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Outer membrane protein A of *Escherichia coli* K1 selectively enhances the expression of intercellular adhesion molecule-1 in brain microvascular endothelial cells

Suresh K. Selvaraj^a, Parameswaran Periandythevar^a, and Nemani V. Prasadara^{a,b,*}

^a Division of Infectious Diseases, The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, CA 90027, USA

^b Keck School of Medicine, University of Southern California, Los Angeles, CA 90027, USA

Abstract

Escherichia coli K1 meningitis is a serious central nervous system disease with unchanged mortality and morbidity rates for last few decades. Intercellular adhesion molecule 1 (ICAM-1) is a cell adhesion molecule involved in leukocyte trafficking toward inflammatory stimuli at the vascular endothelium; however, the effect of *E. coli* invasion of endothelial cells on the expression of ICAM-1 is not known. We demonstrate here that *E. coli* K1 invasion of human brain microvascular endothelial cells (HBMEC) selectively up-regulates the expression of ICAM-1, which occurs only in HBMEC invaded by the bacteria. The interaction of outer membrane protein A (OmpA) of *E. coli* with its receptor, Ecgp, on HBMEC was critical for the up-regulation of ICAM-1 and was depend on PKC- α and PI3-kinase signaling. Of note, the *E. coli*-induced up-regulation of ICAM-1 was not due to the cytokines secreted by HBMEC upon bacterial infection. Activation of NF- κ B was required for *E. coli* mediated expression of ICAM-1, which was significantly inhibited by over-expressing the dominant negative forms of PKC- α and p85 subunit of PI3-kinase. The increased expression of ICAM-1 also enhanced the binding of THP-1 cells to HBMEC. Taken together, these data suggest that localized increase in ICAM-1 expression in HBMEC invaded by *E. coli* requires a novel interaction between OmpA and its receptor, Ecgp.

Keywords

Meningitis; Brain microvascular endothelial cells; Intercellular adhesion molecule-1; Cell adhesion molecules

1. Introduction

Intercellular adhesion molecule-1 (ICAM-1 or CD54), a member of the immunoglobulin supergene family, plays an important role in the immune and inflammatory response [1]. ICAM-1 is a 55-kDa protein that can be expressed on the cell surface or secreted extracellularly by many cell types [2]. The expression of ICAM-1 is strongly up-regulated by several inflammatory mediators, such as cytokines (interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and interferon- γ), oxidative stress, and sheer stress [3]. The primary role of ICAM-1 is the facilitation of the extravasation of leukocytes to migrate towards the sites of infection. In addition, ICAM-1 serves as a receptor for rhinoviruses and is up-regulated by a variety of microorganisms such as *Chlamydia pneumoniae*, *Mycobacterium tuberculosis*, and

* Corresponding author. Division of Infectious Diseases, MS #51, The Saban Research Institute, Children's Hospital Los Angeles, 4650 Sunset Boulevard, Los Angeles, CA 90027, USA. Tel.: +1 323 669 5465; fax: +1 323 660 2661. E-mail address: pnemani@chla.usc.edu (N.V. Prasadara).

Haemophilus influenzae [4–6]. Several studies have shown that transcription factor NF- κ B is the key regulator of ICAM-1 gene expression in endothelial cells [1]. NF- κ B comprises of a family of closely related transcription factors that play a key role in the expression of genes involved in inflammation and immune response [7]. NF- κ B is a dimeric transcription factor composed of homo- or heterodimers of the Rel family of proteins, of which p50/p65 is the most frequent dimer and hence considered the prototype. NF- κ B is usually present in an inactive form in the cytoplasm bound to a member of the I κ B family, generally I κ B- α . In this complex, I κ B blocks the nuclear translocation signal of NF- κ B, and the NF- κ B–I κ B complex needs to be disrupted for NF- κ B to become activated [8]. Specific phosphorylation of serine 32 and 36 on I κ B- α by I κ B kinase (IKK) triggers the disruption of NF- κ B–I κ B complex, which renders I κ B for ubiquitination and subsequent degradation by the proteosomal complex, thereby releasing NF- κ B from I κ B and allowing nuclear translocation [9–11].

Neonatal meningitis is a serious central nervous system disease and *Escherichia coli* K1, a gram-negative bacterium, is the leading cause of this disease [12]. A surge in *E. coli* K1 infections has been reported in recent years surpassing the infections caused by other meningitis associated pathogens such as Group B *Streptococcus* and *Listeria monocytogenes* [13]. The disease is fatal in 5–40% of infected neonates and cause neurological sequelae in up to 30% of survivors, which include deafness, cortical blindness, and mental retardation [12]. Incomplete understanding of the pathogenesis of the disease is attributed to the unchanged mortality and morbidity rates associated with neonatal meningitis for last few decades despite the availability of active antimicrobial agents. For example, the role of bacterial invasion of blood-brain barrier on the expression of cell adhesion molecules is not clearly known. Due to the presence of cytokines in the circulation, it has been speculated that ICAM-1 expression on brain microvascular endothelial cells (BMEC), which constitute the lining of the blood–brain barrier (BBB), might be up-regulated during these bacterial infection [14]. For most Gram-negative organisms, lipopolysaccharide (LPS) serves as a potent pro-inflammatory mediator. One target for LPS to stimulate inflammatory response is the vascular endothelium. LPS, both directly and through the induction of host cytokines, causes endothelial cells to up-regulate expression of adhesion molecules for the recruitment of leukocytes. Although cytokine induced expression of cell adhesion molecules has been well studied, the expression of these molecules and the induction of cytokine profile in HBMEC by *E. coli* infection is poorly understood.

