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TESI DI DOTTORATO

**Molecular Mechanisms Involved in *Escherichia coli* K1 and
Haemophilus influenzae Type a Blood-Brain Barrier impairment**

Dott.ssa Nunzia Caporarello

COORDINATORE

Prof. Massimo Libra

TUTOR

Prof.ssa Gabriella Lupo

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To my niece Aurora

“Se il seme non si lascia aprire da sole, terra, acqua, accogliendo il suo destino, rimane sterile. Se invece trova la ragione per rompere il guscio, si lascia ferire ed entra nel mondo con la sua fioritura e si sperimenta come dono di colori e sapori per gli altri.

Il prezzo da pagare è un dolore, una morte “apparente”, ma in realtà è “più vita””.

(Alessandro D’Avenia)

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ABBREVIATIONS

BBB, Blood-Brain Barrier;

CNS, Central Nervous System;

CSF, Cerebrospinal Fluid;

NVU, Neurovascular Unit;

AJs, Adherens Junctions;

TJs, Tight Junctions;

JAMs, Junctional Adhesion Molecules;

ZO, Zonula Occludens;

TGF- β , Transforming Growth Factor β ;

E.coli, Escherichia coli K1;

EC, Endothelial cells;

PC, Pericytes;

PLA₂, Phospholipase A₂;

VEGF, Vascular Endothelial Growth Factor;

VEGFR, Vascular Endothelial Growth Factor Receptor;

PG, Prostaglandin

BBEC, Bovine Brain Endothelial Cells

BRPC, Bovine, Retina Pericyte Cells

TEER, Transendothelial Electric Resistance

Hia, *Haemophilus influenzae* Type a

BACKGROUND: first study

The Blood-Brain Barrier

The Central Nervous System (CNS), brain and spinal cord, controls the body's response to different stimuli, both internal and external. The physiological functions of CNS are maintained by a fine regulation of its homeostasis thanks to the blood brain barrier (BBB), located at the level of the cerebral microvasculature. In the adult human brain, the total length of capillaries is about 400 miles with a surface area for exchange of 12-18 m² (Abbott *et al*, 2010). The BBB acts as a protective barrier against possible neurotoxic molecules, such as proteins or endogenous metabolites, xenobiotics derived from the environment or ingested with the food. At the same time, BBB selectively allows the cross of ions and nutrients from blood into the CNS by active transport.

History of the BBB

At the end of the 19th century, during an experiment performed administering intravenously water soluble dyes, the German immunologist Paul Ehrlich observed that all tissue outside CNS were stained, whereas brain and cerebrospinal fluid (CSF) had no color (Ehrlich, 1885). At first, this result was related to a lack of chemical affinity for the dyes specific of the brain tissues. Meanwhile, Bield and Kraus noted that, when injected intravenously, both sodium ferro-cyanide and cholic acids had no pharmacological effects on the CNS but, when they were injected intraventricularly, the neurological complications occurred (Ribatti *et al*, 2006) thus providing evidences of the existence of a barrier between CNS and peripheral tissues. In 1890, Max Lewandowsky introduced the term "Blood-Brain Barrier" for the first time. After a few decades, Paul Ehrlich's student Edwin Goldman performed experiments in rabbits and dogs by using trypan blue dye that later clarify Ehrlich's result. Goldman observed that, when administered intravenously, trypan blue was able to stain all tissues throughout the body, except the brain and the spinal cord. Surprisingly, injecting

trypan blue directly into the CSF, through subarachnoid space, the nervous tissue was stained while the peripheral tissues was not (Goldman, 1909). Several decades later, Friedemann performed experiments by using lipid soluble dye and observed that it was able to cross the cerebral microvasculature, staining the brain. The anatomical features of BBB was established only with the advent of electron microscopy, in 1967. By using electron-sense tracer horseradish peroxidase (HRP), Reese and Karnovsky demonstrated that endothelial cells in mouse cerebral capillaries was less permeable to HRP in comparison with endothelial cells arising from heart or skeletal muscle (Reese and Karnovsky, 1967), confirming a structural difference in the junctions between cerebral endothelial cells. Now, we know that the BBB is located in the cerebral microvasculature and that it is characterized by controlled transport across, presence of intercellular tight junctions and a lack of fenestration.

