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

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 (*E. coli*) is the most common Gram-negative causative agent of neonatal meningitis and *E. coli* meningitis is associated with a high morbidity and mortality. Previous research has been carried out with regards 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* invade 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 result 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.

INTRODUCTION

Escherichia coli (*E. 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 extra-intestinal (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 colonization 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 (Dalgakiran *et al.* 2014; Parkkinen *et al.* 1988; Witcomb *et al.* 2015; Zelmer *et al.* 2008).

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 (Kim 2008; Prasadarao *et al.* 1999). 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 (Grundler *et al.* 2013; Huang *et al.* 2001b; Kim *et al.* 2003; Kim 2016; Prasadarao *et al.* 1996; Teng *et al.* 2005; Wang and Kim 2013).

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; Kim 2016; Maruvada and Kim 2012; Wang and Kim 2013)

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-characterized 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 (Feenstra *et al.* 2017; Teng *et al.* 2005; Teng *et al.* 2006). 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 (Confer and Ayalew 2013; Prasadarao *et al.* 1996). 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.* 2003; Kim *et al.* 1992).

The aim of the following paper is to shed light on characteristics of *E. coli* pathogenesis with regards 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

E. 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, E44ΔneuDB, 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. *E. 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 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 (SFM) and diluted to the optical density at 600nm (OD_{600}) of 0.6 for HB101 and 0.3 for all other strains.

PCR-based restriction length di-morphism assay

The 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 an MOI of 10 for 1 h at 37°C. Following the bacterial incubation, 50 µl of the supernatant were 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). 150 ng of the extracted DNA were 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 1min and final extension 72°C for 8 min. After samples were cooled down, 10 µl of PCR product was challenged with FastDigest *Bst*UI (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 bp and 293 bp (phase ON populations of bacteria) and 539 bp 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 100U/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/cm^2, 0.33 cm^2 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 plate 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 Ω × cm^2 the medium was changed to 1% FCS containing culture medium without antibiotics. Cells that had reached a TEER of 250-800 Ω × 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 multiplicities of infection (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 1h 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 permeabilization 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 (DAPI) diluted 1:50000 (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 analogues 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 formular 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, 1024x1024, 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 nonparametric 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 less was 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 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).

E. coli transverse HIBCPP cell layer transcellularly within membrane-bound vacuoles

To investigate cell invasion and adhesion, bacteria were stained for visual analysis with Apoptome 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, 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, f) through the cell. Bacteria were frequently found as groups of two and more bacteria within one vacuole (Fig. 2e).

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 RS218ΔfimH compared to HB101, a non-pathogenic strain in which the *fimH* gene is not expressed ($p=0.2148$; Fisher's test).

E. 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 RS218Δcnf1 strain were statistically significant 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.

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, d and 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* (*N. meninigitidis*), *Streptococcus suis* (*S. suis*) and *Listeria monocytogenes* (*L. monocytogenes*) (Borkowski *et al.* 2014; Dinner *et al.* 2016; Grundler *et al.* 2013; Schwerk *et al.* 2012). 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* (Borkowski *et al.* 2014; Dinner *et al.* 2016; Dinner *et al.* 2017; Grundler *et al.* 2013; Schwerk *et al.* 2012). It calls, however, the previous assumption into question that the occasionally observed choroid plexitis is caused secondarily after CSF infection via the BBB. Our 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 (Dalgakiran *et al.* 2014; Parkkinen *et al.* 1988; Zelmer *et al.* 2008).

Our data indicates 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 (Confer and Ayalew 2013; Huang *et al.* 2001a; Kim et al. 2003; Mokri-Moayyed, Goldsworthy and Khan 2008). 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 (Confer and Ayalew 2013; Wu *et al.* 2009), whereas *ibeA* has been mainly investigated with regards 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.* 2003; Kim *et al.* 1992). 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 (Feenstra *et al.* 2017; Martinez *et al.* 2000; Poole *et al.* 2017; Teng *et al.* 2006). 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 e.g. 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 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 central nervous system.

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REFERENCES

Barichello T, Dagostim VS, Generoso JS *et al.* Neonatal *Escherichia coli* K1 meningitis causes learning and memory impairments in adulthood. *J Neuroimmunol* 2014;**272**:35-41.

Baud O, Aujard Y. Neonatal bacterial meningitis. *Handb Clin Neurol* 2013;**112**:1109-13.

Borkowski J, Li L, Steinmann U *et al. Neisseria meningitidis* elicits a pro-inflammatory response involving IkappaBzeta in a human blood-cerebrospinal fluid barrier model. *J Neuroinflammation* 2014;**11**:163.