Our studies have shown that the expression of outer membrane protein A (OmpA) of *E. coli* K1 is critical for the invasion of cultured human BMEC (HBMEC) [15]. Concurrent to this observation, OmpA+ *E. coli* traverses across the blood brain barrier very efficiently in our newborn rat model of hemogenous meningitis compared to OmpA– *E. coli* despite the presence of equal number of bacteria in the circulation [16]. Subsequently we identified that OmpA interacts with Ecgp, a gp96-like receptor molecule on the surface of HBMEC [17]. Of note, due to the selective expression of Ecgp on HBMEC but not on non-brain endothelial cells, the OmpA-mediated *E. coli* invasion is significantly greater in HBMEC when compared to the invasion of non-brain endothelial cells [17]. The OmpA-mediated entry of *E. coli* into HBMEC increases the activation of PKC- α , which subsequently interacts with caveolin-1, a protein present in caveolae, for the uptake of the bacteria [18,19]. Thus, overexpression of either dominant negative-PKC- α or -caveolin-1 significantly inhibited the entry of *E. coli* into HBMEC. The internalization of *E. coli* into HBMEC also induces the activation of FAK, PLC- γ , and PI3-kinase and over-expression of dominant negative forms of these molecules significantly blocked the invasion [20–22]. In contrast, over-expression of dominant negative-Src kinase had no effect on the invasion (unpublished results). In this study we sought to evaluate the effect OmpA+ *E. coli* entry of HBMEC on the expression of cell adhesion molecules. A better understanding of *E. coli* K1 pathogenesis may lead to novel treatment strategies, thereby significantly reducing the morbidity associated with this disease.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli K1 (serotype O18:K1:H7) strain, RS218, was isolated from the cerebrospinal fluid of a neonate with meningitis and invades HBMEC in a cell culture model [15]. OmpA[−] *E. coli* is a non-invasive mutant lacking the entire OmpA gene. pOmpA⁺ *E. coli* is an OmpA-complemented OmpA[−] *E. coli* and pOmpA[−] *E. coli* is a vector control. All bacteria were grown in brain heart infusion broth with appropriate antibiotics as necessary.

2.2. Antibodies and reagents

Antibodies to ICAM-1, VCAM-1 and β -actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-E-selectin antibody was obtained from Biodesign (Saco, ME). Antibodies to I κ B and phospho-I κ B were obtained from Cell Signaling (Beverly, MA). Cy-2- and Cy-3-conjugated secondary antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA). Normal goat serum and the Vectashield mounting medium with 4,6-diamino-phenylindole were obtained from Vector Laboratories Inc. (Burlingame, CA). Riboquant multiprobe ribosomal protection assay system was obtained from Pharmingen (San Diego, CA). Supersignal chemiluminescence reagent was obtained from Pierce (Rockford, IL). T4 polynucleotide kinase, TriZOL reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). Phorbol myristate acetate (PMA), poly dI-dC, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2.3. *E. coli* invasion assays and transfections

The isolation of HBMEC, maintenance of the cells, and the gentamycin protection assays were described previously [15]. Primary cells and cells transfected with SV-40 large T-antigen (THBMEC) were used between passages 12 and 16 for all experiments. Both primary and transfected HBMEC behave similarly in bacterial invasion assays [23,24]. Primary HBMEC were transfected with mammalian expression vectors using Lipofectamine 2000 according to the manufacturer's instructions. The dominant negative constructs of PKC- α , p85 and c-Src were previously described [18,22].

2.4. Semi-quantitative PCR analysis

The relative abundance of mRNA encoding ICAM-1, VCAM-1 and E-selectin were quantified by RT-PCR. The primers used were as follows: 5'-CAGTGACCATCTA CAGCTTTCCGG-3' and 5'-GCTGCTACCACAGTGAATGA TGACAA-3' for ICAM-1; 5'-GATACAACCGTCTTGGTCA GCCC-3' and 5'-CGCATCCTTCAACTGGCCTT-3' for VCA M-1; 5'-AGAAATATGTGGTTTCCACGATGA-3' and 5'-AA ACTGGAGATTGGTTTCCAATTG-3' for E-selectin.

