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Identification of minimum carbohydrate moiety in N-glycosylation sites of brain endothelial cell glycoprotein 96 for interaction with *Escherichia coli* K1 outer membrane protein A

Subramanian Krishnan^a and Nemani V. Prasadara^{a,b,*}

^aDivision of Infectious Diseases and Department of Pediatrics, Children's Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027, USA

^aDivision of Infectious Diseases and Department of Pediatrics, Children's Hospital Los Angeles and University of Southern California, 4650 Sunset Blvd., Los Angeles, CA 90027, USA

^bDepartment of Surgery, Children's Hospital Los Angeles and University of Southern California, 4650 Sunset Blvd., Los Angeles, CA 90027, USA

Abstract

Bacterial meningitis is a serious central nervous system infection and *Escherichia coli* K1 (*E. coli* K1) is one of the leading etiological agents that cause meningitis in neonates. Outer membrane protein A (OmpA) of *E. coli* K1 is a major virulence factor in the pathogenesis of meningitis, and interacts with human brain microvascular endothelial cells (HBMEC) to cross the blood-brain barrier. Using site-directed mutagenesis, we demonstrate that two N-glycosylation sites (NG1 and NG2) in the extracellular domain of OmpA receptor, Ecgp96 are critical for bacterial binding to HBMEC. *E. coli* invasion assays using CHO-Lec1 cells that express truncated N-glycans, and sequential digestion of HBMEC surface N-glycans using specific glycosidases showed that GlcNAc1-4GlcNAc epitopes are sufficient for OmpA interaction with HBMEC. Lack of NG1 and NG2 sites in Ecgp96 inhibits *E. coli* OmpA induced F-actin polymerization, phosphorylation of protein kinase C- α , and disruption of transendothelial electrical resistance required for efficient invasion of *E. coli* in HBMEC. Furthermore, the microvessels of cortex and hippocampus of the brain sections of *E. coli* K1 infected mice showed increased expression of glycosylated Ecgp96. Therefore, the interface of OmpA and GlcNAc1-4GlcNAc epitope interaction would be a target for preventative strategies against *E. coli* K1 meningitis.

Keywords

Escherichia coli K1; Invasion; brain endothelium; Hsp90; glycoprotein; meningitis

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*To whom correspondence should be addressed: Nemani V. Prasadara, Division of Infectious Diseases, MS #51, Department of Pediatrics and Surgery, Children's Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027, Tel: 323-361-5465; Fax: 323-361-2851, pnemani@chla.usc.edu.

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1. Introduction

Escherichia coli K1 (*E. coli* K1) is one of the leading causes of meningitis in infants within the first month after birth. Neonatal meningitis due to *E. coli* K1, a serious central nervous system disease, results in 5% to 30% mortality and the recent emergence of multi-drug resistant strains could increase these mortality rates further. *E. coli* K1 encounters and endures an arsenal of host defenses including dendritic cells, neutrophils, macrophages, and serum complement to cross the blood-brain barrier (BBB) [1, 2]. The expression of outer membrane protein A (OmpA) in *E. coli* K1 is vital for the bacterium to survive the aforementioned host defenses and reaching high grade bacteremia, a prerequisite for subsequent crossing of the BBB. OmpA interacts with its receptor, endothelial cell glycoprotein 96 (Ecgp96) to invade the human brain microvascular endothelial cells (HBMEC), an *in vitro* model of the BBB [3, 4]. The molecular events and signaling mechanisms underlying this interaction that aid in the invasion process are well-characterized. In HBMEC, Ecgp96, Toll-like receptor 2 (TLR2) and Angiotensin II receptor I (AT1R) are associated with each other at basal levels [5, 6]. The binding of OmpA of *E. coli* K1 to Ecgp96/TLR2/AT1R complex initially sequesters intracellular Ca^{2+} to induce basal level phosphorylation of protein kinase C- α (PKC- α). OmpA binding also stimulates the recruitment of phospho-PKC- α to the Ecgp96/TLR2/AT1R complex, which further signals for nitric oxide (NO) production. NO selectively induces more Ecgp96/TLR2 complexes to the membrane to act as receptor(s) for additional bacteria to bind and invade. Phospho-PKC- α also signals the GTPase activating-like protein, IQGAP1 to dissociate β -catenin from adherens junctions to promote F-actin polymerization beneath the bound bacteria and promotes invasion through active actin remodeling [7–11]. Lack of OmpA impedes all these cellular events in HBMEC as does the overexpression of C-terminal truncated construct of Ecgp96 [10, 12]. Therefore, OmpA-Ecgp96 interaction is critical for the initiation of downstream signaling events partially relayed from the C-terminal of Ecgp96 to promote bacterial invasion.

Ecgp96, also known as Hsp90 β 1, GRP94, gp96, ERp99, TRA-1 and endoplasmic reticulum (ER) paralogue of heat shock protein Hsp90 that acts as a molecular chaperone aiding maturation and compartmentalization of various nascent peptides in the endoplasmic reticulum. Gp96 also acts as a master chaperone for Toll-like receptors (TLRs) and integrins [13, 14]. Though gp96 is predominantly an ER resident chaperone, evidences suggest that it might be surface exposed during infection and in tumor formation [4, 15]. Ecgp96 was implicated for the first time as a bacterial receptor for OmpA of *E. coli* K1 to invade HBMEC [16]. Several studies have now identified gp96, the non-endothelial homologue of Ecgp96, as a receptor for a number of bacteria [17–21]. Our previous studies showed that TLR2 stabilizes Ecgp96 on the membrane of HBMEC to facilitate OmpA binding. Interestingly, another study showed that cell surface expression of TLRs was dependent on N-linked glycosylation of gp96 [22]. Further, gp96 glycosylation is also an indication of the metastatic nature of prostate cancer and down regulation of TNF- α and interleukins [23]. A recent study showed that patients with Alzheimer's disease have elevated levels of glycosylated gp96, showing that N-glycosylation of gp96 is an important marker for the prognosis of various disease conditions [24]. Of interest, *E. coli* K1 OmpA

interacts with GlcNAc1-4GlcNAc (chitobiose) moieties of HBMEC glycoproteins for efficient bacterial invasion [25, 26]. Although Ecgp96 is the primarily responsible for *E. coli* K1 invasion of HBMEC, it is unknown whether N-glycosylated residues in Ecgp96 play any role in OmpA interaction [5, 11]. In this study, we show for the first time using site-directed mutagenesis and sequential enzymatic cleavage that two N-glycosylation sites in the extracellular domain of Ecgp96 are critical for *E. coli* K1 binding to and invasion of HBMEC. By expressing Ecgp96 in CHO-Lec1 cells that do not express certain terminal sugars followed by enzymatic digestion, the minimum sugar moieties required for binding were characterized. We further show that *E. coli* K1 infection of newborn mice induces elevated levels of N-glycosylated Ecgp96 in the brain.