Physiology of the BBB

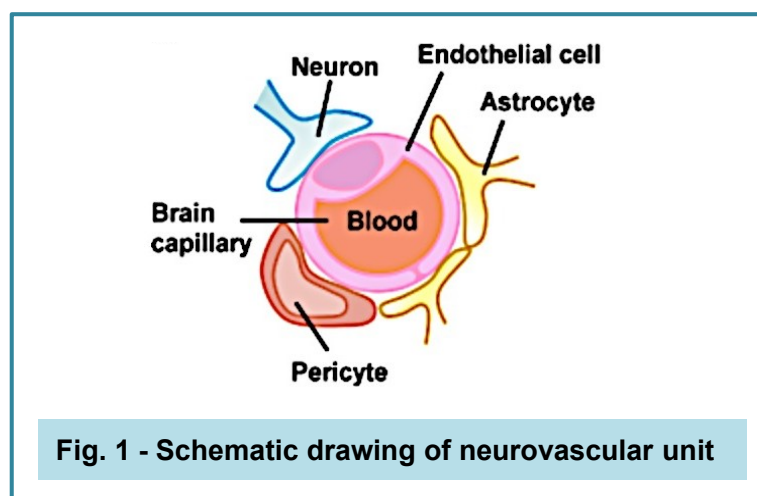
The BBB acts as a filter against all molecules except for small lipophilic molecules with a molecular weight of 400-600 Da (Pardridge, 2001). Nevertheless, it is possible for certain macromolecules to cross the BBB thanks to some receptor-mediated systems, for example GLUT-1 glucose transporter, transferrin receptor, L1 amino acid carrier (Abbott *et al*, 2006).

TJs of the BBB restrict paracellular diffusion of many substances from the blood while lipophilic molecules are able to enter the brain by passively diffusing across the endothelial cell membranes and could be neurotoxic. In order to avoid the crossing of lipophilic xenobiotics, the BBB expresses high levels of ATP-binding cassette (ABC) transporters to pump indeliderate molecules against their concentration gradients utilizing ATP as energy. The most important ABC transporter is P-glycoprotein (Pgp). Although Pgp is expressed both luminally and abluminally, higher levels are expressed on the luminal side (Ronaldson *et al*, 2007). Organic cations, weak organic bases with hydrophobic regions and many commonly prescribed drugs represent substrates for Pgp (Miller, 2010).

Besides the ABC transporters, there are also different enzymes highly expressed at the BBB plasma membrane that act as a metabolic barrier to detoxify endogenous and exogenous molecules. Examples are cytochrome P450, aminopeptidases, endopeptidases and cholinesterase (Dauchy *et al*, 2008).

The Neurovascular Unit

In the brain, the microvessels are phenotypically different from those distributed in the other districts. But how brain microvessels acquire their barrier properties? In 1980s, Stewart and Wiley performed two sets of experiments: they transplanted embryonic brain fragments into the coelomic cavity, in order to expose them to non neural vessels and fragments of the embryonic mesoderm into the brain, in order to expose them to neural vessels (Stewart and Wiley, 1981). They founded that, when vascularizing into the neural *milieu*, the abdominal vessels acquired the characteristic features of the BBB, while the brain vessels insered into the non neural *milieu* were lacking of these features, confirming that the factors present in the neural environment regulate BBB differentiation. BBB is the main component of the Neurovascular Unit (NVU), made of brain microvessels, pericytes, microglia, astrocytes endfeet and neuronal process (Figure 1, Salmeri *et al.*, 2013). All these type of cells are closely connected in order to induce and maintain the physiological function of the BBB (Abbott et al, 2010).



Endothelial cells

Brain endothelium is the first cell type between the blood and the brain and is responsible to bring nutrients to the brain and to avoid the cross of neurotoxic molecules. Each neuron in the human brain has its own capillary (Zlokovic, 2008). Brain endothelial cells are different from peripheric endothelial cells: in fact, they lack fenestrations, present high mitochondrial content to provide energy to support active transport, minimal pinocytotic vesicles and are characterized by the presence of tight interendothelial junctions (Hawkins and Davis, 2005), which confer them a permeability significantly lower than that of peripheric endothelial cells (Tuma and Hubbard, 2003).

