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ORIGINAL INVESTIGATION

Activation of brain endothelium by *Escherichia coli* K1 virulence factor cglD promotes polymorphonuclear leukocyte transendothelial migration

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Abstract
Escherichia coli K1 is the most common Gram-negative bacteria causing neonatal meningitis. Polymorphonuclear leukocyte (PMN) transmigration across the blood–brain barrier (BBB) is the hallmark of bacterial meningitis. Reportedly, the deletion of virulence factor cglD (E44:Δ*cglD*) from E44 is responsible for a less efficient PMN transendothelial migration ability. In the present study, we found that complementation of the *cglD* gene into E44:Δ*cglD* mutant strain might restore the PMN count and myeloperoxidase level in a neonatal mouse meningitis. Using human brain microvascular endothelial cells (HBMECs), the main model of the BBB in vitro, we found that E44:Δ*cglD* mutant strain induced a less efficient PMN adhesion to HBMECs and down-regulated chemokines CXCL1, CXCL6 and CXCL8 and adhesion molecule E-selectin, compared with the E44 strain. Complementation of *cglD* restored the PMN adhesion to HBMECs and the level of these proteins. E44:Δ*cglD* mutant strain also induced a less efficient NF-κB pathway activation in HBMECs and reduced the soluble p65 (sp65) level in the cerebral spinal fluid of newborn mice, compared with the E44 strain. Complementation of *cglD* restored the NF-κB pathway activation and increased the sp65 levels. This suggests that *cglD* in E44 contributes to NF-κB pathway activation in the brain endothelium to promote PMN adhesion to HBMECs and transendothelial migration. Our identified novel requirement of cglD for immune activation and subsequent PMN entry into the central nervous system suggests that therapies directed at neutralising this molecule will be beneficial in preventing bacterial meningitis progression.

Keywords CglD · *Escherichia coli* K1 · Polymorphonuclear leukocyte · Transendothelial migration · Blood–brain barrier

Introduction

Bacterial meningitis is a severe and sometimes fatal infection of the central nervous system (CNS) [1]. *Escherichia coli* K1 (*E. coli* K1) is the most common Gram-negative bacteria causing meningitis during the neonatal period [2]. The pathogenesis of bacterial meningitis is a multistage process requiring the entry of circulating *E. coli* into the CNS by crossing the blood–brain barrier (BBB) [3]. Several *E. coli* virulence factors contributing to bacterial crossing of the BBB have been identified and characterised, including *ibeA, ibeB, ibeC, aslA, fim, traJ* and *ompA* [4]. The subsequent processes underlying the pathogenesis of meningitis following bacterial entry into the cerebral spinal fluid (CSF) remain unknown. Some virulence factors are reportedly involved in the subsequent pathogenesis of *E. coli* meningitis but do not participate in the initial accumulation of bacteria in the CSF. Our previous study showed that the *cglD* gene, which has a 1083-bp open reading frame located in GimA (genetic island of meningitic *E. coli* containing *ibeA*), codes for the cglD protein, which shares high homology with glycerol dehydrogenase and presumably contributes to glycerol metabolism in *E. coli* [5, 6]. CglD is an *E. coli* K1 virulence factor associated with the induction of the inflammatory response and severe damage to the host, but cglD was found not to be related to penetration of the BBB [5].

Polymorphonuclear leukocyte (PMN) transendothelial migration across the BBB is a crucial feature of bacterial

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meningitis as part of the host defence against pathogens but may also cause significant damage to the CNS tissues, resulting in devastating neurologic sequelae [7]. Studies on PMN transmigration across the BBB have been limited to bacterial meningitis caused by *Meningococcus, Pneumococcus* and Group B *Streptococcus* [8–10]. In *E. coli* meningitis, few in vitro or in vivo studies to date have focused on the PMN–endothelial cell interactions in response to the virulence factors of meningitic *E. coli* K1. Our previous study showed that deletion of the *E. coli* K1 virulence factor *cglD* is responsible for a less efficient PMN migration ability [5]. In the present study, we showed that deletion of the *E. coli* K1 virulence factor *cglD* induces a less efficient PMN adhesion to human brain microvascular endothelial cells (HBMECs) by a less effectual activating of the NF-κB pathway and expression of target genes in the brain endothelium. CglD in *E. coli* K1 is associated with PMN–endothelial cell interactions and that eventually promotes PMN transendothelial migration in vitro and across the BBB in vivo in *E. coli* K1 meningitis.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli RS218 (O18: K1: H7) strain was isolated from the CSF of a newborn infant with meningitis, and E44 strain is a spontaneous rifampin-resistant mutant of RS218. The E44:Δ*cglD* mutant strain carries an isogenic in-frame deletion of *cglD* from the E44 strain [5]. Complementation bacteria strains used were the same as those used in a previous publication [5]. Briefly, a 1.3-kb DNA fragment containing the ORF of the *cglD* gene was cloned into a pFN476 vector and named pFN-CglD. The E44:Δ*cglD* strain was transformed by pFN-CglD and pGP1-2 for the expression of CglD (E44:Δ*cglD* + *cglD*). Meanwhile, the E44:Δ*cglD* mutant strain was transformed by empty vector pFN476 and pGP1-2 as control (E44:Δ*cglD* + vector). All bacteria were grown at 37 °C in LB broth (10-g tryptone, 5-g yeast extract and 10-g NaCl/L) supplemented with rifampin (10 μg/mL), and all experiments were conducted with a multiplicity of infection of 100.

Cell culture

HBMECs were a generous gift from Dr. KS Kim (Johns Hopkins University) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% foetal bovine serum (HyClone), 10% Nu-serum (BD Biosciences), 2-mM glutamine, 1-mM sodium pyruvate, 1 × nonessential amino acid and 1 × Gibco® MEM Vitamin Solution (Thermo Fisher Scientific). Cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2/95% air.

