular bacteria could be found when cells were infected apically ($P < 0.0001$; U-test). All *E. coli* strains derived from the RS218 wild-type strain showed higher levels of invasion than the HB101 non-pathogenic laboratory strain ($P < 0.0001$). Compared to the wild type, absence of *ompA*, *ibeA* or *neuDB* proteins significantly reduced the ability of *E. coli* to invade HIBCPP cells. This was most pronounced in the *ompA* deletion mutant, in which invasion was reduced by 62% compared to the wild type ($P < 0.0001$). *IbeA* and *neuDB* deficiency led to a reduction of 50% ($P = 0.0010$) and 53% ($P < 0.0001$), respectively. Also, invasion rates of the RS218Δ*cnf1* strain were statistically significantly different compared to the wild type ($P = 0.0441$). Interestingly, the deletion of *fimH* facilitated invasion into HIBCPP cells, where a 1.88-fold number of bacteria could be found to have invaded the cell in comparison to the wild type ($P = 0.0012$). Analysis of the minimal invasion from the apical side revealed no significant differences between the investigated strains.

Figure 2. Immunofluorescence and electron microscopy of *E. coli* RS218-infected HIBCPP cells. Shown is invasion of HIBCPP cells by *E. coli* RS218 in inverted cell cultures. Assays were carried out after a 1-h incubation period with *E. coli* with an initial MOI of 10. (a, b) Apotome-generated images of HIBCPP monolayers with staining of cell nuclei (blue), actin filaments (purple) and double-immunofluorescent staining of *E. coli* RS218 bacteria (intracellular, green; extracellular, yellow). Panels show an en face view (xy axis) with cross-sections through multiple optical layers (z axis) above the image and to its right. (a) Shown are cells containing intracellular bacteria (green arrows), whereas some bacteria adhered to the cell surface, presumably after cell transversal (red arrows). (b) Intracellular *E. coli* is labelled with green arrows. A red arrow indicates extracellular bacteria at the basolateral cell side, not yet having entered the cell close to bacteria located intracellularly after successful cell invasion. (c–f) Ultrathin section transmission electron microscopic images show *E. coli* adhered to HIBCPP cells at the apical side to microvilli (c, d), and *E. coli* within membrane-bound vacuoles (labelled with arrowheads) inside HIBCPP cells (e, f). Multiple images showed vesicles containing more than one bacterium (e).

For the duration of the experiments, which were performed for evaluation of adhesion and invasion, TEER values remained stable and live/dead assays carried out in parallel assured that cell integrity was not compromised during the time frame studied (Fig. 3c and d; Supplementary Fig. S1).

DISCUSSION

Previous evidence points to the CP as entry point for *E. coli* into the CNS; however, there has been little research carried out concerning this pathway, and so far no *in vitro* model has been established for the investigation of *E. coli* translocation across the BCSFB. Still, a better understanding of the underlying mechanisms is necessary to improve treatment options (Hsieh et al. 2016; Kim 2016). The HIBCPP cell culture has been successfully established as a model for research concerning bacterial invasion at the CP for several bacterial pathogens, including *Neisseria*

meningitidis, *Streptococcus suis* and *Listeria monocytogenes* (Schwerk et al. 2012; Grundler et al. 2013; Borkowski et al. 2014; Dinner et al. 2016). In this study, we show that it is likewise suitable as a model for research regarding *E. coli*.

Our findings show polar toxicity and invasion of pathogenic *E. coli* into HIBCPP cells. Both invasion and cytotoxicity were more pronounced when *E. coli* were primarily applied basolaterally. It is noteworthy that this effect could be demonstrated despite the apically about 10-fold amount of exposed surface area, since the filter only allows limited accessibility to the basolateral cell side, where the pores are the only possible point of contact. This corresponds to previous findings concerning polar invasion of other bacteria such as *N. meningitidis*, *S. suis* and *L. monocytogenes* (Schwerk et al. 2012; Grundler et al. 2013; Borkowski et al. 2014; Dinner et al. 2016, 2017). It calls, however, the previous assumption into question that the occasionally observed choroid plexitis is caused secondarily after CSF infection via the BBB. Our

Figure 3. *Escherichia coli* adhesion to and invasion of HIBCPP cells from the apical (standard culture) and the basolateral side (inverted culture). Apical adhesion of *E. coli* to (a) and apical and basolateral invasion of (b) HIBCPP cell cultures in standard and inverted cell cultures, respectively. Assays were carried out after a 1-h incubation period with *E. coli* with an initial MOI of 10. Values are given as % of bacteria present after 1 h (derived from parallel growth curves). All assays were performed in triplicates and repeated at least five times ($n = 15$). *, significant, $P < 0.05$; **, highly significant, $P < 0.01$; ***, extremely significant, $P < 0.001$. TEER values remain stable when the HIBCPP cells were challenged in the standard culture from the apical side (c) or in the inverted culture from the basolateral side (d) with the indicated *E. coli* strains with an MOI of 10 for 1 h.