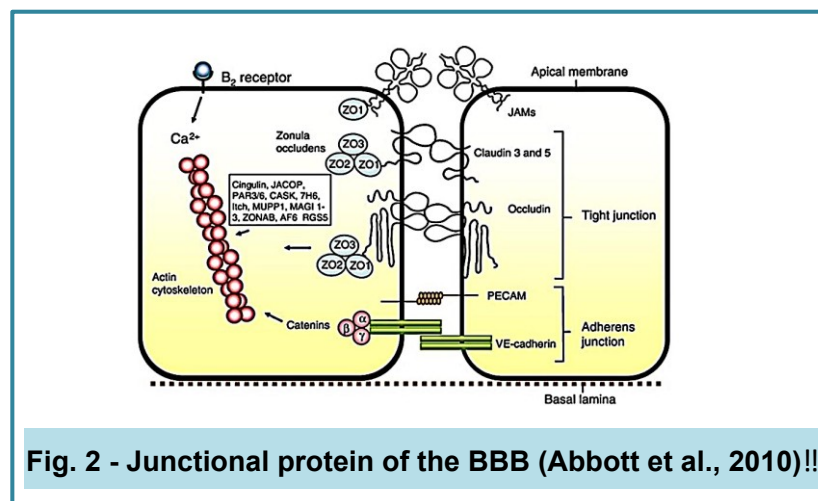
Moreover, brain vasculature is able to increase its diameter *in loco* in order to supply nutrients when neuronal metabolic demand increase, and this contractile ability is related, at least in part, to presence of pericytes, contractile cells wich also secrete various vasoactive mediators (Hamilton *et al*, 2010).

Tight interendothelial junctions are made of adherens junctions (AJ) and tight junctions (TJ) proteins. AJs proteins (such as VE-cadherin and PECAM) give the structural support to the tissue and are critical for tight junctions formation (Abbott *et al*, 2010). TJs include occludin (Hirase *et al*, 1997), claudins (Ohtsuki *et al*, 2007) and junctional adhesion molecules (JAMs) (Jia *et al*, 2013). Occludin was the first TJ protein discovered: it is a 522 amminoacids polypeptide with a molecular mass of 59.1 kDa. Occludin has N-terminal and C-terminal cytoplasmatic tails and 2 extracellular loops. N-terminal tail deletion is involved in loss of barrier property of TJs while C-terminal is important for barrier functions as well as for oligomerization of occludin (Chen *et al*, 1997) and for the interaction with regulatory proteins, such as zonula occludens 1, 2 and 3 (ZO-1, ZO-2 and ZO-3), responsible of anchoring occluding to the actin cytoskeleton (Feldman *et al*, 2005). Occludin has serin, threonine and tyrosine residues as regulatory site. An overall increase in occludin thyrosine phosphorylation lead to an increase in BBB permeability, while serine and threonine phosphorylation are associated with occludin localization within the membrane

(Takenaga *et al*, 2009). The human claudin family is made of 23 proteins with a molecular weight between 20-27 kDa and 4 transmembrane regions, 2 extracellular loops and N- and C-terminal cytoplasmic tails. Claudin-5, showing the highest expression levels of any claudin at the BBB (Lippoldt *et al*, 2000), is found in endothelial and epithelial cells of the choroid plexus.

Depending on the cell type, ZO proteins are located at TJs, adherens junctions (AJ), and/or gap junctions (Bauer *et al*, 2010). The first ZO protein discovered was ZO-1, a 220 kDa protein. (Stevenson *et al*. 1986). ZO-2, a 160 kDa protein which has a high sequence homology to ZO-1 (Gumbiner *et al*, 1991), has been less studied in BBB in comparison to ZO-1 but it is known that alterations in its expression and localization are associated with disruption of BBB (Hom *et al*, 2007). ZO-3 (130 kDa protein) is found at TJs level, but not in endothelial cells (Inoko *et al*, 2003). Junctional adhesion molecule 1 (JAM-1) is a 40 kDa protein which contain two extracellular immunoglobulin-like (Ig-like) loops, a short cytoplasmic tail, a single transmembrane segment, and homodimerizes (Severson *et al.*, 2009).