Confer AW, Ayalew S. The OmpA family of proteins: roles in bacterial pathogenesis and immunity. *Vet Microbiol* 2013;**163**:207-22.

Dalgakiran F, Witcomb LA, McCarthy AJ *et al.* Non-invasive model of neuropathogenic *Escherichia coli* infection in the neonatal rat. *J Vis Exp* 2014, **92**:DOI 10.3791/52018: e52018.

Dinner S, Borkowski J, Stump-Guthier C *et al.* A Choroid Plexus Epithelial Cell-based Model of the Human Blood-Cerebrospinal Fluid Barrier to Study Bacterial Infection from the Basolateral Side. *J Vis Exp* 2016, **111**:DOI 10.3791/54061.

Dinner S, Kaltschmidt J, Stump-Guthier C *et al.* Mitogen-activated protein kinases are required for effective infection of human choroid plexus epithelial cells by *Listeria monocytogenes*. *Microbes Infect* 2017;**19**:18-33.

Eisenstein BI. Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. *Science* 1981;**214**:337-9.

Engelhardt B, Sorokin L. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol* 2009;**31**:497-511.

Fabbri A, Falzano L, Travaglione S *et al.* Rho-activating *Escherichia coli* cytotoxic necrotizing factor 1: macropinocytosis of apoptotic bodies in human epithelial cells. *Int J Med Microbiol* 2002;**291**:551-4.

Feenstra T, Thogersen MS, Wieser E *et al.* Adhesion of *Escherichia coli* under flow conditions reveals potential novel effects of FimH mutations. *Eur J Clin Microbiol Infect Dis* 2017;**36**:467-78.

Flechard M, Cortes MA, Reperant M *et al.* New role for the *ibeA* gene in H2O2 stress resistance of *Escherichia coli*. *J Bacteriol* 2012;**194**:4550-60.

Gaschignard J, Levy C, Romain O *et al.* Neonatal Bacterial Meningitis: 444 Cases in 7 Years. *Pediatr Infect Dis J* 2011;**30**:212-7.

Grundler T, Quednau N, Stump C *et al.* The surface proteins InlA and InlB are interdependently required for polar basolateral invasion by *Listeria monocytogenes* in a human model of the blood-cerebrospinal fluid barrier. *Microbes Infect* 2013;**15**:291-301.

Hsieh WS, Yang YY, Lin PH *et al.* Recombinant OmpA protein fragments mediate interleukin-17 regulation to prevent *Escherichia coli* meningitis. *J Microbiol Immunol Infect* 2016;**49**:843-50.

Huang SH, Chen YH, Kong G *et al.* A novel genetic island of meningitic *Escherichia coli* K1 containing the *ibeA* invasion gene (GimA): functional annotation and carbon-source-regulated invasion of human brain microvascular endothelial cells. *Funct Integr Genomics* 2001a;**1**:312-22.

Huang SH, Wan ZS, Chen YH *et al.* Further characterization of *Escherichia coli* brain microvascular endothelial cell invasion gene *ibeA* by deletion, complementation, and protein expression. *J Infect Dis* 2001b;**183**:1071-8.

Huang SH, Wass C, Fu Q *et al. Escherichia coli* invasion of brain microvascular endothelial cells *in vitro* and *in vivo*: molecular cloning and characterization of invasion gene *ibe10*. *Infect Immun* 1995;**63**:4470-5.

Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2004;**2**:123-40.

Khan NA, Iqbal J, Siddiqui R. *Escherichia coli* K1-induced cytopathogenicity of human brain microvascular endothelial cells. *Microb Pathog* 2012;**53**:269-75.

Khan NA, Wang Y, Kim KJ *et al.* Cytotoxic necrotizing factor-1 contributes to *Escherichia coli* K1 invasion of the central nervous system. *J Biol Chem* 2002;**277**:15607-12.

Kim KJ, Elliott SJ, Di Cello F *et al.* The K1 capsule modulates trafficking of *E. coli*-containing vacuoles and enhances intracellular bacterial survival in human brain microvascular endothelial cells. *Cell Microbiol* 2003;**5**:245-52.

Kim KS. Mechanisms of microbial traversal of the blood-brain barrier. *Nat Rev Microbiol* 2008;**6**:625-34.

Kim KS. Human Meningitis-Associated *Escherichia coli*. *EcoSal Plus* 2016;**7**:DOI 10.1128/ecosalplus.ESP-0015-2015.