2. Materials and Methods

2.1. Bacteria, antibodies and other reagents

E. coli K1 RS218 (serotype O18:K1:H7), a spontaneous rifampin-resistant strain and OmpA- *E. coli*, generated by disrupting the *ompA* gene, have been described previously [10]. Bacteria were grown in Luria-Bertani (LB) broth with appropriate antibiotics. Bacterial media were purchased from Difco laboratories (Detroit, MI). Quikchange site-directed mutagenesis kit was from Agilent technologies (Santa Clara, CA). GFP plasmid pACBB-eGFP was obtained from Addgene (ID#32551). Antibodies to Ecgp96 (GRP94) were from Genetex (Irvine, CA). Antibodies to OmpA were generated as previously described [4]. Concanavalin A coupled to FITC (ConA-FITC) was from MP Biomedicals (Solon, OH). Gp96 siRNA, Lipofectamine for siRNA transfection, Alexa 568 phalloidin for F-actin staining and secondary antibodies coupled to fluorophores Alexa 488 and Alexa 647 were purchased from Life technologies (Grand Island, NY). FuGENE HD and X-tremegene 9 for plasmid transfection were from Roche (Mannheim, Germany). α - and β -mannosidases, and α -methyl manno-pyranoside were from Sigma (St. Louis, MI). Chitin hydrolysate was from Vector labs (Mountain View, CA). PepTag, a non-radioactive protein kinase assay kit, and PNGase-F were from Promega (Madison, WI). Plasma membrane isolation kit was purchased from Biovision (Mountain View, CA). CHO-Lec1 cells, which express truncated N-glycans lacking terminal sugars, and CHO cells were kindly provided by Grace Aldrovandi, Children's Hospital Los Angeles.

2.2. Cell culture, transfection and invasion assays

Human brain microvascular endothelial cells (HBMEC) isolation was described previously [5]. 97% of the cells were positive for Factor VIII-rag and 100% negative for GFAP as assessed by flow cytometry and 99% were positive for Ac-LDL uptake as determined by immuno-cytochemistry. HBMEC were maintained at 37°C in a humidified atmosphere of 5% CO₂ in medium containing M-199/Ham F-12 (1:1 v/v) supplemented with 10% fetal bovine serum, 5% nu-serum, sodium pyruvate and 2 mM glutamine. Cell association and invasion assays using HBMEC were performed as described previously [5]. For transfection experiments, 4 μ g of the respective plasmids were transfected into HBMEC, CHO or CHO-Lec1 cells in Opti-MEM medium (Life technologies) and allowed to recover for at least 24 h before performing cell association or invasion assays. To determine siRNA mediated knockdown of Ecgp96, 33pmol of gp96 siRNA was transfected in CHO cells using

Lipofectamine (Life technologies) and expression levels were analyzed 18–24 h post transfection. For enzyme treatment experiments, 0.1 units each of α - or β -mannosidases in 100 μ l of 50 mM sodium phosphate buffer (pH 5.0) were used to pre-treat all the three cell lines for 15 min at room temperature. The cells were washed three times with PBS and re-suspended in experimental medium for 30 minutes to allow the cells to recover and then infected with bacteria. For PNGase-F, 10U of the enzyme was re-suspended in 500 μ l of PBS and incubated for 1 h at 37°C. The cells were then washed three times with PBS and re-suspended in experimental medium and then infected immediately with bacteria. 10^8 cfu/ml of bacteria were pre-treated with 50 mM final concentration of α -methyl manno-pyranoside in 1 ml of 0.1% BSA/PBS for 30 min in ice and 100 μ l of the suspension were added to cells for assays. For chitin hydrolysate experiments, 10^7 bacteria in 100 μ l of experimental medium were incubated with 2 mg of chitin hydrolysate in ice for 1 h, washed twice with saline and then used to infect the cells. For anti-OmpA antibody inhibition studies, 10^7 bacteria were incubated with 1:1000 dilution of the antibody in PBS on ice for 1 h, washed three times with PBS to remove unbound antibody, and the bacterial pellet was re-suspended in experimental medium before infecting the cells.

2.3. Western blotting

HBMEC were transfected with FLEcgp96 and NG mutants of Ecgp96 as described above, allowed to recover for 24 h, and total cell lysates were prepared using lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EGTA and 1% Triton X-100). Unbroken cells and cell debris were removed by centrifuging the lysates at $700 \times g$ for 10 min at 4°C. The plasma membrane fractions from the lysates were separated using plasma membrane isolation kit (Biovision) and the protein content was estimated using Nanodrop ND-1000 spectrophotometer. 40 μ g of plasma membrane fractions were resolved on a 4%–8% gradient gel, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk in PBS/0.1% Tween-20 (PBS/T) for 1 h at room temperature. After three washes with PBS/T, the blot was incubated with 1:1000 anti-Ecgp96 antibodies for 2 h. The membrane was washed extensively for 15 min with TBST and incubated with anti-rabbit secondary antibody conjugated to horse radish peroxidase for 1 h. The membrane was finally washed for 15 min with TBST and developed with Super Signal chemiluminescence substrate (Pierce, Rockford, IL) and exposed to X-ray film for protein visualization.

2.4. Flow cytometry

To detect the surface expression of Ecgp96, cells (HBMEC/CHO/CHO-Lec1) were infected with *E. coli* K1 for different time points. The cells were washed three times with PBS and then detached with TrypLE express (Life technologies) from the plates. The cells were fixed using BD cytofix for 15 min, washed and pre-incubated for 30 min with blocking/wash buffer (PBS+3% normal goat serum) to mask non-specific binding sites. Cells were then incubated with anti-Ecgp96 antibody or an isotype-matched control antibody for 30 min at 4°C and washed with buffer. Then FITC-conjugated secondary antibody was added, incubated for 20 min at 4°C and washed with the buffer. The stained cells were then analyzed by four-color flow cytometry using FACS Calibur Cell Quest Pro software (BD Biosciences, San Diego, CA) and at least 10,000 events were collected for analysis. Results are expressed as histogram overlays with respect to isotype matched antibody controls. For

detection of mannose cleavage using ConA-FITC, cells were treated with β -mannosidase, and probed with 1:1000 dilution of ConA-FITC for 30 min at 4°C, washed, analyzed by flow cytometry and represented as histogram overlays.

2.5. PepTag assay to determine PKC activity

The PepTag (Promega) assay to detect PKC activity was performed as described previously [5]. The PepTag assay uses brightly colored, fluorescent peptide substrates that are highly specific for PKC. Phosphorylation of the peptide alters the net charge from +1 to -1. This change in the net charge allows the phosphorylated and non-phosphorylated versions of the substrate to be rapidly separated on an agarose gel at neutral pH. Control HBMEC and HBMEC transfected with FLEcgp96 or the NG mutants were infected with *E. coli* K1 for 15 min and cell lysates from each well were used for determining PKC activity.

2.6. Endothelial cell permeability assay

HBMEC were seeded onto filter inserts (polycarbonate filters with 4 micron pore size, Costar, NY, USA) in 200 μ l of medium, and the lower compartment was filled with 600 ml of the same medium. HBMEC were seeded at an appropriate density so that they attained 90–95% confluence the next day. All wells except control cells were transfected with the respective plasmids carrying FLEcgp96 or the NG mutants. After 24 h, the medium in the upper compartment was carefully removed and replaced with 200 μ l of fresh medium alone for uninfected control or medium containing bacteria (MOI 1:100). The lower compartment was also refilled with fresh medium. TER (transendothelial electrical resistance) was measured at 15, 30, 60 and 90 min post infection with a Millipore ERS apparatus, according to the manufacturer's protocol.