findings suggest that another likely route of CP infection would be from the basolateral cell side facing the bloodstream, as the basolateral side is more vulnerable to *E. coli* invasion. This coincides with other studies that suggest the BCSFB as another important possible bacterial point of entry (Parkkinen et al. 1988; Zelmer et al. 2008; Dalgakiran et al. 2014).

Our data indicate that several of the described virulence factors of *E. coli* are likely involved in BCSFB transversal. Basolateral cell invasion was decreased for *ompA*, *ibeA* and *neuDB*-deficient strains by 62%, 50% and 53%, respectively, compared to the RS218 wild type. This observation is in accord with what has been recognised in an array of previous experiments that have shown these factors to be involved in cell invasion and the ability to cause meningitis (Huang et al. 2001a; Kim et al. 2003; Mokri-Moayyed, Goldsworthy and Khan 2008; Confer and Ayalew 2013). We observed a decrease of the phase-ON status of type 1 fimbrial expression in the *ompA* deletion mutant, which has been described before (Teng et al. 2006). This could additionally affect adhesion and invasion of the *ompA*-deficient strain, although adhesion to the apical side of HIBCPP cells is not changed compared to the wild-type strain, and invasion from the basolateral side is strongly inhibited, whereas deletion of *fimH* enhances invasion. Noteworthy, a contribution of *ompA* during adhesion to HBMEC has been proposed (Teng et al. 2006). Also, for *ompA* the role in invasion has been demonstrated for various cell lines, including astrocytes and macrophages (Wu et al. 2009; Confer and

Ayalew 2013), whereas *ibeA* has been mainly investigated with regard to the brain endothelium (Flechard et al. 2012). The K1 capsule phenotype is known to be a prerequisite for meningitis to occur (Kim et al. 1992, 2003). Though *E. coli* with other capsular types can traverse brain microvascular endothelial cells *in vitro* and enter the CNS *in vivo*, only infections caused by K1 strains result in positive CSF cultures. Whether the decrease in intracellular bacteria is ascribable to intracellular cell death and degradation or is caused by lessened invasion will need to be specified in further studies.

The deletion of *fimH* had an unanticipated effect on bacterial invasion. FimH is well-characterised for its adhesion properties and has been shown to mediate invasion into epithelial cells (Martinez et al. 2000; Teng et al. 2006; Feenstra et al. 2017; Poole et al. 2017). In our experiments we found apical adhesion to be drastically reduced in the *fimH*-deficient strain, but basolateral invasion was consistently increased compared to the wild-type strain. It has to be considered that in the adhesion assays bacteria were exposed to the apical cell side; invasion, however, only occurred from the basolateral cell side. We were not able to evaluate adhesion to the basolateral cell side due to the limited accessibility caused by the filter. As HIBCPP cells display a clear polarity, a polar distribution of surface targets for FimH could explain the observation of decreased apical adhesion and increased basolateral invasion. Identification of receptors involved in FimH-mediated adhesion as well as a possible

polarity of expression in HIBCPP cells could act as the next step in characterising invasion across the BCSFB.

By measuring TEER values and employing live/dead assays, we could not discern a difference in cytotoxicity between the wild-type RS218 strain and the derived mutant strains, which concurs with previous findings that have described cytotoxicity to be independent of known virulence factors (Khan, Iqbal and Siddiqui 2012).

In summary, to the best of our knowledge, this is the first study using a functional human BCSFB model to investigate the role of the CP in the pathogenesis of *E. coli* meningitis. Our data show polar bacterial invasion into HIBCPP cells, occurring exclusively from the basolateral cell side. Furthermore, cytotoxicity and loss of barrier function is also more pronounced when exposure occurs from the basolateral cell side. Finally, we demonstrate the role of several known virulence factors. We show that *ompA*, *ibeA* and *neuDB* facilitate infection, whereas *fimH*, though during apical infection we confirm it to be a key factor for cell adhesion, its absence led to an increased number of basolaterally invaded bacteria. These findings elucidate the previously underestimated relevance of the BCSFB in the pathogenetic mechanism of *E. coli* meningitis and serve as attest for the necessity of further research concerning the mechanisms and entry points of *E. coli* into the CNS.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](#) online.

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Conflicts of interests. None declared.

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