All these proteins play a pivotal role in the restrictive BBB features.



Pericytes

Pericytes were for the first time described by Rouget in 1879, and for this reason they were named “Rouget cells”. Due to their location on the outer surface of blood capillaries and their strict

interaction with the underlying endothelial cells, with which they share the basement membrane, in 1923 Zimmermann renamed them “Pericytes” (*peri*: around; *cyte*: cell) (Zimmermann, 1923). Pericytes cover between 22 and 37% of the cerebral capillary surface and present processes from their cell body that are in contact with more than one endothelial cell. The ratio endothelial cells/pericytes in the CNS is between 1:1 and 1:3 (Haddad-Tòvolli *et al*, 2017). Direct physical connection between pericytes and endothelial cells is provided by gap junctions, which enable the exchange of small molecules and ions. Pericytes are anchored to endothelial cells through adhesion plaques, while peg and socket contacts allow the cells to penetrate inside discontinuities in the basement membrane of vessels in contact with each other (Bergers *et al*, 2005). These junction complexes allow the transmission of mechanical contractile forces from pericytes to endothelial cells, and include cell-adhesion molecules, N-cadherin/ β -catenin based adherent junctions and extracellular matrix protein, e.g. fibronectin (Gerhardt *et al*, 2003).

The role of pericytes in maintaining a proper BBB function was discovered for the first time considering the increased ratio of pericytes to endothelial cells present in the CNS in comparison to other body regions (Díaz Flores *et al*, 2009).

Using transgenic mice PDGFR β knockdown (in order to downregulate pericyte generation), it has been shown that pericytes are necessary for the formation of the BBB (Daneman *et al.*, 2010) and that the vascular permeability is due to pericyte coverage of a capillary (Armulik *et al*, 2010).

In addition to the role on BBB development, pericytes are also involved in maintenance of the BBB during adult and age life: in fact, the number of pericytes decreases with aging while, at the same time, vascular permeability increases (Bell *et al*, 2010).

Microglia cells

Microglia are cells deputies to immune response within the CNS. In physiological conditions, microglia have small cellular bodies with long processes in direct contact with endothelial cells (Han and Suk, 2005). Under pathological conditions, microglia become activated, retract their long

processes and enlarge their cellular bodies. Once activated, microglia may secrete inflammatory cytokines and may become reactive, exhibiting phagocytic activity and acting as antigen presenting cells (Zlokovic, 2008).

Astrocytes

The most important function of perivascular astrocyte endfeet, wrapping endothelial cells, is to regulate water transport through aquaporin-4 (Tait *et al*, 2008). Astrocytes are able to induce barrier properties in endothelial cells both *in vivo* (Janzer *et al*, 1987) and *in vitro* (Rubin *et al*, 1991). Moreover, astrocytes secrete chemical mediators able to promote the BBB phenotype, such as basic fibroblast growth factor (bFGF), transforming growth factor β (TGF- β) and glial derived neurotrophic factor (GDNF). On the other hand, endothelial cells secrete mediators that promote growth and differentiation of astrocytes, thus underlying the close relation between these two type of cell (Mi *et al*, 2001).

Bacterial Meningitis

Bacterial Meningitis is a common and severe CNS infection, which is often fatal and over 50% of the survivors develop neurological complications, such as epilepsy, increased intra-cranial pressure, stroke and educational deficits in later life (Hoffman O *et al*, 2009). There are different pathogens associated with bacterial meningitis: *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Escherichia coli K1* and *Haemophilus influenzae type B*. The bacteria responsible of meningitis are often member of the human microbiome of gut, skin and mucous membranes of healthy humans (Cogen *et al.*, 2008) which can gain virulence factors lead to penetration of epithelial and endothelial barriers and invasive disease. Before to infect CNS, bacteria must survive and persist within the blood circulation because only the bacteria that survive in the bloodstream possess high penetration rate across the brain endothelium (Dando *et al.*,

2014). Through the years, bacterial meningitis has remained an infection with a high mortality rate, particularly in very young and elderly patients, despite advances in antimicrobial therapy (Kim, 2008). The reason for the poor outcome has been attributed to limited knowledge of pathogenesis and pathophysiology of the disease.