2.5. Preparation of cell lysates, Western blot analysis, immunocytochemistry, and ELISA

Confluent HBMEC cultures in 100 mm dishes were incubated with either OmpA⁺ or OmpA[−] *E. coli* (MOI of 1:100, cell: bacteria ratio) for 1.5 h at 37 °C in experimental medium without antibiotics. The cells were then washed with RPMI and further incubated with experimental medium containing 20 μ g/ml of gentamycin for varying periods. After indicated periods of incubation, cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting. Immunocytochemistry of infected monolayers of HBMEC grown in eight-well chamber slides was performed by differential staining as previously described [18]. Briefly, HBMEC monolayers were incubated with OmpA⁺ or OmpA[−] *E. coli* for 1.5 h as described. The monolayers were then washed and further incubated in medium containing 30 μ g/ml gentamycin to prevent the growth of extracellular bacteria for 8 and 12 h. The eight-well chamber slides were washed with cold RPMI and incubated with anti-S-fimbria antibody for

1 h at 4 °C. The monolayers were further washed and incubated with excess amounts of secondary antibody coupled to horseradish peroxidase for 1 h. The chamber slides were washed, brought to room temperature, and fixed. The invaded OmpA+ *E. coli* were then stained with the same antibody after permeabilization of the monolayers with 1% Triton X-100, followed by secondary antibody coupled to Cy3. The slides were mounted in Vectashield anti-fade solution containing 4,6-diamidino-2-phenylindole. Cells were viewed with a Leica DMRA microscope (Wetzlar, Germany). The levels of E-selectin expression were assessed by ELISA using HBMEC plated in 96 well plates as described previously [25].

2.6. Enzyme mobility shift assay (EMSA) and ribonuclease protection assays (RPA)

Nuclear extracts were prepared from cells treated with bacteria for different periods and were analyzed by EMSA, essentially as described [26]. RPA analysis was performed using a Riboquant multiprobe system with the total RNA isolated from infected HBMEC. In brief, the isolated RNA (10 µg) was hybridized with ³²P-labeled probes overnight at 56 °C followed by RNase digestion, according to the manufacturer's instructions. After digestion, the protected fragments were resolved on a 5% denaturing polyacrylamide gel, dried and later exposed to X-ray film. The intensity of bands corresponding to TNF-α, MCP-1, IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was analyzed using ImageJ software.

2.7. THP-1 binding assay

Binding of THP-1 monocytes to activated endothelial cells was performed as described previously [27]. HBMEC in eight-well chamber slides (Nunc Inc., Naperville, IL) were treated with *E. coli* for 12 h, washed and 500 µl fresh experimental media containing 2×10^5 THP-1 monocytes expressing GFP were added to each well. After 30 min incubation at room temperature with continuous rocking to allow binding, the top portion of the chambers and the gasket were removed. The slide was then dipped twice in HEPES-buffered saline to remove unbound cells and mounted. Slides were visualized under both fluorescence and light microscope and the average number of cells bound in 10 fields in triplicate wells was determined.

2.8. Determination of cytokine levels

HBMEC in 24-well culture plates were infected with bacteria for different periods and the supernatants were collected, centrifuged to remove bacteria, and then stored at -80 °C until further use. Levels of TNF-α and IL-8 in the clarified supernatants were assayed using a specific DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

3. Results

3.1. Selective up-regulation of ICAM-1 expression in HBMEC infected with OmpA+ *E. coli*

The endothelium expresses multiple leukocyte adhesion molecules when activated by inflammatory mediators [14]. This phenomenon promotes transmigration of leukocytes, which in conjunction with other host mediators cause neuronal death. However, it is not clear whether the bacteria themselves can induce the expression of cell adhesion molecules on endothelial cells. Therefore, the effect of *E. coli* invasion of HBMEC on the expression of ICAM-1, VCAM-1, and E-selectin was examined. The total cell lysates of HBMEC monolayers infected with either OmpA+ or OmpA- *E. coli* were subjected to Western blotting using anti-ICAM-1 antibody. HBMEC infected with OmpA+ *E. coli* showed an increase in ICAM-1 expression 4 h post-infection with a peak at 8 h well above background (Fig. 1A). OmpA- *E. coli* infected cells also showed a slight increase in the expression of ICAM-1 at 4 h when compared to control but without further increase at later time points. PMA, which robustly induced the expression

of ICAM-1 at all time points of incubation, was used as a positive control. Densitometric analysis of the ICAM-1 bands normalized to β -actin from three individual experiments showed that ICAM-1 expression induced by OmpA+ *E. coli* was ten-fold higher than control cells and two fold that of induced by OmpA- *E. coli* (Fig. 1B, $P < 0.05$ by Student's *t*-test). VCAM-1 expression was also up-regulated, however, similarly by both OmpA+ and OmpA- *E. coli* (Fig. 1C). Of note, PMA did not cause up-regulation of VCAM-1 in HBMEC beyond the control levels. The expression of another cell adhesion molecule, E-selectin, was assessed by cell-based ELISA as the anti-E-selectin antibody does not recognize the denatured molecule. E-selectin expression in cells inoculated with either OmpA+ or OmpA- *E. coli* showed no significant differences at any time point (Fig. 1D). PMA also showed similar increase in the expression of E-selectin.