Kim KS, Itabashi H, Gemski P *et al.* The K1 capsule is the critical determinant in the development of *Escherichia coli* meningitis in the rat. *J Clin Invest* 1992;**90**:897-905.

Kline KA, Falker S, Dahlberg S *et al.* Bacterial adhesins in host-microbe interactions. *Cell Host Microbe* 2009;**5**:580-92.

Krishnan S, Chang AC, Stoltz BM *et al. Escherichia coli* K1 Modulates Peroxisome Proliferator-Activated Receptor gamma and Glucose Transporter 1 at the Blood-Brain Barrier in Neonatal Meningitis. *J Infect Dis* 2016;**214**:1092-104.

Martinez JJ, Mulvey MA, Schilling JD *et al.* Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *EMBO J* 2000;**19**:2803-12.

Maruvada R, Kim KS. IbeA and OmpA of *Escherichia coli* K1 exploit Rac1 activation for invasion of human brain microvascular endothelial cells. *Infect Immun* 2012;**80**:2035-41.

Mokri-Moayyed B, Goldsworthy GJ, Khan NA. Development of a novel *ex vivo* insect model for studying virulence determinants of *Escherichia coli* K1. *J Med Microbiol* 2008;**57**:106-10.

Parkkinen J, Korhonen TK, Pere A *et al.* Binding sites in the rat brain for *Escherichia coli* S fimbriae associated with neonatal meningitis. *J Clin Invest* 1988;**81**:860-5.

Poole NM, Green SI, Rajan A *et al.* Role for FimH in Extraintestinal Pathogenic *Escherichia coli* Invasion and Translocation through the Intestinal Epithelium. *Infect Immun* 2017;**85**: DOI 10.1128/IAI.00581-17pii: e00581-17.

Prasadarao NV, Wass CA, Stins MF *et al.* Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun* 1999;**67**:5775-83.

Prasadarao NV, Wass CA, Weiser JN *et al.* Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* 1996;**64**:146-53.

Schwerk C, Papandreou T, Schuhmann D *et al.* Polar Invasion and Translocation of *Neisseria meningitidis* and *Streptococcus suis* in a Novel Human Model of the Blood-Cerebrospinal Fluid Barrier. *PLoS One* 2012;**7**:e30069.

Smith SG, Dorman CJ. Functional analysis of the FimE integrase of *Escherichia coli* K-12: isolation of mutant derivatives with altered DNA inversion preferences. *Mol Microbiol* 1999;**34**:965-79.

Teng CH, Cai M, Shin S *et al. Escherichia coli* K1 RS218 interacts with human brain microvascular endothelial cells via type 1 fimbria bacteria in the fimbriated state. *Infect Immun* 2005;**73**:2923-31.

Teng CH, Xie Y, Shin S *et al.* Effects of *ompA* deletion on expression of type 1 fimbriae in *Escherichia coli* K1 strain RS218 and on the association of *E. coli* with human brain microvascular endothelial cells. *Infect Immun* 2006;**74**:5609-16.

Wang MH, Kim KS. Cytotoxic necrotizing factor 1 contributes to *Escherichia coli* meningitis. *Toxins (Basel)* 2013;**5**:2270-80.

Wijetunge DS, Katani R, Kapur V *et al.* Complete Genome Sequence of *Escherichia coli* Strain RS218 (O18:H7:K1), Associated with Neonatal Meningitis. *Genome Announc* 2015;**3**:e00804-15.

Witcomb LA, Collins JW, McCarthy AJ *et al.* Bioluminescent imaging reveals novel patterns of colonization and invasion in systemic *Escherichia coli* K1 experimental infection in the neonatal rat. *Infect Immun* 2015;**83**:4528-40.

Wood MW, Breitschwerdt EB, Nordone SK *et al.* Uropathogenic *E. coli* promote a paracellular urothelial barrier defect characterized by altered tight junction integrity, epithelial cell sloughing and cytokine release. *J Comp Pathol* 2012;**147**:11-9.

Wu HH, Yang YY, Hsieh WS *et al.* OmpA is the critical component for *Escherichia coli* invasion-induced astrocyte activation. *J Neuropathol Exp Neurol* 2009;**68**:677-90.

Zelmer A, Bowen M, Jokilammi A *et al.* Differential expression of the polysialyl capsule during blood-to-brain transit of neuropathogenic *Escherichia coli* K1. *Microbiology* 2008;**154**:2522-32.

Zhao WD, Liu DX, Wei JY *et al.* Caspr1 is a host receptor for meningitis-causing *Escherichia coli*. *Nat Commun* 2018;**9**:2296.