Cell adhesion assay

In total, 2×10^5 HBMECs were cultured on glass coverslips. Before the cell adhesion assay, the HBMECs were stained with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) fluorescent dye (Thermo Fisher Scientific), in accordance with the manufacturer's instructions, and then pre-treated with bacteria for 1 h. Human PMNs were isolated with the PMN Separation Kit (Genmed Scientifics Inc), according to the manufacturer's instructions, and then the erythrocytes were lysed. In total, 2×10^6 PMNs labelled with CellTracker™ Red CMPTX (5-chloromethylfluorescein diacetate) fluorescent dye (Thermo Fisher Scientific) were co-cultured with HBMECs for an additional 1 h and then gently washed with phosphate-buffered saline three times to remove the non-adhered PMNs. Fluorescent-labelled PMNs adhered to HBMEC monolayers were counted under a fluorescence microscope from ten random fields. The adhesion rate was calculated as the ratio of the adhered cells to the input cells. Results are presented as relative percent adhesion of the parent *E. coli* K1 strain E44 infected group, which is defined as 100%.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

HBMECs were infected with the bacteria at the indicated times. Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific) and reverse transcribed into cDNA using Moloney murine leukaemia virus reverse transcriptase (Promega Corporation). RT-PCR was performed on an ABI 7500 RT-PCR system (Applied Biosystems) with an SYBR premix Ex Taq Kit (Takara Biotechnology), according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as the internal control. The amplification conditions were as follows: 95 °C for 10 s, and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The comparative cycle threshold ($2^{-\Delta\Delta CT}$) method was used to calculate relative gene expression levels, with GAPDH as the internal control. RT-PCR products were separated by agarose gel electrophoresis and verified by DNA sequencing.

Chemokine expression

The concentrations of cytokines and chemokines in HBMEC supernatants collected at 1 and 2 h post-infection with bacteria were measured using enzyme-linked immunosorbent assay (ELISA) for CXCL1, CXCL6 and CXCL8 (SRB Technology), according to the manufacturer's instructions.

Western blotting

Confluent HBMECs were infected with bacteria at the indicated times, and cells were washed three times with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (50-mM Tris–HCl, 150-mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecylsulfate) or lysed in TritonX-100 buffer (25-mM HEPES, 150-mM NaCl, 4-mM EDTA, 1% TritonX-100) containing protease inhibitor cocktail (Roche). The samples were subjected to SDS-PAGE and then transferred to a PVDF membrane (Millipore). The PVDF membrane was blocked with 5% non-fat milk and probed with the indicated antibodies at 4 °C overnight. The primary antibodies against E-selectin (ab18981), P-selectin (ab6632), ICAM-1 (ab2213), VCAM-1 (ab98954) and PECAM-1 (ab24590) were obtained from Abcam. Then, the blots were incubated with an HRP-conjugated secondary antibody (Santa Cruz Biotech.) for 1 h at room temperature. Immunoreactive bands were visualised by SuperSignal West Pico Chemiluminescent Substrate (Pierce) using a LAS-3000 mini analyser (Fuji Film). For quantitative analysis, the mean density of each band was measured by Multi Gauge V3.1 software, and the band density of target proteins was divided by the band density of the internal reference protein to obtain the normalised band density.

Nuclear factor (NF)-κB localization by immunofluorescence

NF-κB activation and nuclear translocation were monitored using the NF-κB Activation and Nuclear Translocation Assay Kit (Beyotime Institute of Biotechnology), in accordance with the manufacturer's instructions. Briefly, HBMECs cultured on the glass coverslips were pre-treated with bacteria for 1 h, fixed with 4% paraformaldehyde for 5 min, permeabilized with 0.2% Triton X-100 for 5 min and then blocked with 5% bovine serum albumin for 1 h at room temperature. Next, the HBMECs were incubated with NF-κB p65 antibody overnight at 4 °C and then incubated with Cy3-labelled secondary antibody for 1 h at room temperature. Afterward, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (Thermo Fisher Scientific) was used to stain the cell nuclei. NF-κB activation and nuclear translocation were observed under a laser scanning confocal microscope from ten random fields.

Nuclear and cytoplasmic protein extraction

To determine the activation status of NF-κB in HBMECs induced by bacteria, HBMECs were invaded with bacteria for 1 h. Then, the nuclear and cytoplasmic proteins of the HBMECs were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. The phosphorylated ikb-α (Abcam) and ikk α/β (Cell Signalling Technology) cytoplasmic proteins were identified by western blot analysis, with β-tubulin (ProteinTech Group) as the internal control. The nuclear extracts were identified by the expression of p65 (Abcam), with the histone H3 (ProteinTech Group) as an internal control.

Mouse model of *E. coli* meningitis

All animal experiments were performed according to the Guidelines for Animal Care in China Medical University (CMU) and were approved by the CMU Animal Care and Use Committee. *E. coli* meningitis was induced in 5-day-old Kunming mice of both sexes (CMU). Neonatal mice were randomly divided into four groups, and each pup was intraperitoneally injected with 1×10^5 colony-forming units (CFU) of bacteria. At 24 h after *E. coli* inoculation, the mice were anaesthetised and CSF samples were obtained as described previously [5]. The PMN count was determined using a hemocytometer. The CSF samples were subjected to ELISA to quantify the level of myeloperoxidase (MPO), chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 (SRB Technology) and soluble NF-κB (sp65) (Boster Biological Technology), according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± standard deviation (SD), and comparisons between experimental groups were analysed using SPSS statistical software. One-way ANOVA and post-hoc Holm–Sidak method were used for statistical analyses of data. Student's *t* test was used for direct comparisons between two groups. A probability (p) value of < 0.05 was considered significant.