We have previously demonstrated that inhibitors of actin polymerization, PKC- α , or PI3-kinase significantly reduced *E. coli* invasion of HBMEC [18,22]. Therefore, to examine whether invasion of HBMEC by *E. coli* is necessary for the up-regulation of ICAM-1, confluent monolayers of HBMEC were treated with cytochalasin D (an actin polymerization inhibitor) or a cell permeable PKC- α inhibitory peptide or Wortmannin (an inhibitor of PI3-kinase) prior to performing invasion assays. An inhibitor of Src kinase, PP1, which had no effect on the invasion of *E. coli*, was also used as a control. Interestingly, inhibition of invasion by cytochalasin D or PKC- α inhibitory peptide or Wortmannin significantly reduced the up-regulation of ICAM-1 by OmpA+ *E. coli*, whereas PP1 did not show any effect (Fig. 1E), suggesting that the invasion of *E. coli* is important for increased expression of ICAM-1. Our previous studies also demonstrated that OmpA+ *E. coli* invades only a certain population of HBMEC [17]. To examine whether the ICAM-1 expression occurs only in those cells infected by the bacteria, immunocytochemistry of infected HBMEC was carried out. The intracellular bacteria were stained in the experiments infected with OmpA+ *E. coli*, whereas total cell-associated bacteria were stained in OmpA- *E. coli* infected HBMEC (since no invasion occurs with OmpA- *E. coli*) with anti-S-fimbria antibody by differential staining. As shown in Fig. 2, only HBMEC infected with OmpA+ *E. coli* expressed higher levels of ICAM-1 when compared to the non-infected surrounding HBMEC (Fig. 2C,D), whereas ICAM-1 immunostaining was slightly increased in HBMEC infected with OmpA- *E. coli* (Fig. 2E,F). These data indicate that OmpA+ *E. coli* selectively up-regulates the expression of ICAM-1 in HBMEC only invaded by the bacteria and significantly greater than OmpA- *E. coli* induced ICAM-1 expression.

3.2. OmpA expression in *E. coli* and its interaction with the HBMEC-specific gp96-like receptor, Ecgp, is critical for up-regulation of ICAM-1

Our previous studies have established that the expression of OmpA on the surface of *E. coli* K1 is one of the critical features to invade HBMEC by interacting with a specific receptor, Ecgp [17]. Therefore, first, to determine whether the presence of OmpA is necessary for ICAM-1 expression in HBMEC, OmpA- *E. coli* was complemented with a plasmid containing *ompA* gene. The strain, designated as pOmpA+ *E. coli*, expresses similar amounts of OmpA as the wild-type bacteria, as shown by dot-blot analysis [16]. Infection of HBMEC with pOmpA+ *E. coli* exhibited an increase in ICAM-1 expression similar to the levels observed in HBMEC infected with wild-type *E. coli* and two-fold greater than the pOmpA- *E. coli* infected cells (Fig. 3A,B). In contrast, the VCAM-1 expression is similar in HBMEC treated with either pOmpA+ or pOmpA- *E. coli*. Of note, invasion assays performed in the absence of serum also revealed up-regulation of ICAM-1 by OmpA+ *E. coli* suggesting that LPS may not be playing a major role (data not shown). Additionally, to demonstrate that OmpA interaction with Ecgp is critical, either the bacteria or HBMEC were pre-incubated with antibodies to OmpA or Ecgp, respectively for 1 h prior to infection. As expected, both antibodies reduced the up-regulation of ICAM-1 to background levels, whereas control isotype matched antibodies had no effect

(Fig. 3C). Pretreatment of HBMEC with anti-Ecgp antibody alone or in the presence of PMA did not induce the up-regulation of ICAM-1. These data suggest that the anti-Ecgp antibody is a blocking antibody and that the signaling pathways involved in OmpA-Ecgp mediated ICAM-1 expression might be different than those induced by PMA. Taken together, these results demonstrate that OmpA-Ecgp interaction is involved for the selective up-regulation of ICAM-1.