***Escherichia coli* K1**

Regarding *Escherichia coli*, more than 80% of the neuropathogenic strains express the K1 capsule. This capsule is made of homopolymer of α -2,8-linked N-acetylneuraminic acid. Although most cases of *Escherichia coli* meningitis occur via haematogenous spread, microbial and host factors responsible for the ability of neurotropic strains of *Escherichia coli* to cross the BBB are still missing. After infection, release of pro-inflammatory mediators such as cytokines and prostaglandins (PGs) by leucocytes, endothelial cells, astrocytes, microglial cells and other cells in the central nervous system was stimulated, leading to an increase in the permeability of the BBB (Engblom *et al.*, 2002; Zhu *et al.*, 2010). *E. coli* K1 invasion of brain EC is promoted by IQGAP1 (Ras GTPase-activating-like protein) (Krishnan *et al.*, 2012) and is responsible for TJs disruption.

Do the PC have a role in *Escherichia coli* crossing the BBB?

PCs are structural cells that helps to promote vascular integrity and their loss or dysfunction could play a critical role in the pathogenesis of meningitis.

PC are the cells closest brain EC, with which they share a common basement membrane, but they have not been investigated in a co-culture BBB model understanding the molecular mechanism of bacterial invasion. PC are able to secrete soluble factors, leading to the upregulation of BBB functions (Hori *et al.*, 2004; Dohgu *et al.*, 2005; 2011; Takata *et al.*, 2007; Nakagawa *et al.*, 2009). Recently, it has been reported that lipopolysaccharide-induced sepsis in mice is responsible for the detachment of brain PC from the basal lamina with disruption of the BBB (Nishioku *et al.*, 2009).

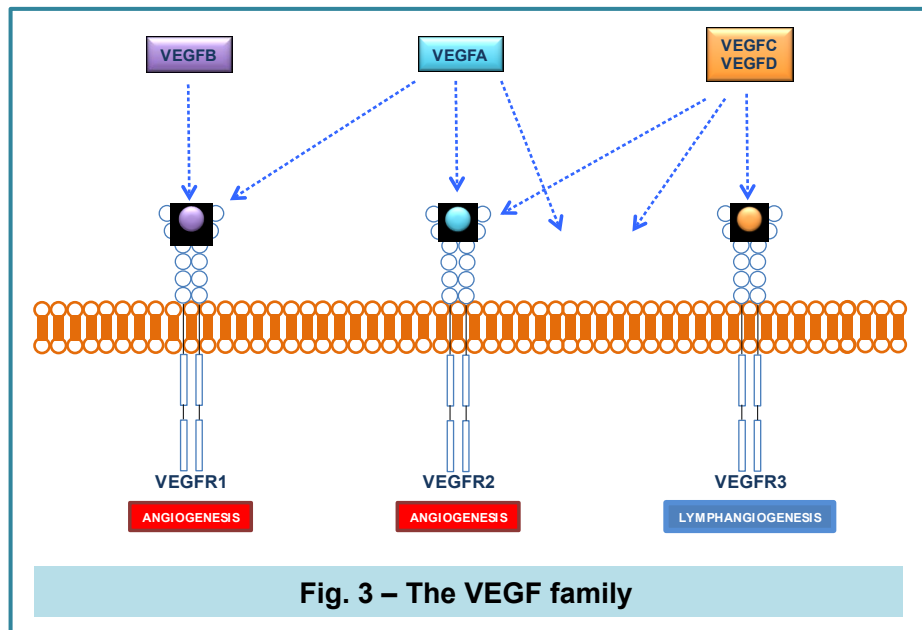
Other studies have revealed that PC deficiency in the CNS leads to BBB breakdown and brain hypoperfusion resulting in secondary neurodegenerative changes (Winkler *et al.*, 2011) and that loss of pericytal function can result in development of CNS disease (Bonkowski *et al.*, 2011).