Results

Complementation of *cglD* contributed to the promotion of PMN across the BBB in a mouse model of neonatal meningitis

Our previous study showed that deletion of the virulence factor *cglD* from *E. coli* K1 induces a less efficient PMN transendothelial migration ability [5]. To determine whether *cglD* is associated with PMN migration across the BBB in vivo, a *cglD* complementation strain (E44:Δ*cglD* + *cglD*) was used to infect neonatal mice [5]. PMN counts in the CSF of the E44:Δ*cglD* infection group were significantly lower than those in the CSF of the E44 infection group, suggesting that the complementation of *cglD* had restored

the number of PMNs in the CSF (Fig. 1a). MPO, which serves as an effective indicator of leukocyte infiltration, was detected in the CSF of newborn mice with suspected meningitis [3]. The results showed that the levels of MPO were lower after infection with the E44:Δ*cglD* mutant strain than after infection with the E44 strain, which could be restored in the E44:Δ*cglD* + *cglD* infection group (Fig. 1b). These results indicated that *cglD* in *E. coli* K1 contributes to PMN across the BBB in vivo.

CglD deletion from *E. coli* K1 induced a less efficient PMN adhesion to HBMECs

Endothelial cells lining the cerebral blood vessels are responsible for the formation and maintenance of the BBB, which normally restricts the movement of white blood cells into the CNS [11, 12]. PMNs undergo a strong adhesion reaction to HBMECs before transendothelial migration. We first examined the PMN adhesion to HBMECs stimulated by the E44 strain or the E44:Δ*cglD* mutant strain. The results showed that the PMN adhesion to HBMECs was significantly decreased when infected by the E44:Δ*cglD* mutant strain, compared with the E44 strain (Fig. 2a, b). We next used the E44:Δ*cglD* + *cglD* strain to identify the role of *cglD* in PMN adhesion to HBMECs. The results showed that complementation of *cglD* into the E44:Δ*cglD* mutant strain may restore the PMN adhesion to HBMECs (Fig. 2a, b). These results suggest that *cglD* deletion from *E. coli* K1 induced a less efficient PMN adhesion to HBMECs, which may be the major factor inducing impairment of PMN transendothelial migration.

The E44:Δ*cglD* mutant strain induced a less effectual expression of leukocyte chemotaxis in vitro and in vivo than the E44 strain

The process of transendothelial migration involves PMN attraction, activation and adhesion [13]. Leukocyte attraction to the site of infection is regulated by chemotactic substances, such as complement factor C5a, platelet-activating factor and various chemokines [11, 14]. Chemokines, which can be subdivided into several families according to structural features related to the relative positions of their cysteine residues, are produced at sites of inflammation and are then presented on the luminal side of endothelial cells [14]. The main chemokines that stimulate leukocyte chemotaxis include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8. Therefore, we examined whether the virulence factor *cglD* gene was involved in the expression of these chemokines in HBMECs. RT-PCR was used to detect the mRNA expression levels of the CXC family members in HBMECs infected with bacteria. The results showed that the mRNA expression levels of CXCL1, CXCL6 and CXCL8 were significantly increased in HBMECs infected with bacteria compared with the uninfected control cells. However, expression levels were significantly lower in HBMECs infected with the E44:Δ*cglD* mutant strain than in those infected with E44 strain (Fig. 3a, e, g), whereas CXCL2, CXCL3, CXCL5 and CXCL7 induced no obvious change (Fig. 3b–d, f). In addition, the secretion of CXCL1, CXCL6 and CXCL8 by HBMECs challenged with the E44:Δ*cglD* mutant strain was markedly reduced compared with that of cells infected with the E44 strain (Fig. 3h–j). Furthermore, the secretion of CXCL1, CXCL6 and CXCL8 was restored

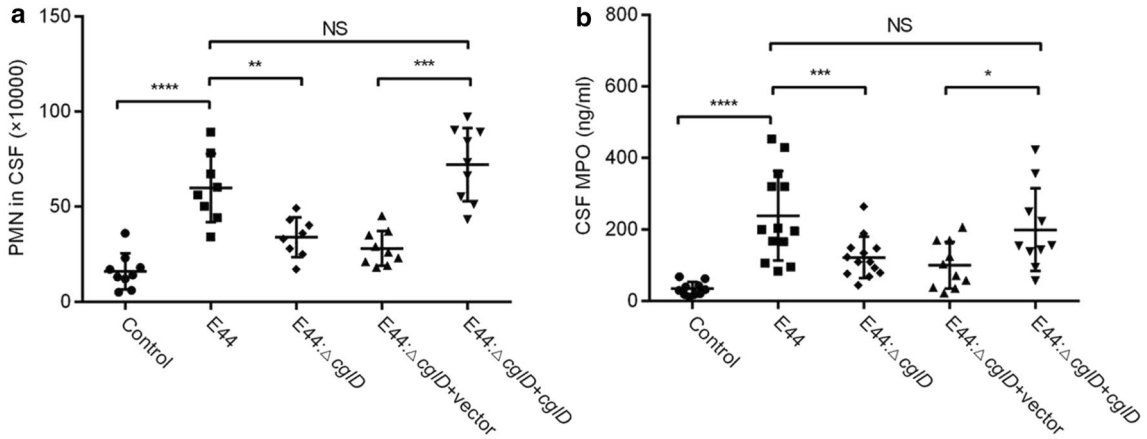

Fig. 1 Complementation of *cglD* contributes to the promotion of PMN across the BBB in a mouse model of neonatal meningitis. 5-day-old mice were inoculated with 1×10^5 CFU of bacteria and killed at 24 h post-injection. CSF samples were collected 24 h post-inoculation. The PMNs in the CSF were counted with a hemocytometer under a light microscope; E44 and E44:Δ*cglD* group: $n = 8$; control, E44:Δ*cglD* + vector; E44:Δ*cglD* + *cglD* group: $n = 9$ (**a**). MPO in the CSF were determined using ELISA; E44 and E44:Δ*cglD* group: $n = 13$; control, E44:Δ*cglD* + vector; E44:Δ*cglD* + *cglD* group: $n = 10$ (**b**). Data are presented as the mean ± SD. The results shown are representative of three independent experiments. Statistical analyses were performed with one-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