3.3. Cytokines produced by HBMEC upon infection with OmpA+ *E. coli* are not responsible for the up-regulation of ICAM-1

Cytokines have been shown to elicit multiple biological functions during the inflammatory response including ICAM-1 expression on endothelial cells [6,28]. Therefore, the cytokine and chemokine profile induced by *E. coli* infection of HBMEC was examined by RPA using RNA isolated from cells infected with *E. coli*. A commercially available riboquant probe set for the inflammatory genes TNF- α , IL-1 β , IL-6, MIP-1 β , IL-8, MCP-1, and RANTES was used. The experiments were carried out at least three times and a representative picture is shown in Fig. 3D. OmpA+ *E. coli* infected HBMEC showed a significant increase in the mRNA levels of TNF- α , MCP-1 and IL-8 over time when compared to the uninfected controls. No increase of other cytokines and chemokines was observed. Of note, no significant differences in the mRNA levels of cytokines and chemokines were observed between OmpA+ and OmpA- *E. coli*, as determined by densitometric analysis of the blot. The supernatants of *E. coli* infected HBMEC after different periods of infection were collected and subjected to a sandwich ELISA to estimate the amount of cytokines secreted (TNF- α and IL-8). OmpA+ *E. coli* induced increased production of TNF- α (15–45 pg/ml) with time when compared to uninfected controls. Similar quantities of TNF- α were also observed with OmpA- *E. coli*. Both OmpA+ and OmpA- *E. coli* induced the secretion of IL-8 with similar peak levels at 16 h (~400 pg/ml). Since we did not find any significant difference in the cytokine secretion profile between OmpA+ and OmpA- *E. coli*, it is assumed that the secretion of TNF- α and IL-8 by infected HBMEC does not contribute to the difference in ICAM-1 expression between the two strains.

3.4. OmpA+ *E. coli* induces ICAM-1 expression in HBMEC by activating NF- κ B

To examine the role of NF- κ B in *E. coli*-mediated increase in ICAM-1 expression, nuclear extracts were prepared from *E. coli* infected cells and were subjected to EMSA. As expected, OmpA+ *E. coli* induced a 2.5-fold increase in NF- κ B activity by two-hour post-infection when compared to untreated cells while OmpA- *E. coli* induced NF- κ B activity was 1.5-fold lower than that of the OmpA+ *E. coli* (Fig. 4A). To further characterize the NF- κ B subunits involved, we added antibodies against the p50 and p65 NF- κ B subunits to the reaction mixture before mixing with the labeled probe. Addition of both antibodies resulted in super shift of the NF- κ B bands, indicating that both p50 and p65 subunits are involved (Fig. 4A).

Phosphorylation of inhibitory kappa B (I κ B) by I κ B-kinase results in its ubiquitination and subsequent degradation, and allowing the translocation of NF- κ B to the nucleus [11]. Hence, I κ B phosphorylation and degradation in the lysates of HBMEC infected with *E. coli* were also analyzed. Fig. 4B shows that I κ B phosphorylation increases in OmpA+ *E. coli* infected HBMEC, which correlated with greater I κ B degradation with time. In contrast, OmpA- *E. coli* infection did not result in major changes in either I κ B phosphorylation or degradation. Densitometric analysis of phospho-I κ B and I κ B bands on the blots revealed a two-fold increase in the phosphorylation and degradation of I κ B upon incubation of HBMEC with OmpA+ *E. coli* (Fig. 4C). To determine the upstream signaling components in OmpA+ *E. coli* induced NF- κ B activation, we analyzed ICAM-1 protein expression in HBMEC transiently transfected with dominant negative NIK, IKK- α and IKK- β expressing plasmids. The transfected cells were treated with *E. coli* for different periods and the lysates were subjected to Western blotting for ICAM-1. As shown in Fig. 4D, the expression of ICAM-1 was significantly reduced in

HBMEC expressing dominant negative (DN) IKK- β . In contrast, neither DN IKK- α nor DN NIK showed any effect on the expression of ICAM-1 induced by OmpA+ *E. coli*, suggesting that IKK- β plays a major role in this process. Since equal number of OmpA+ or OmpA- *E. coli* was associated with the cells tested, the differences observed in NF- κ B or I κ B activation is not due to unequal binding of bacteria. Altogether, these experiments further indicate that NF- κ B plays a role in *E. coli*-induced ICAM-1 expression in HBMEC.

3.5. PKC- α and PI3-K dependent but Src-independent induction of ICAM-1 expression by OmpA+ *E. coli*

Our previous studies have demonstrated that OmpA+ *E. coli* invasion requires the activation of PKC- α and PI3-K, which are downstream of Ecgp interaction with OmpA [18,22]. Therefore, to examine whether PKC- α and/or PI3-K are required for ICAM-1 expression upon infection with the bacteria, HBMEC were transfected with dominant negative constructs of PKC- α and the p85 subunit of PI3-K. Both PKC- α and PI3-K are shown to be necessary for *E. coli* invasion of HBMEC [10,14]. In addition, HBMEC were also transfected with kinase-dead Src dominant negative construct. Both DN-PKC- α and DN-PI3-K/HBMEC showed substantial inhibition of ICAM-1 expression when infected with OmpA+ *E. coli* at all the time points (Fig. 5A). In contrast, DN-Src/HBMEC showed no such decrease in the ICAM-1 expression, suggesting that OmpA+ *E. coli* activates ICAM-1 expression via PKC- α and PI3-K pathways. To examine whether PKC- α or PI3-K inhibition results in inhibition of NF- κ B activity induced by *E. coli*, EMSA were performed with nuclear extracts from DN-transfected HBMEC. The densities of NF- κ B bands were determined from three independent experiments and expressed in a graph. As seen in Fig. 5B, no increase in NF- κ B activity was observed in both DN-PKC- α /HBMEC and DN-p85/HBMEC infected with OmpA+ *E. coli*. However, in DN-Src/HBMEC, substantial increase in NF- κ B activity was detected. These results indicate that PKC- α and PI3-K-dependent pathways are involved in NF- κ B activation in OmpA+ *E. coli* infected HBMEC.