Role of phospholipases A₂ on *E. coli* K1 invasion of the brain

PGs and leukotrienes (LTs) are arachidonate metabolites and contribute to *E. coli* K1 invasion of microvascular EC and crossing of BBB (Zhu *et al.*, 2010). Arachidonic acid (AA) derives from phospholipids by the action of different isoforms of PLA₂s and is converted to PGs or LTs by the action of cyclooxygenase (COX) and 5-lipoxygenase, respectively. There are 3 different PLA₂s isoforms: cytosolic PLA₂ (cPLA₂), Ca²⁺-independent intracellular PLA₂ (iPLA₂) and Ca²⁺-dependent secretory PLA₂ (sPLA₂) that differ from each other in terms of substrate specificity, Ca²⁺ requirement, lipid modification, translocation to cellular membranes and AA release (Alberghina, 2010).

The VEGF family

The vascular endothelial growth factor (VEGF) is a critical regulator of vasculogenesis, angiogenesis, lymphangiogenesis and vascular permeability in vertebrates. The family is made of VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PlGF). There are 2 VEGFA isoforms: VEGF₁₂₁ and VEGF₁₆₅, and both are principal mediators of tumour angiogenesis. The main signaling tyrosine kinase receptor (TKR) is VEGF receptor 2 (VEGFR2; FLK, KDR in humans), followed by VEGFR1 (FLT1) and VEGFR3 (FLT3). VEGFR1 is a “decoy” receptor, being its affinity for the growth factor high, whose kinase activity is weak, preventing VEGF from binding to VEGFR-2. VEGFR3 is mostly involved in lymphangiogenesis (Ferrara *et al.*, 2005). The PlGF and VEGFB (VEGF-related molecules) bind selectively to VEGFR-1, while VEGFA binds VEGFR-1 and VEGFR-2. VEGFC and -D bind to VEGFR3, and, following a proteolytic processing, activate VEGFR-2 after binding (Ferrara *et al.*, 2016).



VEGFR-1 is a positive regulator of angiogenesis and inflammatory responses in several human diseases such as rheumatoid arthritis (Kong *et al.*, 2011), cancer (Subramanian *et al.*, 2010) and bacterial meningitis. It was demonstrated that *E. coli* K1 has a critical role in the promotion of the physical association between phosphorylated VEGFR-1 and p85 subunit of PI3K, suggesting the involvement of VEGFR-1 in *E. coli* K1 invasion of microvascular ECs (Zhao *et al.*, 2010).

High VEGF levels have been measured in the cerebrospinal fluid (CSF) of patients affected by bacterial meningitis with severe BBB disruption, and VEGF immunoreactivity was found in endothelium and smooth-muscle cells arising from brain specimens of patients who died of bacterial meningitis (van der Flier *et al.*, 2001).

VEGF is able to stimulate PGI₂ synthesis via cPLA₂-mediated AA release via activation of p42/p44 MAP kinases (Wheeler-Jones *et al.*, 1997). So, PKC may be a key mediator of VEGF-induced activation of the ERK pathway via increased association with Raf-1 (Gliki *et al.*, 2001).

AIM OF THE STUDY

The aim of the present study was to understand how *E. coli* K1 strain is able to cross BBB. To achieve this goal, an *in vitro* model of BBB obtained by co-culturing BBEC and BRPC in transwell insert was used.

Additionally, in order to identify the contribute of PC in this process, the investigation was also extent to evaluation of the role of the pericytal VEGFR-1.