2.7. Immunofluorescence and confocal microscopy

HBMEC grown in 8-well chamber slides were transfected with the respective plasmids, and 24 h after transfection, infected with GFP+*E. coli* K1 for 30 min. The cells were then washed with RPMI-1640, and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. The cells were washed and blocked with PBS containing 3% normal goat serum for 30 min at room temperature. The non-permeabilized cells were probed with anti-Ecgp96 antibodies (1:250 dilution) for 1 h, washed and incubated with FITC-coupled secondary antibodies (1:500 dilution) to detect surface expression of Ecgp96. The cells were then permeabilized with PBS/0.05 % Triton X-100 for 15 min at room temperature and incubated with Alexa 568-phalloidin (1:250 dilution in sterile PBS) for 20 min and washed three times with sterile PBS. Finally, the slides were washed and mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Slides were imaged with a DM RXA microscope equipped with interference contrast optics and HCX PL APO 63 $\times$ /1.4 oil immersion objective lens (Leica Microsystems, Wetzlar, Germany). Fluorescence was imaged using excitation light from a Lambda LS300 xenon lamp (Sutter Instrument, Novato, CA) and the following excitation/emission filter sets: 480/535 for FITC and 665/725 for Alexa 647 (Chroma Technology Corp., Rockingham, VT). Images were captured with a MicroMax 1300Y cooled charge coupled device (CCD) camera (Princeton Instruments,

Trenton, NJ). During image acquisition the microscope and camera were controlled by Micro-Manager 1.4 software.

2.8. Newborn mouse model of meningitis and tissue immunostaining

The animal studies were approved by the Institutional Animal Care and Use Committee of the Saban Research Institute at Children's Hospital Los Angeles (CHLA) (Protocol #59-11) and followed National Institutes of Health guidelines for the performance of animal experiments. Breeding pairs of C57BL/6 wild-type mice were obtained from Jackson Laboratories. Three-day-old mouse pups were randomly divided into two groups ($n=5$) and infected intranasally with 10^3 colony forming units (CFU) of *E. coli* K1 or received pyrogen free saline. 72 h post-infection, pups were sacrificed and the brains were removed. One half of each brain was homogenized and plated on LB agar containing rifampicin to determine the brain bacterial load. The other half of the brain from control and infected pups was embedded in paraffin for tissue sectioning followed by immunostaining. Sections of paraffinized brain sections from control and infected pups were sequentially rehydrated using xylene, 100% ethanol, 95% ethanol, 50% ethanol and water. The slides were trypsinized (0.5% trypsin) for 20 min at room temperature for antigen unmasking. The sections were blocked in PBS containing 3% normal goat serum for 30 min at room temperature, stained with 1:500 dilution of ConA-FITC, followed by 1:250 dilution of anti-Ecgp96 antibody. The sections were further stained with Alexa 647 secondary antibody specific to Ecgp96 and visualized by confocal microscopy as mentioned above.

2.9. Statistical analysis

Results were analyzed using Student's unpaired *t*-test (<http://www.graphpad.com/quickcalcs/ttest1.cfm>) and *P* values <0.05 were considered statistically significant.

3. Results

3.1. Mutation of two asparagines bearing N-glycans in Ecgp96 inhibits total cell association and invasion of *E. coli* K1 in HBMEC

Previous studies have shown that OmpA of *E. coli* K1 interacts with Ecgp96 to invade HBMEC and the N-terminal portion of Ecgp96 is partly exposed to the extracellular milieu [4]. Additionally, removal of N-glycans from the surface of HBMEC by PNGase-F treatment blocked the *E. coli* K1 invasion [25]. Nonetheless, it is unclear whether N-glycosylation (NG) sites in the N-terminus of Ecgp96 are critical for invasion process. Although we showed previously that Ecgp96 may be partially exposed to extracellular milieu at the cell surface, the exact peptide sequence exposed is unknown. Recently, Vip, an important virulent factor in *Listeria monocytogenes* was shown to bind surface exposed gp96, which was identified as a 390 amino acid sequence in the N-terminal domain. This information is in contrary to the results obtained from transmembrane prediction server, TMPred, which predicted a 172 amino acid extracellular domain in gp96 [21]. Since the presence of an extracellular 390 amino acid sequence was experimentally verified, we analyzed the sequences downstream of this region for a putative transmembrane sequence. We identified a consensus motif GVVDS (blue color), which agrees with the GXXXG motif rule, complied by protein transmembrane helices [27], suggesting that a putative

transmembrane domain might be present after the N-terminal 390 amino acids of Ecgp96 (Fig. 1A). In addition, three of six NG sites were found to be present in the extracellular domain based on the typical glycosylation consensus sequence, asn-X-Ser/Thr (yellow color) [28]. A simplistic version of N-glycosylation pattern is shown in Fig. 1B, in which carbohydrate cleavage sites by specific enzymes (used in subsequent experiments) are indicated by dotted arrows.

By site-directed mutagenesis, the three extracellular NG sites were mutated to alanines utilizing specific primer sequences (Table 1). The pcDNA 3.0 plasmid carrying the full length Ecgp96 cDNA (FLEcgp96) was used for mutagenesis and the NG sites were named as NG0, NG1, and NG2 based on their order of presence in the N-terminus. All the mutations were verified by sequencing. Next, the respective plasmids, including FLEcgp96, were independently overexpressed in HBMEC and their expression levels, compared to endogenous Ecgp96, were verified by flow cytometry using an anti-Ecgp96 antibody. The expression of Ecgp96 was considerably higher in transfected HBMEC compared to control HBMEC (Fig. 1C). The total cell associated and invasion assays performed with *E. coli* K1 in HBMEC, transfected with the various NG-mutants, showed that HBMEC/NG1 and NG2 prevented the total cell associated bacteria by 25% ($6 \pm 0.35 \times 10^6$ for control versus $4.5 \pm 0.27 \times 10^6$ for NG1 and NG2) and invasion by 45% compared to untransfected cells ($7.5 \pm 0.22 \times 10^3$ for control versus $3.5 \pm 0.23 \times 10^3$ for NG1 and NG2). Interestingly, FLEcgp96 expression allowed the bacteria to associate and invade more than untransfected cells (122% and 139%, respectively), while the expression of NG0 mutant in HBMEC yielded patterns similar to that of FLEcgp96 (Fig. 1D). Based on this observation, a double mutant (NG1/2) was generated and tested whether there would be an additive effect on the invasion. Indeed, NG1/2 expressing HBMEC inhibited the cell association and invasion of *E. coli* K1 by 30% ($6 \pm 0.35 \times 10^6$ for control versus $4.2 \pm 0.34 \times 10^6$ for NG1/2) and 70% ($7.5 \pm 0.22 \times 10^3$ for control versus $1.4 \pm 0.25 \times 10^3$ for NG1/2), respectively. Our previous studies showed that OmpA of *E. coli* K1 interacts directly with Ecgp96 and blocking OmpA or Ecgp96 using specific antibodies prevents invasion [3, 4]. Therefore, cell association and invasion experiments using *E. coli* K1 pre-incubated with anti-OmpA antibody were performed in parallel. The data showed that the cell association and invasion in both control and transfected HBMEC was significantly reduced (Fig. 1D). To ascertain whether the mutated NG sites actually carried N-glycans, Western blot analysis of membrane preparations from HBMEC expressing the NG-mutants was performed using anti-Ecgp96 antibodies in a 4–8% gradient gel. Lack of one NG site changes the migration of Ecgp96 on the gel by ~3 kDa. NG1 and NG2 mutations resulted in Ecgp96 migrating as ~93kDa bands, whereas NG1/2 mutant migrated as a ~90 kDa band, confirming that lack of NG sites decreased the size of Ecgp96 on SDS-PAGE. A faint band corresponding to the native 96 kDa Ecgp96 was observed in all three lanes (NG1, NG2 and NG1/2), showing that the expression levels of NG-mutant Ecgp96 were greater than the expression levels of endogenous Ecgp96. β -actin expression served as a loading control (Fig. 1E). However, Ecgp96 expression from untransfected control, FLEcgp96 and NG0 expressing HBMEC membrane fractions showed a normal 96 kDa protein, indicating that NG0 was not a true NG site. It also explains the reason why *E. coli* K1 cell association and invasion patterns in HBMEC overexpressing NG0 resembled that of HBMEC/FLEcgp96. These results suggest that interaction of OmpA

with two N-glycosylation sites in the N-terminus of Ecgp96 contribute to the cell association and invasion of *E. coli* K1 in HBMEC.