3.6. *E. coli*-induced ICAM-1 expression results in increased adhesion of THP-1 monocytes

To investigate whether *E. coli* induced ICAM-1 expression had an effect on the adhesive properties of HBMEC, we performed adhesion experiments using the monocytic THP-1 cells transfected with GFP plasmid. HBMEC were treated with *E. coli* for 8 h before incubation with GFP-expressing THP-1 cells for 30 min. Non-adherent cells were removed by washing, mounted in DAPI, and GFP-fluorescent cells were counted. The binding of THP-1 to OmpA+ *E. coli* infected HBMEC was significantly greater than the control and OmpA- *E. coli* infected cells (Fig. 6A,B, $P < 0.01$ by Student's *t*-test). The increased binding of THP-1 cells is not due to increased association of OmpA+ *E. coli* with HBMEC as our previous studies showed that overexpression of ICAM-1 had no effect on the adherence of the bacteria to HBMEC [17]. In addition, THP-1 adherence to HBMEC transfected with DN-PKC- α and DN-PI3-K constructs was reduced by 50% and 70%, respectively ($P < 0.05$, Fig. 6C). These results are in agreement with the ICAM-1 expression data and further substantiate the role of PKC- α and PI3-K in the expression of ICAM-1 induced by *E. coli*. They also suggest the biological relevance of *E. coli* induced ICAM-1 expression in recruiting monocytes during HBMEC infection.

4. Discussion

An increasing body of evidence suggests that bacterial invasion of endothelial cells can initiate profound alterations in their functions. For example, several pathogens known to invade the central nervous system (CNS), including *Listeria* and *Borrelia*, have been shown to up-regulate the expression of adhesion molecules in HUVEC and to stimulate leukocyte adhesion [29, 30]. For *E. coli* K1, we have previously shown that it is far more efficient at invading HBMEC

than HUVEC, mainly because of the critical interaction between OmpA and its receptor, Ecgp, which is expressed in HBMEC but not in HUVEC [17]. Furthermore, we demonstrated that the tight junctions of only those HBMEC invaded by OmpA+ *E. coli* were selectively disrupted but not HUVEC infected with the same bacteria [31]. In agreement with the *in vitro* data, leakage of injected horseradish peroxidase in newborn rats infected with OmpA+ *E. coli* was observed only in certain areas of the brain, suggesting that some parts of the brain are more affected (unpublished results). Therefore, we speculate that the invasion of *E. coli* in selective areas of the blood–brain barrier might increase the expression of cell adhesion molecules to which sites larger numbers of leukocytes would be recruited. The transmigration of leukocytes might contribute to the damage to these areas of brain such as hippocampal dentate gyrus and leakage of the blood–brain barrier.

Here, we demonstrate that OmpA+ *E. coli* invasion of HBMEC selectively up-regulates the expression of ICAM-1, but not other cell adhesion molecules tested, VCAM-1 or E-selectin. Studies on *F. tularensis* LVS [32] reveal a similar selective expression of ICAM-1 and VCAM-1 without affecting E-selectin, whereas periodontopathic bacteria, *Porphyromonas gingivalis* in cultured HEP-2 epithelial cells induces the expression of ICAM-1 but not VCAM-1 [33]. The LPS present on Gram-negative bacteria has been shown to be a major factor in inducing cell adhesion molecule expression in endothelial cells. However, OmpA– *E. coli* despite the presence of LPS could not induce ICAM-1 expression parallel to the levels induced by OmpA+ *E. coli* regardless of similar binding to HBMEC, indicating that invasion of *E. coli* is critical for selective expression of ICAM-1. Of note, the enhanced expression of ICAM-1 induced by OmpA+ *E. coli* either in the absence or presence of serum in the incubation medium, implying that LPS plays a very minimal role (data not shown). Other *E. coli* K1 proteins besides OmpA have been shown to play a role in the invasion of bacteria into HBMEC, such as IbeA, IbeB, and CNF, although neither their cognate receptors nor modes of action are fully characterized yet [34]. Nonetheless, antibodies to OmpA or Ecgp significantly inhibited the OmpA+ *E. coli* induced expression of ICAM-1, demonstrating the requirement of OmpA–Ecgp interaction for the selective expression of ICAM-1. These findings indicate a novel bacterial ligand-HBMEC receptor interaction for the up-regulation of ICAM-1 expression.