MATERIALS AND METHODS

Chemicals, antibodies, bacteria

Phospholipase A2 inhibitors, arachidonoyl trifluoromethyl ketone (AACOCF₃), bromoenol lactone (BEL) and VEGF A were purchased from Calbiochem (La Jolla, CA). NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), a selective cyclooxygenase-2 inhibitor, and rabbit polyclonal against iPLA2 antibody were from Cayman Chemical (Ann Arbor, Michigan). Rabbit polyclonal against von Willebrandt factor antibody, mouse monoclonal against cPLA2, VEGFR-2, α -actin and GAPDH antibodies, rabbit polyclonal against VEGFR-1 antibody, were from Santa Cruz Biotechnology (CA). Rabbit polyclonal anti-phospho-VEGFR-2 Tyr1175 was from Cell Signaling Technologies and rabbit polyclonal anti-phospho-VEGFR-1 Tyr1333 was from Sigma Aldrich (St. Louis, MO, USA).

A rifampicin-resistant mutant of *E. coli* K1 strain (DSMZ 10723) was used for invasion of BBEC and BRPC.

Cell cultures

Primary microvascular endothelial cells from bovine brain (BBEC) were purchased from European Collection of Cell Cultures (ECACC) fed with Ham's F-10 while pure microvessel pericytes cultures were prepared from bovine retinas. After isolation, cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Morphological changes and cell viability was determined by MTT test. Pericytes were characterized by their large size and branched morphology, positive immunostaining for α -smooth muscle actin, NG2 chondroitin sulfate proteoglycan and absence of von Willebrand factor and glial fibrillary acidic protein (GFAP) staining.

Construction of *in vitro* BBB model

Transwell inserts (Transwells, Corning, Corning, NY) were coated on the upper and bottom side with 2 mg/ml solution of rat tail collagen before being placed in complete medium (half and half DMEM/HAM's). To construct an *in vitro* model of BBB, BRPC (2×10^4 cells cm^2) were first plated on the outside of the Transwell inserts membrane and placed upside down in the well culture plate to allow adhesion. After adhesion, the Transwells were inverted and BBEC (2×10^4 cells cm^2) were seeded on the top surface of the insert (Fig. 5). Under these conditions, the two type of cells on direct contact established an *in vitro* BBB model within 3 days.

Electron microscopy

For Scanning Electron Microscopy (SEM) preparations, cells grown in co-culture on filters were fixed with 1.5% glutaraldehyde overnight, following by an additional fix in 1% OsO₄ (1 h). Then, cells on the membrane were dehydrated in graded ethanol, critical point dried, sputtered with 5 nm gold layer using an Emscope SM 300 (Emscope Laboratories, Ashford, UK) and observed using a Hitachi S-4000 (Hitachi High-Technologies America, Schaumburg, IL) field emission scanning electron microscope.

For Transmission Electron Microscopy (TEM), after dehydrating in a graded series of acetone, cells were embedded in Durcupan ACM (Fluka Chemika-Biochemika, Buchs, Switzerland). Ultrathin sections were cut using a Reichert Ultracut E microtome and double stained with uranyl acetate and lead citrate. Observations were carried out using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Evaluation of the barrier integrity

TEER was measured using a Millicell-ERS (Millipore). Background resistance was measured using

collagen-treated Transwell inserts without cells. Values were expressed as $\omega \times \text{cm}^2$ and were calculated using the formula: (the average resistance of experimental wells – the average resistance of blank wells)*0.33 (the area of the transwell membrane).

For the determination of the flux of sodium fluorescein (Na-F) across endothelial monolayer, inserts containing cells were transferred to 12- well plates containing 1.5 ml of Ringer-Hepes buffer (136 mM NaCl, 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , 10 mM NaH_2PO_4 , 25 mM glucose and 10 mM Hepes, pH 7.4) in the lower compartments (abluminal). In the inserts (luminal compartment), culture medium was replaced by 0.5 ml of buffer containing $10 \mu\text{g ml}^{-1}$ Na-F. The inserts were transferred at 5, 15 and 30 min to a new well containing Ringer-Hepes buffer. The concentrations of the marker molecule in samples were determined by fluorescence multiwell plate reader (PerkinElmer; excitation wavelenght: 485 nm, emission wavelenght: 535 nm). Transendothelial permeability coefficient (P_e) was calculated measuring flux across cell-free inserts.