3.2. Overexpression of Ecgp96 that carry NG mutations in CHO cells prevents the invasion of *E. coli* K1

Overexpression of Ecgp96 allows *E. coli* K1 to invade CHO cells efficiently [16]. To determine whether the two N-glycosylation sites in Ecgp96 are critical of *E. coli* K1 binding and invasion, we used CHO cells to determine the total cell association and invasion patterns by overexpressing Ecgp96 containing NG mutants. Cell association and invasion frequencies of *E. coli* K1 were significantly lower in CHO transfected with NG1, NG2 and NG1/2 compared to the levels in FLEcgp96 and NG0 transfected cells, similar to HBMEC (Fig. 2A). Flow cytometry analysis also revealed Ecgp96 expression levels in transfected CHO cells were similar to the levels expressed in HBMEC on the cell surface as shown previously, suggesting that the altered invasion is not due to differences in the surface expression of Ecgp96 (Fig. 2B) [5]. Next, to confirm whether Ecgp96 is responsible for *E. coli* K1 entry into CHO cells, we knocked down Ecgp96 expression in CHO cells using siRNA and performed the invasion assays. Knockdown of Ecgp96 inhibited the bacterial cell association by ~35% and invasion by ~70% in CHO cells, indicating that Ecgp96 interaction with *E. coli* K1 is mainly contributing to the invasion in CHO cells (Fig. 2C). Flow cytometry analysis showed that Ecgp96 siRNA suppressed Ecgp96 expression by more than 80% in CHO cells (Fig. 2D). These results suggest that Ecgp96 also acts as a receptor in CHO cells for *E. coli* K1 binding and invasion.

3.3 Terminal sugars in the N-glycans hinder *E. coli* K1 invasion in HBMEC

Our results in this study show that *E. coli* K1 requires N-glycosylation of Ecgp96 for effective cell association and invasion in both HBMEC and CHO cells. However, *E. coli* K1 expresses two other adhesins besides OmpA that bind to different sugar epitopes. Those are S-fimbriae that bind to terminal sialyl-lactosamine and type-1 fimbriae specific to mannose. Our previous studies showed that both S-fimbriae and type-1 fimbriae play no role in the invasion of *E. coli* K1 in HBMEC [29]. However, studies by other investigators showed that knocking out the *fimH* gene, which encodes the tip adhesin of type-1 fimbriae, significantly reduced the invasion in HBMEC [30, 31]. Therefore, to determine the role of individual sugars in the N-glycans necessary for this process, we initially performed invasion assays in CHO-Lec1 cells that lack the terminal sialic acid, galactose and GlcNAc owing to lack of UDP-GlcNAc (α -D-mannoside- β -1,2-N-acetylglucosaminyl transferase) which results in the N-glycans terminating after Man5-GlcNAc2-Asn [32]. Hence, the sugars beginning with the distal GlcNAc are not expressed (refer Fig. 1B). However, the core GlcNAc1-4GlcNAc expression is not affected since it is a β -1,4 linkage as opposed to the β -1,2 linkage between mannose and the distal GlcNAc. The total cell association of *E. coli* K1 was increased by 7% in CHO-Lec1 cells, whereas the invasion in CHO-Lec1 was 50% more than in HBMEC and CHO cells (Fig. 3A). A marginal increase in the binding of *E. coli* to CHO-Lec1 cells is sufficient to enhance the invasion by 50%. This shows that terminal sialic acid, galactose and GlcNAc in the N-glycans hinder the interaction of *E. coli* K1 with Ecgp96 for bacterial invasion. Next, to examine the role of core mannose residues, HBMEC were pre-treated with α -mannosidase, which cleaves branched mannoses; β -mannosidase, which cleaves the

mannose attached to GlcNAc1-4GlcNAc moieties, and PNGase-F that cleaves the core chitobiose (GlcNAc1-4GlcNAc) attached to asparagine residues (Fig. 1B). The cells were then used for total cell associated and invasion assays. In addition, *E. coli* K1 was pre-treated with α -methyl manno-pyranoside or chito-oligosaccharides (chitin hydrolysate) to block the mannose binding sites of type-1 fimbriae or GlcNAc1-4GlcNAc binding sites of OmpA, respectively and then allowed to infect HBMEC. Of note, α -mannosidase pre-treatment increased the invasion of *E. coli* K1 by ~40% and β -mannosidase pre-treatment enhanced about ~48 % compared to untreated cells (Fig. 3B). However, PNGase-F treatment significantly inhibited both cell association and invasion of *E. coli* K1 in HBMEC confirming our previous results that GlcNAc1-4GlcNAc epitopes are critical for *E. coli* K1 invasion [25]. Although pre-treatment with α -methyl manno-pyranoside prevented type-1 fimbriae mediated *Saccharomyces* agglutination (data not shown), the total cell associated or intracellular bacteria was not affected by this treatment. In contrast, pre-treating *E. coli* K1 with chitin hydrolysate significantly prevented the cell association and invasion (Fig. 3B). Similar patterns were also observed in CHO cells and CHO-Lec1 cells (Fig. 3C and D). It should be noted here that although the cell association and invasion of *E. coli* K1 in control CHO-Lec1 cells (Fig. 3D) is taken as 100%, it is actually 50% more than that observed with HBMEC or CHO cells. Hence, there is only an insignificant increase in cell association/invasion after α -mannosidase or β -mannosidase pre-treatment in CHO-Lec1 cells. Previous studies from this lab demonstrated that OmpA has specific affinity for GlcNAc1-4GlcNAc moieties [4]. To confirm whether OmpA specifically binds GlcNAc1-4GlcNAc epitopes on Ecgp96, HBMEC expressing FLEcgp96 and treated with β -mannosidase were infected with *E. coli* K1 with or without pre-incubation with anti-OmpA antibody. As observed earlier, β -mannosidase treatment did not alter infection patterns. However, pre-incubation of *E. coli* K1 with anti-OmpA antibody inhibited both cell association and invasion (Fig. 3E). Experiments performed with CHO and CHO-Lec1 cells also exhibited similar results (data not shown). To ensure optimal enzyme activity in these experiments, HBMEC, CHO and CHO-Lec1 cells pre-treated with β -mannosidase were subjected to flow cytometry analysis using concanavalin A-FITC (ConA-FITC). The results show that 85–90% of mannose was cleaved based on lack of ConA-FITC binding to each of the cell type (Fig. 3F). These results demonstrate that removal of mannose from N-glycans does not affect the invasion process. Since pre-incubation of bacteria with either chitin hydrolysate or anti-OmpA antibody inhibits the invasion process, the interaction of OmpA with GlcNAc1-4GlcNAc epitopes on N-glycosylation sites 1 and 2 of Ecgp96 appears to be important for the invasion process.