Several pathogens also have been shown to induce expression of pro-inflammatory cytokines in endothelial cells, which subsequently contribute to the up-regulation of ICAM-1, VCAM-1 and E-selectin [35]. Our data suggest that both OmpA+ and OmpA– *E. coli* K1 induce the production of similar levels of cytokines. Thus, pro-inflammatory cytokines secreted by HBMEC infected with the bacteria play an insignificant role in enhanced expression of ICAM-1, at least in culture and OmpA has a marginal role in inducing cytokine production. The expression of ICAM-1 occurs only in HBMEC infected with OmpA+ *E. coli* but not in all cells further suggesting that the released cytokines might be playing no role in increased ICAM-1 expression. NF- κ B elements within the proximal ICAM-1 promoter region mediate the increased expression of ICAM-1 in HUVEC and epithelial cells exposed to cytokines (TNF- α and IL-1 β) [36]. Similarly, our results demonstrate that OmpA+ *E. coli* induced the activation of NF- κ B when compared to OmpA– *E. coli* for up-regulation of ICAM-1 expression. In the classical or canonical pathway, activation of the IKK complex leads to the phosphorylation at the serine or tyrosine residues of the IKK- β subunit, which in turn phosphorylates two N-terminal specific serines in IKK- β , thus targeting it for ubiquitination. Our studies with transiently transfected NIK, IKK- α and IKK- β dominant negative HBMEC revealed that activation of NF- κ B was reliant on IKK- β and not on NIK and IKK- α . Elewaut et al. showed that the inhibition of IKK- β alone was sufficient to block NF- κ B activity induced by enteroinvasive bacteria in intestinal epithelial cells [37]. Thus, there is precedence for a key role for IKK- β acting upstream of ICAM-1 expression initiated by bacterial invasion. We further demonstrated using dominant negative constructs, that PKC- α and PI3-kinase participate in the signaling pathways leading to OmpA+ *E. coli*-induced up-regulation of

ICAM-1 expression while Src-kinase does not. In contrast, PMA induced activation of ICAM-1 was blocked by dominant negative constructs of PKC- α and Src-kinase (data not shown). Previous studies have reported that both TNF- α and interferon- γ mediates ICAM-1 up-regulation, which is dependent on PKC- α and Src-kinase activity [28,38]. In contrast, OmpA–Ecgp interaction, which is required for invasion of HBMEC, appears to trigger specific signaling pathways leading to up-regulation of ICAM-1 and therefore may differ from the signaling events induced by PMA and cytokines.

There are two salient features of this study. First, the invasion of *E. coli* into HBMEC is a requisite for selective up-regulation of ICAM-1 expression. Second, there is a requirement for PKC- α and PI3-kinase signaling, which are stimulated proximally to Ecgp after interacting with OmpA for up-regulation of ICAM-1 expression. Other intermediate signaling molecules that relay the signals for the up-regulation of ICAM-1 are not clearly known at present; however, they appear to be distinct from that of either TNF- α or PMA-induced activation of ICAM-1. Our present data suggest that selective enhanced expression of ICAM-1 in HBMEC invaded by *E. coli* could behave as “hot spots” for the recruitment of greater number of leukocytes, which in turn cause damage to the brain. In addition, the cumulative effect of leukocyte-induced and *E. coli*-induced blood–brain barrier disruption might also be responsible for the damage of selective areas in the brain. Therefore, prevention of *E. coli* K1 invasion-induced recruitment of leukocytes seemed partially important for the control of the morbidity associated with neonatal meningitis.

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Abbreviations

OmpA	outer membrane protein A
HBMEC	human brain microvascular endothelial cells
Ecgp	endothelial cell glycoprotein
PKC-α	protein kinase C- α
PI3-K	phosphatidylinositol 3-kinase
NF-κB	nuclear factor- κ B

Fig 1.

Increased expression of ICAM-1 in HBMEC infected with OmpA+ *E. coli*. Confluent HBMEC monolayers were treated with either OmpA+ or OmpA- *E. coli* or PMA for indicated periods. Total cell lysates were prepared, separated by SDS polyacrylamide gel electrophoresis and immunoblotted with anti-ICAM-1 (A) or anti-VCAM-1 (C) antibodies. The lower portion of the blot was used to probe with anti-β-actin antibody. The bands on the blot were scanned, normalized to actin, and plotted for ICAM-1 (B). In separate experiments, HBMEC monolayers in 96-well plates were incubated with either bacteria or PMA. E-selectin expression was measured using an ELISA kit (D). For inhibition studies, HBMEC were pre-treated with cytochalasin D (CytD) or a cell permeable peptide inhibitor for PKC-α (PKC-α-IP), or Wortmannin or PP1 for 1 h prior to performing the invasion assays. The total cell lysates were then subjected to immunoblotting with anti-ICAM-1 or β-actin antibody (E). All experiments were performed at least three times and the values represent the mean ± SD. The densities of ICAM-1 bands in OmpA+ *E. coli* infected cell lysates were considered significant when compared to OmpA- *E. coli* infected cell lysates. * $P < 0.05$ by Student's *t*-test.