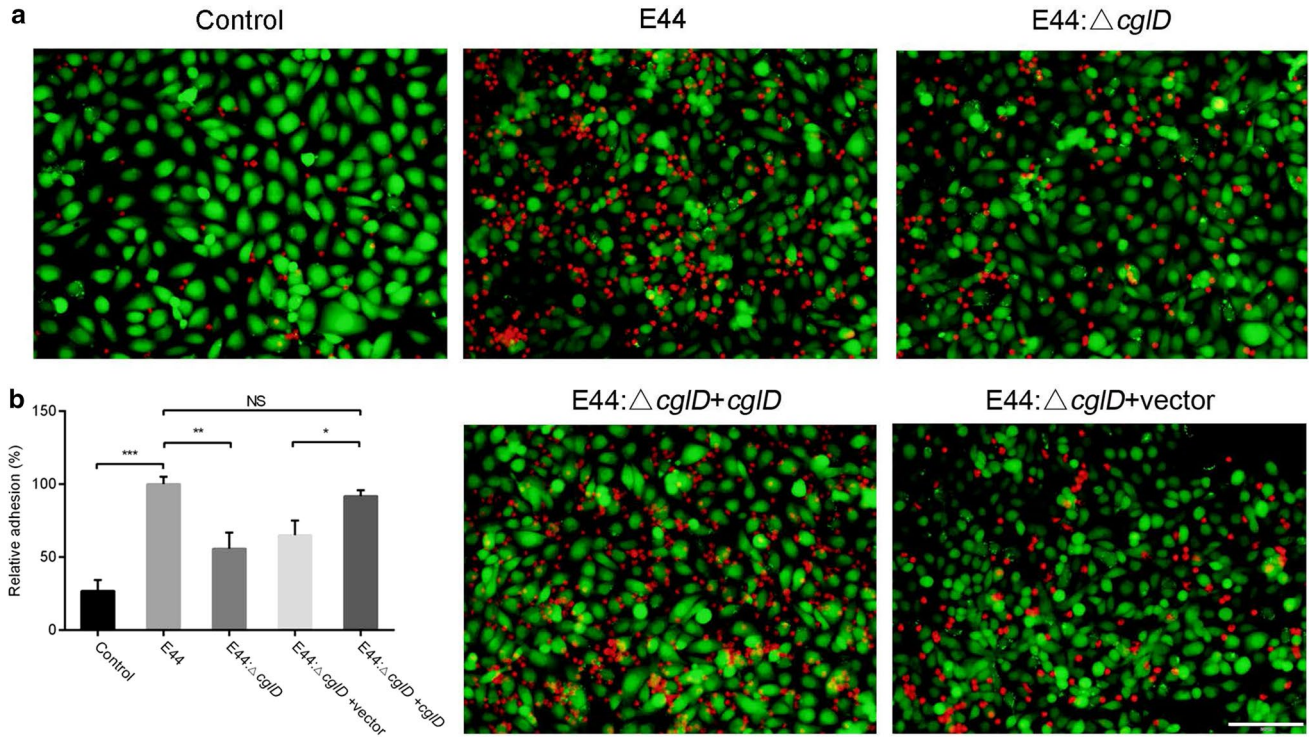

Fig. 2 Deletion of *cglD* from *E. coli* K1 induces a less efficient PMN adhesion to HBMECs. HBMECs on glass coverslips were stained with CellTracker™ Green CMFDA dye before pre-treatment with bacteria as indicated for 1 h. PMNs labelled with CellTracker™ Red CMPTX dyes were co-cultured with HBMECs for an additional 1 h. Cells were observed under a fluorescent microscope and counted from ten random fields (**a, b**). The adhesion rate was calculated as the ratio of the adhesion cells to the input cells. Results are presented as relative percent adhesion of the parent *E. coli* K1 strain E44 infected group, which is defined as 100%. Data are presented as the mean ± SD. The results shown are representative of three independent experiments. Statistical analyses were performed with one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Scale 200 μm

in HBMECs infected with the E44:Δ*cglD*+*cglD* strain at 1 h compared with those infected with the E44:Δ*cglD*+vector strain (Fig. 3k). In addition, we measured the expression levels of CXCL1, CXCL6 and CXCL8 in a mouse model of neonatal meningitis and found obvious less efficient elevation in the expression levels of the investigated chemokines in the CSF of the E44:Δ*cglD* mutant strain infection group, and that complementation of *cglD* might restore chemokine secretion following infection with the E44:Δ*cglD*+*cglD* strain (Fig. 3l–n). We also examined the levels of CXCL2, CXCL3, CXCL5 and CXCL7 in the CSF of the infected mice and found no obvious differences between the E44 strain, E44:Δ*cglD* mutant strain (Fig. 3o–r). These results suggest that CXCL1, CXCL6 and CXCL8 were the major chemokines responsible for attracting PMN to HBMECs induced by the *cglD* gene in *E. coli* K1.

The E44:Δ*cglD* mutant strain had an inadequate capability to induce expression of adhesion molecules in HBMECs compared with the E44 strain

Leukocytes attracted to the endothelium are first observed to roll along the endothelium of postcapillary venules adjacent to the extravascular site of inflammation [12, 13]. Subsequently, some of the rolling leukocytes adhere firmly. Selectin-mediated rolling of leukocytes is essential for the transendothelial migration of leukocytes, and the immunoglobulin-like domains of adhesion proteins are involved in firm adhesion [12, 13]. We detected the expression of cell adhesion molecules in HBMECs following bacterial challenge. The RT-PCR and western blot results showed that the mRNA and protein expression levels of E-selectin were down-regulated in HBMECs infected with the E44:Δ*cglD* mutant strain (Fig. 4a, f, g) compared with those infected with the E44 strain. Complementation of *cglD* into the E44:Δ*cglD* mutant strain was found to restore the expression of E-selectin (Fig. 4l). However, the expression levels of other cell adhesion molecules, such as P-selectin, VCAM-1, ICAM-1 and PECAM-1, were not influenced by deletion of the *cglD* gene (Fig. 4b–f, h–k). These results suggest that the less efficient expression of E-selectin is a major reason for the impaired ability of PMNs to adhere to HBMECs infected with the E44:Δ*cglD* mutant strain.