3.4. *E. coli* K1 fails to induce cellular events necessary for invasion in HBMEC overexpressing Ecgp96 with N-glycosylation mutations

E. coli K1 manipulates PKC- α phosphorylation to stimulate nitric oxide (NO) production as well as F-actin polymerization to invade HBMEC. While NO promotes translocation of Ecgp96 to the membrane to act as receptor for OmpA, F-actin accumulates beneath bound bacteria and pulls the bacteria inside [5, 8, 10, 11]. To examine whether NG mutations influence F-actin polymerization, HBMEC overexpressing NG mutants of Ecgp96 were subsequently infected with GFP+ *E. coli* K1 and actin accumulation patterns were analyzed by confocal microscopy. Prior to F-actin staining, the cells were stained with anti-Ecgp96 antibody without permeabilization to examine the surface expression pattern of Ecgp96 in

relation to F-actin accumulation. Overexpression of FLEcgp96 or NG0 plasmids in HBMEC induced F-actin accumulation beneath invading bacteria, whereas NG2 and NG1/2 mutations drastically reduced F-actin polymerization (Fig. 4A). NG1 mutation exhibited bacterial binding patterns very similar to NG2 (data not shown). Of note, the Ecgp96 in FLEcgp96/HBMEC and co-localized with bacterial binding sites whereas, in NG2- and NG1/2-HBMEC, Ecgp96 was distributed at random without specific accumulation beneath bound bacteria. These results suggest that *E. coli* K1 binding to FLEcgp96 induces recruitment of additional Ecgp96 to the bacterial binding sites to induce F-actin remodeling. Lack of N-glycosylation on sites 1 and 2 prevents specific binding to HBMEC and therefore, could not induce either Ecgp96 recruitment to infection sites or F-actin accumulation.

Similarly, phosphorylation of PKC- α , which is central in *E. coli* K1 induced signaling events was also significantly reduced in HBMEC overexpressing NG1, NG2 or NG1/2 despite infection with the bacteria (Fig. 4B). Another important event in the infection process is the disassembly of adherens junction molecules to rearrange F-actin to the sites of *E. coli* K1 binding, which is responsible for HBMEC permeability [10, 33]. Therefore, we also examined the permeability of HBMEC (measured as transendothelial electrical resistance or TER) overexpressing the NG-mutant Ecgp96 after infection with *E. coli* K1. As shown in Fig. 4C, overexpression of FLEcgp96 or NG0-Ecgp96 showed a 60% drop in TER compared with uninfected control HBMEC (Fig. 4C). In contrast, overexpression of NG1, NG2 or NG1/2 in HBMEC prevented the *E. coli* K1 induced permeability of the monolayers. Taken together, these results suggest that the binding of *E. coli* K1 to Ecgp96 N-glycosylation sites is critical for subsequent invasion process.

3.5. *E. coli* K1 induces N-glycosylated Ecgp96 expression in the brain of newborn mice

Although our studies have demonstrated that Ecgp96 expression increases in HBMEC upon infection with *E. coli* K1 [4], it is unclear whether the expression of Ecgp96 is altered in the brains of infected newborn mice. To examine the expression of Ecgp96 in the brains after *E. coli* K1 infection, we used a clinically relevant newborn mouse model of meningitis, which exhibits several similarities to human disease [5]. The brain sections from control and infected mouse pups were subjected to immunofluorescence staining with ConA-FITC and anti-Ecgp96 antibodies. As shown in Fig. 5, control cells showed basal levels of mannose on the surface of microvessels in the cortex, hippocampus and meninges with little or no Ecgp96 expression. In contrast, *E. coli* K1 infected brain showed higher levels of ConA (green) and Ecgp96 (red) staining. Of note, in several areas both ConA reactivity and Ecgp96 staining was co-localized (yellow), indicating that glycosylated Ecgp96 is expressed in the brains of *E. coli* K1 infected mouse pups.

4. Discussion

Bacterial pathogens, in the process of interacting with host tissues for colonization, utilize their surface proteins called adhesins [34]. *E. coli* K1, the second most common bacterium that causes meningitis in neonatal populations exhibits neurotropism to establish the infection [35]. Our studies have shown that the non-fimbrial adhesin, OmpA is a major virulence component of *E. coli* K1 and that it interacts with Ecgp96 on the surface of HBMEC to bind to and invade [3]. A recent study also showed that Vip, a major virulence

factor in *Listeria monocytogenes* binds to the N-terminal extracellular domain of gp96 (a homologue of Ecgp96) in epithelial cells [21]. Gp96 also acts as a receptor for a wide variety of pathogens, including *Clostridium difficile*, adherent-invasive *E. coli*, *Neisseria gonorrhoeae* and *Staphylococcus aureus* and for bovine adeno-associated virus (BAAV) [17–20, 36, 37]. BAAV binds GlcNAc moieties in gp96 for transcytosis, suggesting that N-glycosylation of gp96 might play a general role in pathogen binding/uptake by host cells. However, the interactions of several other adhesins with specific carbohydrate moieties present on receptors are not well characterized for many bacterial pathogens.

We have demonstrated that OmpA interacts with GlcNAc1-4GlcNAc epitopes and Ecgp96 for invasion of *E. coli* into HBMEC [16, 25]. Here, the observations reveal that GlcNAc1-4GlcNAc epitopes present in NG1 and NG2 sites of Ecgp96 are important for bacterial binding and invasion. This is supported by (i) Mutation of N-glycosylated asparagines blocks the invasion by ~75%, (ii) Suppression of Ecgp96 using specific siRNA blocks invasion in CHO cells, implying that gp96 is the primary receptor for OmpA in these cells (iii) Confirmation that these putative sites are indeed glycosylated by Western blotting, (iv) Enzymatic removal of GlcNAc1-4GlcNAc, but not mannose in Ecgp96, inhibits bacterial binding and invasion, and (v) Pre-incubation of bacteria with either chitin hydrolysate or anti-OmpA antibody significantly attenuates bacterial adhesion and invasion, which corroborates our previous observation that OmpA binds GlcNAc1-4GlcNAc moieties. Based on experimental evidence provided by Martins et al., Ecgp96 seems to have a larger extracellular domain (390 amino acids) than a 172 amino acid extracellular domain predicted using transmembrane prediction server, TMPred [21]. A highly correlative statistical analysis of helix-helix interactions in membrane proteins reveal a common GXXXG motif, which could represent a transmembrane region [27], and we found the sequence ‘GVVDS’ downstream of extracellular domain in Ecgp96 that concurred with the GXXXG motif rule [27]. This suggests that ‘GVVDS’ could be a potential transmembrane region of Ecgp96, which however, warrants further investigation. With the establishment of the actual extracellular loop region of Ecgp96, this region was scanned for potential NG sites. We found three NG sites in the extracellular domain of Ecgp96, although NG0 site is not glycosylated in HBMEC as shown in this study. This may be due to differences observed in N-glycosylation patterns of gp96 in various cell types [38]. However, the biological relevance of this lack of N-glycosylation is currently unknown. NG1 and NG2 sites were found to be critical for OmpA binding as mutation of these residues to alanines significantly reduced *E. coli* K1 binding to and invasion in HBMEC.