Fig 2.

Immunocytochemistry of ICAM-1 expression on HBMEC infected with *E. coli*. Confluent monolayers of HBMEC were either uninfected (A, B), infected with OmpA+ *E. coli* (C, D), or OmpA- *E. coli* (E, F) and then subjected to differential staining as described in Section 2 using anti-ICAM-1 antibodies followed by Cy2 conjugated secondary antibody (green). The invaded OmpA+ *E. coli* were stained with anti-S-fimbria antibody followed by Cy3 conjugated secondary antibody, which were indicated by arrows (C, D). For OmpA- *E. coli*, the total cell associated bacteria were stained with anti-S-fimbria antibody (E, F).

Fig 3.

OmpA interaction with its cellular receptor, Ecgp, is involved for *E. coli* K1 induced ICAM-1 expression. (A) HBMEC were infected with OmpA+ *E. coli*, OmpA- *E. coli*, OmpA- *E. coli* transformed with a plasmid containing *ompA* gene (pOmpA+) or PMA for 8 h. Total cell lysates were prepared and analyzed by Western blotting with antibodies to ICAM-1 or VCAM-1 or β-actin. (B) Densities of ICAM-1 and VCAM-1 bands on the blot, normalized to actin, are shown. (C) In some experiments, cells were pre-incubated with antibodies to Ecgp or *E. coli* was pre-incubated with anti-OmpA antibodies before infection. Cell lysates were then analyzed by Western blotting for ICAM-1 expression. A non-specific antibody was used as a control. (D) HBMEC were infected with *E. coli* or pOmpA+ *E. coli* for 1, 2 and 4 h. RNA was isolated from the cells and then subjected to ribosomal protection assays for the indicated cytokines and chemokines as described in Section 2. The figure is a representative of three individual experiments.

Fig 4.

OmpA+ *E. coli* induces ICAM-1 expression in HBMEC by activating NF-κB. (A) HBMEC were infected with *E. coli* for different time periods, nuclear extracts were prepared, and subjected to EMSA using radiolabeled probe for NF-κB. In certain experiments, the nuclear extracts were pre-incubated with antibodies to p50 and p65 subunits of NF-κB before the addition of the labeled probe. Increase in NF-κB activity is indicated as -fold increase at each time point when compared to control. (B) Phosphorylation and degradation of IkB-α was assessed in cells treated with *E. coli* for different time periods by immunoblotting the total cell lysates with anti-phospho-IkB and anti-IkB antibodies. β-Actin was used as a loading control. (C) The data represent densities of phospho-IkB and IkB bands on the blot normalized to β-actin. (D) The endothelial cells at 30% confluence were transiently transfected with dominant negative plasmids of NIK, IKK-α, and IKK-β. The cells were then infected with *E. coli*, total cell lysates prepared, and were then subjected to immunoblotting with anti-ICAM-1 antibody. β-Actin was used as a loading control.

Fig 5. PKC- α and P13-K but not Src-kinase signaling is required for the induction of ICAM-1 expression by OmpA+ *E. coli*. (A) HBMEC were transfected with dominant negative constructs of PKC- α , p85 subunit of PI3-K, and a kinase dead Src in pcDNA3 vectors containing the neomycin resistance gene. As a control, cells were transfected with an empty vector. Cells were infected with *E. coli* and the total cell lysates analyzed by Western blotting for ICAM-1 expression. (B) EMSA were performed using HBMEC transfectants after infection with OmpA+ *E. coli* for varying periods, the bands were scanned and graphed. The experiments were performed three times and the error bars represent means $\pm$ SD. The NF- κ B activation was significantly lower than the activation in DN-Src/HBMEC, * $P < 0.05$ by Student's *t*-test.

Fig 6.

E. coli induced ICAM-1 expression results in increased adhesion of THP-1 monocytes. (A) Non-transfected HBMEC were plated in eight-well chamber slides. After reaching confluence, the cells were treated with *E. coli* as described in Section 2. The cells were washed and 2×10^5 THP-1 monocytes expressing GFP were added to each well and allowed to bind for 30 min. After the incubation period, unbound cells were washed, mounted in Vectashield, and the bound cells were visualized under a fluorescence microscope. (B) The average number of cells bound in 10 fields in triplicate wells was determined and the results from three independent experiments were plotted. * $P < 0.01$ by Student's *t*-test. (C) HBMEC transfected with dominant negative mutants of PKC- α , p85 subunit of PI3-kinase or Src-kinase were plated in eight-well chamber slides and THP-1 adhesion assays were carried out as described in (A).