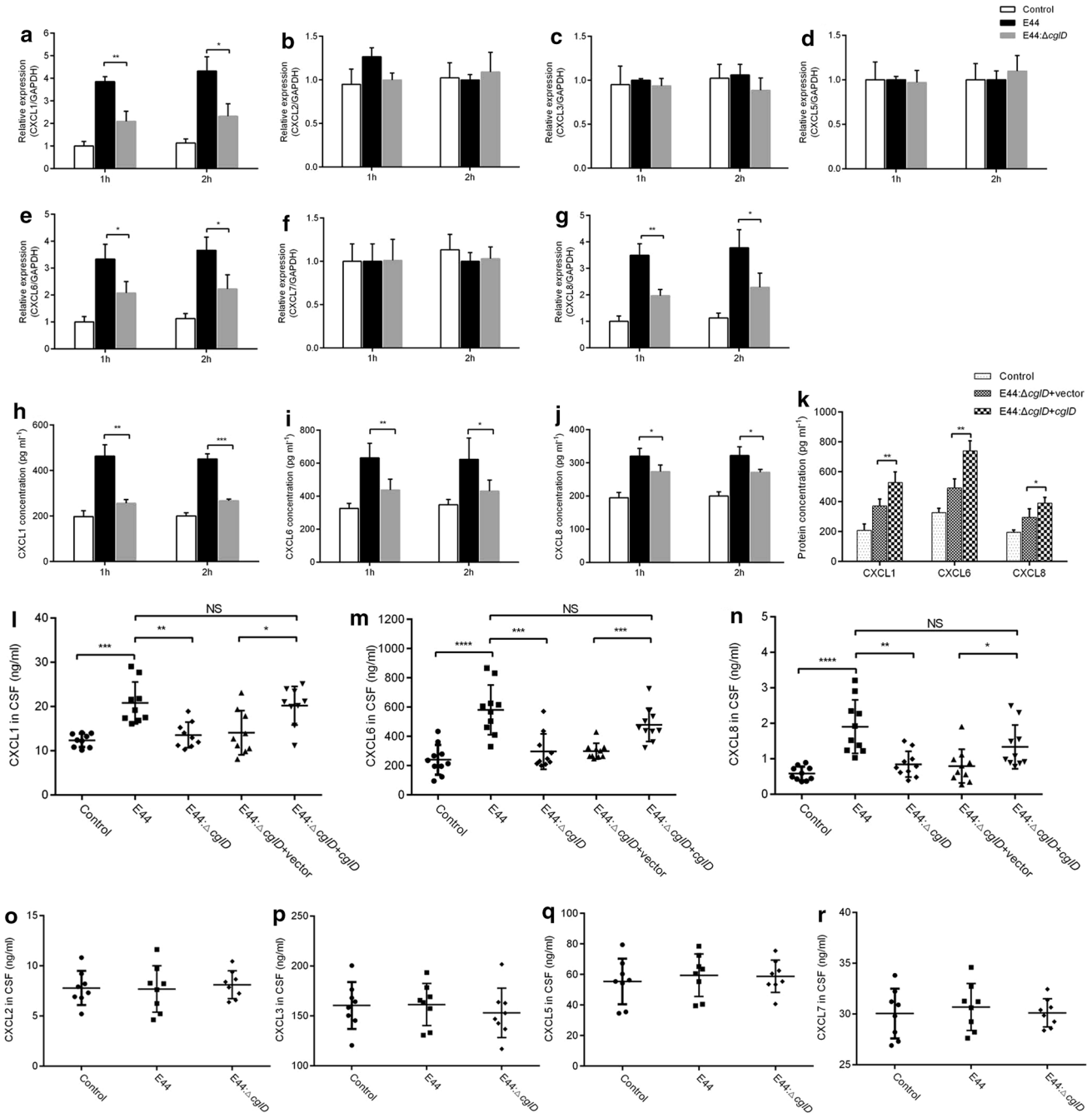

Fig. 3 Analysis of the expression of chemokines following bacterial infection. HBMECs were infected with bacteria for 1 or 2 h. For complementation analysis, HBMECs were infected with the E44:Δ*cglD* + vector strain or the E44:Δ*cglD* + *cglD* strain for 1 h. RT-PCR was used to quantify the mRNA expression of chemokines (**a**–**g**). ELISA was used to detect the secreted protein levels of chemokines in the culture supernatant of HBMECs infected with bacteria (**h**–**k**). Five-day-old mice were inoculated with 1×10^5 CFU of bacteria and then killed 24 h post-injection. CSF was obtained and the levels of chemokines were quantified using ELISA (**l**–**r**). CXCL1 ($n = 9$, each group); CXCL6 and CXCL8 ($n = 10$, each group); CXCL2, CXCL3, CXCL5 and CXCL7 ($n = 8$, each group). Data are presented as the mean ± SD. The results shown are representative of three independent experiments. The data in **a**–**j** were analysed with the *t* test between the E44 strain and the E44:Δ*cglD* mutant strain group; the data in **k** were analysed with the *t* test between the E44:Δ*cglD* + vector strain and the E44:Δ*cglD* + *cglD* strain group; the data in **l**–**r** were analysed with one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