Apart from OmpA, *E. coli* K1 expresses two important fimbrial adhesins that bind sugar residues, S-fimbriae and type-1 fimbriae, which are specific to NeuAc2-3Gal1-3GlcNAc and mannose residues on the surface expressed N-glycans in host cells, respectively [39]. S-fimbriae, which comprises several subunits, also binds sulfated galactosyl ceramide via a major subunit SfaA in ELISA and immuno-TLC assays, whereas SfaS present at the tip of the adhesin binds NeuAc2-3Gal1-3GlcNAc residues [40]. However, it was demonstrated that S-fimbriae play an insignificant role in the invasion of *E. coli* K1 in HBMEC [23]. In contrast, subsequent studies utilizing mutation of *fimH* gene that encodes the adhesin portion of type-1 fimbriae revealed that this adhesin also plays a role in *E. coli* K1 invasion process

[30]. These studies also showed that the expression of type-1 fimbriae increases in *E. coli* K1 upon interaction with HBMEC. Notably, lack of OmpA expression in *E. coli* K1 partially suppresses type-1 fimbriation. Hence, there might be a regulation of other adhesins in the absence of OmpA. However, when the promoter of *fimH* operon was kept in 'ON' phase in an OmpA negative background, *E. coli* K1 still showed significantly less binding to HBMEC, although type-1 fimbriae expression was almost similar to that of wild type. This suggests that, even if there was an optimal expression of type-1 fimbriae achieved by genetic manipulation, lack of OmpA still severely attenuates *E. coli* K1 binding to HBMEC [31, 41]. In addition, *E. coli* K1 that do not express OmpA could not cause meningitis in a biologically relevant mouse model of meningitis while no such studies have been shown with *fimH*⁻ *E. coli* K1. Therefore, based on the current understanding about *E. coli* K1 pathogenesis, OmpA expression is essential for *E. coli* K1 virulence.

We moved on to identify minimum carbohydrate structure required for the binding to and invasion of the bacterium in HBMEC using various enzyme treatments. The data obtained in this study clearly demonstrate that terminal Neu5Ac residues (for S-fimbriae) are not necessary for binding of the bacterium and subsequent invasion, which confirms our previous results [23]. Furthermore, assays using CHO-Lec1 cells and sequential enzymatic digestion clearly show that GlcNAc1-4GlcNAc epitopes linked to asparagines are a minimum requirement for *E. coli* K1 interaction with Ecgp96 in HBMEC via OmpA. Computer modeling studies of OmpA interaction with GlcNAc1-4GlcNAc revealed that OmpA interacts with two chitobiose moieties, one at the top of loops 1 and 2, and the other one at the barrel formed by loops 1 and 4 [26]. Therefore, it is possible that NG1 and NG2 glycans may interact with these loops in OmpA. This might also be a reason why the mutation of these two asparagine residues reduced the invasion of *E. coli* K1 by >75% in HBMEC compared to mutation of individual N-glycosylation sites. In agreement, mutation of specific amino acids in the extracellular loops 1 and 2 of OmpA significantly prevented *E. coli* K1 invasion of HBMEC [42]. To conclude, GlcNAc1-4GlcNAc moieties present in two specific N-glycosylation sites in the extracellular region of Ecgp96 are critical for OmpA binding, which further aids in the invasion of *E. coli* K1 into HBMEC. Since *E. coli* K1 OmpA has sequence similarity to the allele *ompA2* present in pathogenic bacteria [43], the interface of OmpA-Ecgp96 interaction could be an attractive target for preventing *E. coli* K1 meningitis.

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References

1. Mittal R, Krishnan S, Gonzalez-Gomez I, Prasadarao NV. Deciphering the roles of outer membrane protein A extracellular loops in the pathogenesis of *Escherichia coli* K1 meningitis. *J Biol Chem.* 2010; 286:2183–2193. [PubMed: 21071448]
2. Krishnan S, Prasadarao NV. Outer membrane protein A and OprF: versatile roles in Gram-negative bacterial infections. *FEBS J.* 2010; 279:919–931. [PubMed: 22240162]

3. Prasadarao NV, Wass CA, Weiser JN, Stins MF, Huang SH, Kim KS. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun*. 1996; 64:146–153. [PubMed: 8557332]
4. Prasadarao NV. Identification of *Escherichia coli* outer membrane protein A receptor on human brain microvascular endothelial cells. *Infect Immun*. 2002; 70:4556–4563. [PubMed: 12117968]
5. Krishnan S, Chen S, Turcatel G, Arditi M, Prasadarao NV. Regulation of Toll-like receptor 2 interaction with Ecgp96 controls *Escherichia coli* K1 invasion of brain endothelial cells. *Cell Microbiol*. 2013; 15:63–81. [PubMed: 22963587]
6. Krishnan S, Shanmuganathan MV, Behenna D, Stoltz BM, Prasadarao NV. Angiotensin II Receptor Type 1--A Novel Target for Preventing Neonatal Meningitis in Mice by *Escherichia coli* K1. *J Infect Dis*. 2014; 209:409–419. [PubMed: 24041786]
7. Sukumaran SK, Quon MJ, Prasadarao NV. *Escherichia coli* K1 internalization via caveolae requires caveolin-1 and protein kinase C alpha interaction in human brain microvascular endothelial cells. *J Biol Chem*. 2002; 277:50716–50724. [PubMed: 12386163]
8. Sukumaran SK, Prasadarao NV. Regulation of protein kinase C in *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *J Biol Chem*. 2002; 277:12253–12262. [PubMed: 11805101]
9. Sukumaran SK, McNamara G, Prasadarao NV. *Escherichia coli* K-1 interaction with human brain micro-vascular endothelial cells triggers phospholipase C-gamma1 activation downstream of phosphatidylinositol 3-kinase. *J Biol Chem*. 2003; 278:45753–45762. [PubMed: 12952950]
10. Krishnan S, Fernandez GE, Sacks DB, Prasadarao NV. IQGAP1 mediates the disruption of adherens junctions to promote *Escherichia coli* K1 invasion of brain endothelial cells. *Cell Microbiol*. 2012; 14:1415–1433. [PubMed: 22519731]
11. Mittal R, Prasadarao NV. Nitric oxide/cGMP signalling induces *Escherichia coli* K1 receptor expression and modulates the permeability in human brain endothelial cell monolayers during invasion. *Cell Microbiol*. 2010; 12:67–83. [PubMed: 19732056]
12. Maruvada R, Argon Y, Prasadarao NV. *Escherichia coli* interaction with human brain microvascular endothelial cells induces signal transducer and activator of transcription 3 association with the C-terminal domain of Ec-gp96, the outer membrane protein A receptor for invasion. *Cell Microbiol*. 2008; 10:2326–2338. [PubMed: 18662321]
13. Yang Y, Li Z. Roles of heat shock protein gp96 in the ER quality control: redundant or unique function? *Mol Cells*. 2005; 20:173–182. [PubMed: 16267390]
14. Wu S, Hong F, Gewirth D, Guo B, Liu B, Li Z. The molecular chaperone gp96/GRP94 interacts with Toll-like receptors and integrins via its C-terminal hydrophobic domain. *J Biol Chem*. 2012; 287:6735–6742. [PubMed: 22223641]
15. Altmeyer A, Maki RG, Feldweg AM, Heike M, Protopopov VP, Masur SK, Srivastava PK. Tumor-specific cell surface expression of the-KDEL containing, endoplasmic reticular heat shock protein gp96. *Int J Cancer*. 1996; 69:340–349. [PubMed: 8797880]
16. Prasadarao NV, Srivastava PK, Rudrabhatla RS, Kim KS, Huang SH, Sukumaran SK. Cloning and expression of the *Escherichia coli* K1 outer membrane protein A receptor, a gp96 homologue. *Infect Immun*. 2003; 71:1680–1688. [PubMed: 12654781]
17. Cabanes D, Sousa S, Cebria A, Lecuit M, Garcia-del Portillo F, Cossart P. Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface protein. *EMBO J*. 2005; 24:2827–2838. [PubMed: 16015374]
18. Na X, Kim H, Moyer MP, Pothoulakis C, LaMont JT. gp96 is a human colonocyte plasma membrane binding protein for *Clostridium difficile* toxin A. *Infect Immun*. 2008; 76:2862–2871. [PubMed: 18411291]
19. Rolhion N, Barnich N, Bringer MA, Glasser AL, Ranc J, Hebuterne X, et al. Abnormally expressed ER stress response chaperone Gp96 in CD favours adherent-invasive *Escherichia coli* invasion. *Gut*. 2010; 59:1355–1362. [PubMed: 20587550]
20. Valle J, Latasa C, Gil C, Toledo-Arana A, Solano C, Penades JR, et al. Bap, a biofilm matrix protein of *Staphylococcus aureus* prevents cellular internalization through binding to GP96 host receptor. *PLoS Pathog*. 2012; 8:e1002843. [PubMed: 22876182]