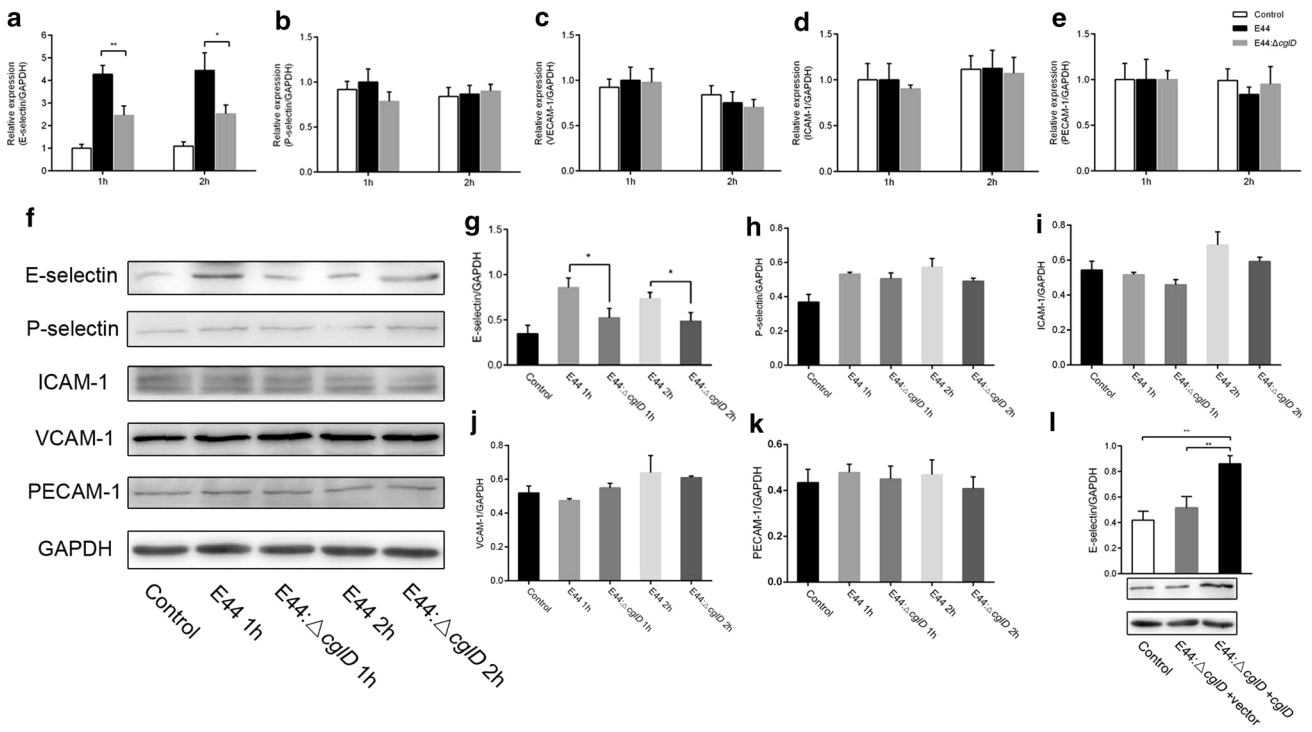

Fig. 4 Analysis of the expression of adhesion molecules by infected HBMECs. HBMECs were infected with the E44 strain or the E44:Δ*cglD* mutant strain for 1 or 2 h. For complementation analysis, HBMECs were infected with the E44:Δ*cglD* + vector strain or the E44:Δ*cglD* + *cglD* strain for 1 h. RT-PCR was used to detect the mRNA expression of adhesion molecules (**a**–**e**). Western blot analysis was performed to detect the protein expression of adhesion molecules (**f**–**l**). Data are presented as the mean ± SD. The results shown were representative of three independent experiments. All data were analysed using the *t* test. $*p < 0.05$, $**p < 0.01$

Deletion of the *cglD* gene from *E. coli* K1 inhibited activation of the NF-κB pathway

The results of a previous study showed that activation of the NF-κB pathway was essential for PMN transmigration across the BBB during the onset of bacterial meningitis [7]. The *cglD*-related genes CXCL1, CXCL6, CXCL8 and E-selectin are target genes that are regulated via the NF-κB pathway. The NF-κB pathway is controlled by the proteolysis of the phosphorylated Ikb α protein [15–18]. Therefore, we assumed that the *cglD* gene of *E. coli* K1 is involved in the inhibition of the activation of the NF-κB pathway. Hence, we attempted to identify factors involved in the activation of the NF-κB pathway in HBMECs infected with the E44 strain or the E44:Δ*cglD* mutant strain. Laser scanning confocal microscopy was used to examine the localization of NF-κB in HBMECs challenged with bacteria. As shown in Fig. 5a, b, NF-κB translocation (p65) was very low in HBMECs at 1 h after infection with the E44:Δ*cglD* mutant strain, whereas NF-κB (p65) translocation to the nucleus was strongly up-regulated in cells infected with the E44 strain (Fig. 5a, b). Complementation of *cglD* into the E44:Δ*cglD* mutant strain might rescue p65 translocation in the cell nucleus (Fig. 5a, b). NF-κB activity was lower in HBMECs infected with the E44:Δ*cglD* mutant strain than in those infected with the E44 strain, as demonstrated by the phosphorylation of ikk α/β (p-ikk α/β) and ikb α (p-ikb α) in the cytoplasm, whereas the nuclear expression of p65 was less efficient in HBMECs (Fig. 5c–f). Complementation of *cglD* into the E44:Δ*cglD* mutant strain may restore the cytoplasmic expression of p-ikk α/β and p-ikb α and the nuclear expression of p65 in HBMECs, compared with the E44:Δ*cglD* + vector control (Fig. 5c–f). The results of a previous study showed that the level of p65 was increased in wild-type mice infected with the E44 strain [19] and that p65 was released in the CSF. Next, we investigated whether *cglD* of *E. coli* K1 contributes to the activation of the NF-κB pathway in vivo. The level of soluble p65 (sp65) in the CSF of newborn mice infected with the E44:Δ*cglD* mutant strain have a significant decreasing compared with the E44 strain infection group (Fig. 5g). Complementation of *cglD* into the E44:Δ*cglD* mutant strain may restore the level of sp65 (Fig. 5g) in the CSF of newborn mice infected with bacteria. These results show that the deletion of *cglD* from *E. coli* K1 inhibits activation of the NF-κB pathway both in vitro and in vivo, which might explain the less efficient expression of these identified target genes.

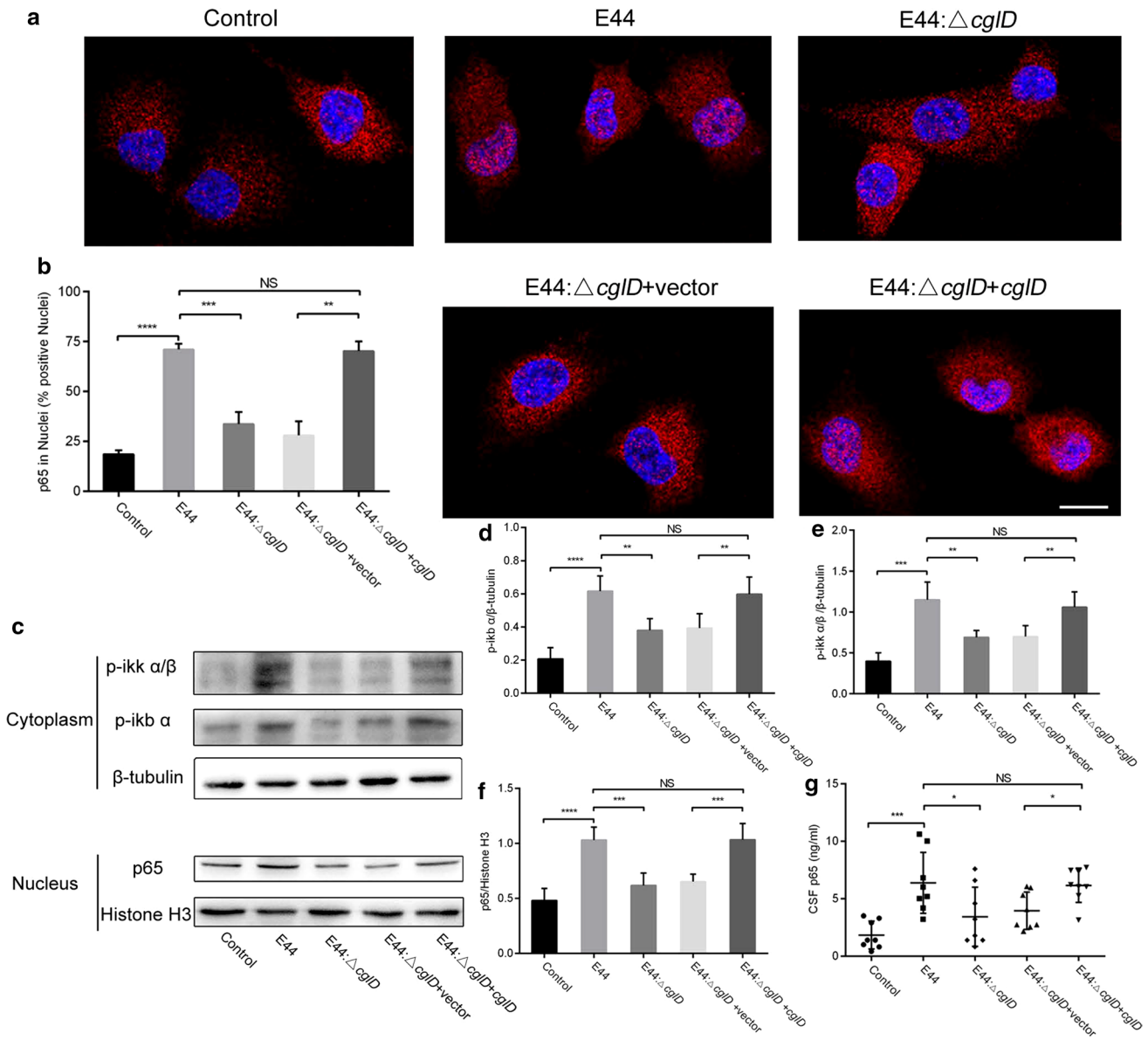

Fig. 5 Deletion of the *cglD* gene from *E. coli* K1 inhibits activation of the NF-κB pathway. HBMECs were infected with bacteria for 1 h. The p65 of HBMECs was stained with a Cy3-labelled secondary antibody (red), and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) (**a, b**). Nuclear and cytoplasmic protein extraction was performed as described in the "Materials and methods" section. Western blot analysis was used to detect the expression of the NF-κB pathway proteins (**c**–**f**). 5-day-old mice were inoculated with 1×10^5 CFU of bacteria, and CSF samples were collected at 24 h after inoculation. The level of soluble p65 was determined using ELISA ($n = 8$, each group) (**g**). The results shown are representative of three independent experiments. All data were analysed using one-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Scale 10 μm

Discussion

PMN transmigration across the BBB is the hallmark of bacterial meningitis. The recruitment of PMNs into the CNS is a 'double-edge sword' because it not only is crucial for host defence against meningitic bacterial pathogens but also causes significant CNS tissue damage, which results in devastating neurologic sequelae [7]. In a previous report, we found that deletion of the *cglD* virulence factor induced a less efficient PMN transendothelial migration in vitro [5]. In the present study, we further identified the role of *cglD* in PMN migration across the BBB using a complementation strain constructed by delivery of the *cglD* gene into the E44:Δ*cglD* mutant strain (E44:Δ*cglD* + *cglD*). The results showed that complementation of *cglD* could restore the ability of PMNs to cross the BBB in a mouse model of neonatal meningitis and that the level of MPO is an effective indicator of PMN in vivo. We further attempted to identify the

molecular mechanism underlying the ability of the *E. coli* K1 virulence factor *cglD* to mediate PMN migration across the BBB and found that the activation of the brain endothelium by *cglD* in *E. coli* K1 was one of the main reasons responsibly.