21. Martins M, Custodio R, Camejo A, Almeida MT, Cabanes D, Sousa S. *Listeria monocytogenes* triggers the cell surface expression of Gp96 protein and interacts with its N terminus to support cellular infection. *J Biol Chem.* 2012; 287:43083–43093. [PubMed: 23109341]
22. Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, Lefrancois L, et al. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity.* 2007; 26:215–226. [PubMed: 17275357]
23. Suriano R, Ghosh SK, Chaudhuri D, Mittelman A, Banerjee A, Tiwari RK. Sialic acid content of tissue-specific gp96 and its potential role in modulating gp96-macrophage interactions. *Glycobiology.* 2009; 19:1427–1435. [PubMed: 19578160]
24. Di Domenico F, Owen JB, Sultana R, Sowell RA, Perluigi M, Cini C, et al. The wheat germ agglutinin-fractionated proteome of subjects with Alzheimer's disease and mild cognitive impairment hippocampus and inferior parietal lobule: Implications for disease pathogenesis and progression. *J Neurosci Res.* 2010; 88:3566–3577. [PubMed: 20936705]
25. Prasadarao NV, Wass CA, Kim KS. Endothelial cell GlcNAc beta 1-4GlcNAc epitopes for outer membrane protein A enhance traversal of *Escherichia coli* across the blood-brain barrier. *Infect Immun.* 1996; 64:154–160. [PubMed: 8557333]
26. Datta D, Vaidehi N, Floriano WB, Kim KS, Prasadarao NV, Goddard WA 3rd. Interaction of *E. coli* outer-membrane protein A with sugars on the receptors of the brain microvascular endothelial cells. *Proteins.* 2003; 50:213–221. [PubMed: 12486715]
27. Senes A, Gerstein M, Engelman DM. Statistical analysis of amino acid patterns in transmembrane helices: the GxxxG motif occurs frequently and in association with beta-branched residues at neighboring positions. *J Mol Biol.* 2000; 296:921–936. [PubMed: 10677292]
28. Mellquist JL, Kasturi L, Spitalnik SL, Shakin-Eshleman SH. The amino acid following an asn-X-Ser/Thr sequon is an important determinant of N-linked core glycosylation efficiency. *Biochemistry.* 1998; 37:6833–6837. [PubMed: 9578569]
29. Wang Y, Wen ZG, Kim KS. Role of S fimbriae in *Escherichia coli* K1 binding to brain microvascular endothelial cells in vitro and penetration into the central nervous system in vivo. *Microb Pathog.* 2004; 37:287–293. [PubMed: 15619424]
30. Khan NA, Kim Y, Shin S, Kim KS. FimH-mediated *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *Cell Microbiol.* 2007; 9:169–178. [PubMed: 17222190]
31. Teng CH, Cai M, Shin S, Xie Y, Kim KJ, Khan NA, 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; 73:2923–2931. [PubMed: 15845498]
32. Kumar R, Stanley P. Transfection of a human gene that corrects the Lec1 glycosylation defect: evidence for transfer of the structural gene for N-acetylglucosaminyltransferase I. *Mol Cell Biol.* 1989; 9:5713–5717. [PubMed: 2531285]
33. Sukumaran SK, Prasadarao NV. *Escherichia coli* K1 invasion increases human brain microvascular endothelial cell monolayer permeability by disassembling vascular-endothelial cadherins at tight junctions. *J Infect Dis.* 2003; 188:1295–1309. [PubMed: 14593586]
34. Klemm P, Schembri MA. Bacterial adhesins: function and structure. *Int J Med Microbiol.* 2000; 290:27–35. [PubMed: 11043979]
35. Moissenet D, Salauze B, Clermont O, Bingen E, Arlet G, Denamur E, et al. Meningitis caused by *Escherichia coli* producing TEM-52 extended-spectrum beta-lactamase within an extensive outbreak in a neonatal ward: epidemiological investigation and characterization of the strain. *J Clin Microbiol.* 2010; 48:2459–2463. [PubMed: 20519482]
36. Rechner C, Kuhlewein C, Muller A, Schild H, Rudel T. Host glycoprotein Gp96 and scavenger receptor SREC interact with PorB of disseminating *Neisseria gonorrhoeae* in an epithelial invasion pathway. *Cell Host Microbe.* 2007; 2:393–403. [PubMed: 18078691]
37. Di Pasquale G, Kaludov N, Agbandje-McKenna M, Chiorini JA. BAAV transcytosis requires an interaction with beta-1-4 linked- glucosamine and gp96. *PLoS One.* 2010; 5:e9336. [PubMed: 20231878]
38. Suriano R, Ghosh SK, Ashok BT, Mittelman A, Chen Y, Banerjee A, et al. Differences in glycosylation patterns of heat shock protein, gp96: implications for prostate cancer prevention. *Cancer Res.* 2005; 65:6466–6475. [PubMed: 16024652]

39. Saukkonen KM, Nowicki B, Leinonen M. Role of type 1 and S fimbriae in the pathogenesis of *Escherichia coli* O18:K1 bacteremia and meningitis in the infant rat. *Infect Immun*. 1988; 56:892–897. [PubMed: 2894363]
40. Prasadarao NV, Wass CA, Hacker J, Jann K, Kim KS. Adhesion of S-fimbriated *Escherichia coli* to brain glycolipids mediated by *sfaA* gene-encoded protein of S-fimbriae. *J Biol Chem*. 1993; 268:10356–10363. [PubMed: 8098039]
41. Teng CH, Xie Y, Shin S, Di Cello F, Paul-Satyaseela M, Cai M, 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; 74:5609–5616. [PubMed: 16988236]
42. Pascal TA, Abrol R, Mittal R, Wang Y, Prasadarao NV, Goddard WA 3rd. Experimental validation of the predicted binding site of *Escherichia coli* K1 outer membrane protein A to human brain microvascular endothelial cells: identification of critical mutations that prevent *E. coli* meningitis. *J Biol Chem*. 2010; 285:37753–37761. [PubMed: 20851887]
43. Smith SG, Mahon V, Lambert MA, Fagan RP. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett*. 2007; 273:1–11. [PubMed: 17559395]

Fig. 1. Prediction and verification of N-glycosylation sites in Ecgp96, and their role in *E. coli* K1 invasion of HBMEC