PMN adhesion to HBMECs is a prerequisite step for transendothelial migration. Our results showed that deletion of the *cglD* gene from *E. coli* K1 may induce a less efficient PMN adhesion to HBMECs, possibly because of impaired PMN transendothelial migration ability in response to infection with the E44:Δ*cglD* mutant strain. PMNs must establish strong adhesion to HBMECs before transendothelial migration. Activation of the brain endothelium stimulated by bacteria may release various chemokines that may play a role in the regulation of immune cell migration. Some virulence factors of pathogenic bacteria have been associated with chemokine secretion by the brain endothelium. Isogenic NanA-deficient *Streptococcus pneumoniae* were shown to significantly reduce secretion of the chemoattractants IL-8, CXCL1, CXCL2 and CCL20 in the brain endothelium [9]. In *E. coli* K1 bacterial meningitis, *E. coli* K1 induces IL-8 expression in HBMECs [20]. Our results showed that deletion of the *cglD* gene from *E. coli* K1 may significantly induce less efficient expression levels of CXCL1, CXCL6 and CXCL8 in HBMECs as well as the CSF of neonatal mice with meningitis compared with the E44 strain infection. In addition, complementation of *cglD* into the E44:Δ*cglD* mutant strain may restore the levels of these chemokines, which mainly participate in leukocyte chemotaxis. *CglD* in *E. coli* K1 may be necessary and sufficient to activate these chemokines in HBMECs.

Leukocyte migration into the CSF involves the interaction of leukocytes with the vascular endothelium via several sets of surface adhesion molecules. Initially, the leukocytes slow and roll down the endothelium and then interact with endothelial E-selectin, P-selectin and L-selectin. Chemokine binding to receptors on leukocytes activates leukocyte adhesins of the integrin family, such as the $\beta_2\alpha_M$ integrin CD18/CD11b [13]. Simultaneously, mediators, such as IL-8, upregulate the endothelial adhesion molecules of the immunoglobulin superfamily (e.g. ICAM-1), which bind to CD18 on the leukocyte surface [13]. In bacterial meningitis, ompA of *E. coli* K1 selectively enhances the expression of ICAM-1 in HBMECs, which is dependent on PKC-α and PI3-kinase signalling to enhance the binding of THP-1 cells to HBMECs [21]. *E. coli* K1 IbeA-induced PMN is correlated with the up-regulation of endothelial cell expression of ICAM-1 and CD44 through proteasomal regulation of NF-κB activity [22, 23]. Our data showed that the mRNA and protein levels of E-selectin were obviously down-regulated in HBMECs infected with the E44:Δ*cglD* mutant strain compared with those infected with the E44 strain. However, the expression levels of other cell adhesion molecules, such as P-selectin, VCAM-1, ICAM-1 and PECAM-1, were not influenced by deletion of the *cglD* gene. Complementation of *cglD* into the E44:Δ*cglD* mutant strain may restore the expression of E-selectin. E-selectin was initially described as a 115-kD antigen that was induced in cultured human umbilical vein endothelial cells after stimulation with interleukin-l and was involved in the adhesion of leukocytes and several leukaemic cell lines [12, 18]. It is possible that the *cglD* gene of *E. coli* K1 contributes to the expression of E-selectin and promotes the interaction between PMNs and the endothelium at the initial step of rolling down.

Among known pathogens that invade the local tissue barrier and the BBB, most can activate the transcription factor NF-κB, which is another hallmark feature of bacterial meningitis [7]. NF-κB has often been called a central mediator of the human immune response because many microbial pathogens, including meningitic bacteria, can activate this transcription factor, which regulates the expression of inflammatory cytokines, chemokines, immune receptors and cell adhesion molecules [19]. The NF-κB protein family comprises five different members, p65/RelA, c-Rel, RelB, NF-κB2/p52 and NF-κB1/p50, which share a Rel homology domain that mediates DNA binding and dimerisation [24]. In resting cells, NF-κB is trapped in the cytoplasm by inhibitory IκB proteins. The NF-κB activation process is induced by phosphorylation of the serine residues of IκB proteins, which are then subjected to ubiquitination and proteasomal degradation [23]. In *E. coli* K1 meningitis, IbeA induces PMN migration through the proteasomal regulation of NF-κB activity [22, 23, 25]. Our results showed that deletion of the *cglD* gene might induce a significant less efficient in the expression profiles of CXCL1, CXCL6, CXCL8 and E-selectin, which are the target genes of NF-κB. Therefore, we investigated whether NF-κB activation in HBMECs was induced by the E44 strain or the E44:Δ*cglD* mutant strain. Deletion of the *cglD* gene from *E. coli* K1 inhibited activation of the NF-κB pathway in HBMEC, compared with challenged with the E44 strain. Our in vivo data showed that the levels of soluble NF-κB (sp65) in the CSF are lower in newborn mice infected with the E44:Δ*cglD* mutant strain those infected with the E44 strain. Moreover, complementation of the *cglD* gene restores not only NF-κB activation but also the p65 levels in the CSF of newborn mice.

Based on these data, we deduced that the *cglD* gene is an important virulence factor of *E. coli* K1 that participates in the activation of the NF-κB pathway in HBMECs, which results in the release of chemotactic molecules in leukocytes, including CXCL1, CXCL6 and CXCL8, and increased levels of E-selectin, which ultimately promotes PMN adhesion to HBMECs to promote transendothelial migration across the BBB into the brain. Although we showed that the PMNs in the CSF had a less efficient elevation in the neonatal meningitis mice infected with the E44:Δ*cglD* mutant strain, there

are also other ways for immune cells to enter the CNS, such as the choroid plexus pathway [26]. Further study should focus on whether cglD as glycerol dehydrogenase in *E. coli* K1 is associated with glycerol metabolism contributing to PMN transmigration. Our discovery of the novel requirement of cglD for immune activation and subsequent PMN entry into the CNS suggests that therapies directed at neutralising this molecule are beneficial in preventing the progression of bacterial meningitis.

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