Complete protein sequence of heat shock protein 90kDa beta (Grp94), member 1 [Homo sapiens] (GenBank: AAH66656.1) showing the signal sequence (underlined). Extracellular domain experimentally verified by Martins et al., is highlighted in grey. Putative N-glycosylation sites identified based on consensus sequence asn-X-Ser/Thr are highlighted in yellow. The GVVDS sequence highlighted in blue is hypothesized to be the putative transmembrane domain based on the GXXXG motif rule (A). A typical N-glycosylation pattern is depicted with various enzymatic cleavage sites (B). HBMEC grown to ~90 % confluence in 6 well plates, transfected with FLEcgp96 and the NG mutants and after 24 h, were infected with *E. coli* K1 for 1 h and subjected to flow cytometry using anti-Ecgp96 primary antibody followed by Alexa 488 secondary antibody (C). HBMEC grown to ~90 % confluence in 24 well plates were transfected with FLEcgp96 and the NG mutants, and subjected to total cell associated and intracellular bacteria determination in the absence (–) or presence (+) of anti-OmpA antibody. The results are presented as percent invasion (means ± SD) considering the total cell associated or intracellular bacteria of control HBMEC as 100%. Increased cell association/invasion of *E. coli* K1 in HBMEC/FLEcgp96 and HBMEC/NG0-FLEcgp96 as well as decreased cell association/invasion of *E. coli* K1 in HBMEC/NG1-FLEcgp96, HBMEC/NG2-FLEcgp96 and HBMEC/NG1/2-FLEcgp96 (*) and inhibition of cell association/invasion in the presence of anti-OmpA antibody (**) in control HBMEC/FLEcgp96 and HBMEC/NG0-FLEcgp96 was statistically significant, while inhibition of cell association/invasion in HBMEC/NG1-FLEcgp96, HBMEC/NG2-FLEcgp96 and HBMEC/NG1/2-FLEcgp96 was not significant (ns) (D). HBMEC were

cultured in 6 well plates and transfected as mentioned in panel B. The cells were then lysed 24 h post transfection, plasma membranes were prepared and 40 µg of membrane fractions were separated on a 4%–8% gradient SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with anti-Ecgp96 antibodies followed by HRP coupled secondary antibodies. Experiments in C, D, and E were performed at least three times independently.

Fig. 2. N-glycosylation of Ecgp96 in CHO cells is critical for *E. coli* K1 invasion

CHO cells were grown to ~90 % confluence in 24 well plates were transfected with FLEcgp96 and NG mutants, infected with *E. coli* K1 for 90 min and subjected to total cell association/invasion assays. Increased cell association/invasion of *E. coli* K1 in CHO/FLEcgp96 and CHO/NG0-FLEcgp96 as well as decreased cell association/invasion of *E. coli* K1 in CHO/NG1-FLEcgp96, CHO/NG2-FLEcgp96 and CHO/NG1/2-FLEcgp96 was statistically significant (* and ** respectively) (A). HBMEC grown to ~90 % confluence in 6 well plates, transfected with FLEcgp96 and the NG mutants and after 24 h, were infected with *E. coli* K1 for 15, 30, 60 and 90 min and subjected to flow cytometry using anti-Ecgp96 primary antibody and Alexa 488 secondary antibody. Overlay plot shown is a representative of 3 different experiments (B). CHO cells were grown to ~85% confluence and transfected with gp96 siRNA, allowed to recover for 24 h, and cell association/invasion assays were performed. The values represent percent means $\pm$ SD compared to control values taken as 100% from three different experiments. Reduction in cell association and invasion was statistically significant (* and ** respectively) (C). To verify knockdown efficiency of siRNA, CHO cells grown in 6 well plates were transfected with gp96 siRNA and subjected to flow cytometry using anti-Ecgp96 primary antibody and Alexa 488 secondary antibody. Overlay plot shown is a representative of three different experiments (D).

Fig. 3. Lack of mannose residues in N-glycans in HBMEC and CHO cells did not affect the total cell association and invasion of *E. coli* K1

Confluent monolayers of HBMEC, CHO and CHO Lec1 cells were subjected to total cell associated and invasion assays with *E. coli* K1 (A). In separate experiments, the monolayers of HBMEC (B), CHO cells (C) or CHO-lec1 cells (D) were treated with different enzymes as described in Materials and Methods. Similarly, *E. coli* K1 was incubated with α -methyl mannopyranoside or chitin hydrolysate on ice for 1 h and then added to the confluent monolayers. The cells were then used for total cell associated and invasion assays. Untreated or β -mannosidase treated HBMEC/FLEcgp96 were subjected to cell association/invasion assays with *E. coli* K1 that was either untreated or pre-incubated with anti-OmpA antibody. Untransfected HBMEC served as a control. Increased cell association/invasion of *E. coli* K1 in untreated and β -mannosidase treated HBMEC/FLEcgp96 compared to control was statistically significant (*). Reduction in cell association/invasion of *E. coli* K1 treated with

anti-OmpA antibody was also statistically significant compared to untreated bacteria (**). The cells after treatment with β -mannosidase were stained with FITC-ConA and then analyzed by flow cytometry (F). In these experiments, isotype matched secondary antibody was used as a control. All the experiments in A–D and F were performed at least three times and the values are presented as % means $\pm$ SD compared to control values taken as 100%. Statistical significance is as indicated.

Fig. 4. Overexpression of Ecgp96 containing NG2 or NG1/2 mutations prevent cellular events required for *E. coli* K1 invasion in HBMEC

(A) HBMEC/FLEcgp96, HBMEC/NG2-FLEcgp96 and HBMEC/NG1/2-FLEcgp96 were infected with GFP+ *E. coli* K1 for 1 h. The monolayers were fixed and incubated with anti-Ecgp96 antibody followed by secondary antibody coupled to Alexa 647, permeabilized and then stained with Alexa 568-phalloidin to stain F-actin. Arrows indicate the location of *E. coli* K1 or F-actin polymerization. GFP and Ecgp96 expression were pseudo-colored (pale yellow and cyan respectively) to clearly distinguish color patterns and to eliminate color bleed-through. Scale = 10 μ m (B) HBMEC transfected with FLEcgp96 or various other constructs were infected with *E. coli* K1 for 15 min, total cell lysates prepared and then subjected to PepTag non-radioactive assay for PKC phosphorylation activity. (C) HBMEC transfected with various Ecgp96 constructs were seeded onto filter inserts and after becoming confluent, the monolayers were infected with *E. coli* K1 for varying periods. TER was measured using a Millicell apparatus (Millipore). The experiments were performed at three times in triplicate and the values represent means $\pm$ SD. FuGENE represents the transfection reagent control.

Control -Cortex/
Hippocampus

Infected -Cortex/
Hippocampus

Fig. 5. *E. coli* K1 induces the expression of glycosylated Ecgp96 in the brains of newborn pups
Three day old mice were infected with 10^3 CFU of *E. coli* K1 via intranasal route and after 72 h, the infected animals were sacrificed. Brains were removed and fixed in formalin. Tissues sections were made as described in the Materials and Methods and then stained with FITC-ConA and anti-Ecgp96 antibodies followed by Alexa 647 secondary antibodies. Arrows indicate the co-localization of FITC-ConA staining and Ecgp96 staining on the microvessels present in cortex and hippocampus. The experiments were performed with at least three different brain specimens from control and infected pups. Scale=100 μ m.

Table 1

List of primer sequences used for site-directed mutagenesis.

GP96NG0-F GP96NG0-R	GTTGGATGGATTAGCAGCAGCAGCAATAAGAGAACTTAG CTAAGTTCTCTTATTGCTGCTGCTAATCCATCCAAC
GP96NG1-F GP96NG1-R	CCTGAGAGAACTGATTTTCAGCAGCTTCTGATGCTTTAG CTAAAGCATCAGAAGCTGCTGAAATCAGTTCTCTCAGG
GP96NG2-F GP96NG2-R	CTTCAAAACACAACGCAGATACCCAGCACATCTGG CCAGATGTGCTGGGTATC7GCGTTGTGTTTGAAG