Bacterial invasion and adhesion assays

Bacteria (10^7 cfu per well) were added to confluent cells in mono-culture or in co-culture on Transwell inserts (37°C, 60 min). The number of intracellular bacteria was determined after incubation with gentamicin (100 $\mu\text{g/ml}$) for 1 h at 37°C to kill extracellular bacteria. Cells were washed and lysed with 0.5% Triton X-100. The released intracellular bacteria were evaluated by seeding on LB agar plates and counting the number of colonies the day after. In duplicate experiments, the total cell-associated bacteria were determined as described for invasion, except that the gentamicin step was omitted (in order to include extracellular, adherent to membrane bacteria). Results were expressed as per cent invasion [$100 \times (\text{number of intracellular bacteria recovered})/(\text{number of bacteria inoculated})$].

Cell viability

After incubation of BBEC/BRPC in co-culture with *E. coli* K1 (60 min), cells from inserts were trypsinized separately, cell suspensions were mixed with a 0.4% (w/v) trypan blue solution, and the number of live cells was determined using a haemocytometer. Cells failing to exclude the dye were considered non-viable. Each infection was performed in triplicate and counted four times each.

Immunoblotting

The lysates of BBEC incubated with *E. coli* strains for 60 min were prepared for Western blotting (Giurdanella *et al.*, 2011). Membranes were incubated overnight at 4°C with primary antibodies against cPLA₂, iPLA₂, VEGFR-1, VEGFR-2, phospho-VEGFR-2 Tyr1175 or phospho-VEGFR-1 Tyr1333 (dilution, 1:1000) and then incubated with secondary antibodies (dilution, 1200) for 1 h at room temperature.

Phospholipase A₂ assay

BBEC and BRPC in mono- or in co-culture were pre-incubated for 60 min in culture medium in the absence or presence of either 50 µM AACOCF₃ or 2.5 µM BEL. The cells were then refed with fresh culture medium containing the inhibitors in presence or in absence of *E. coli* K1 for 60 min. Controls were performed by incubation of co-cultures with inhibitors for 120 min in absence of bacteria. After incubations, cells grown on both sides of the inserts were scraped separately, lysed (Anfuso *et al.*, 2007) and equal amounts of cell lysates were incubated in a 96-well plate with the substrate arachidonoyl-thio-phosphatidylcholine (ATPC), using cPLA₂ assay kit (Cayman Chemicals, Ann Arbor, MI, USA) according to manufacturer's instructions.

Determination of PGE₂ and VEGF production

BBEC and BRPC in mono- or in co-culture were pre-incubated for 60 min in the absence or

presence of 50 μ M AACOCF₃ or 2.5 μ M BEL or 5 μ M NS-398. The cells were then refed with fresh culture medium containing the inhibitors in presence or in absence of *E. coli* K1 for 60 min. Supernatants were collected and used for PGE₂ determination (Cayman Chemicals, Ann Arbor, MI, USA). Conditioned medium was removed from mono- and co-cultures and analysed for VEGF by ELISA (R&D Systems, Minneapolis, MN, USA), as specified by the manufacturer's instructions.

VEGFR-1 blockade experiments

For BBEC/BRPC simultaneous blockade experiments, co-culture were treated with VEGFR-1 Ab (2 μ g ml⁻¹) for 60 min before treatment with *E. coli* K1 for 60 min. Parallel co-cultures without VEGFR-1 blockade were performed.

For BBEC (but not BRPC) VEGFR-1 blockade experiments, BBEC were grown on the top surface of the Transwell insert and incubated with VEGFR-1 Ab. Then, inserts were inverted and BRPC were seeded on the outside of the insert. After BRPC adhered, the inserts were reinserted into six-well plates and the *in vitro* BBB model, in which BBEC were blocked by VEGFR-1 Ab, was established within 3 days.

For BRPC (but not BBEC) VEGFR-1 blockade experiments, BRPC were first plated on the outside of the Transwell inserts. After adhesion of BRPC, the inserts were reinserted into six-well and cells were incubated with VEGFR-1 Ab. Then, BBEC were seeded on the top surface of the insert and the *in vitro* BBB model, in which BRPC were blocked by VEGFR-1 Ab, was established within 3 days.

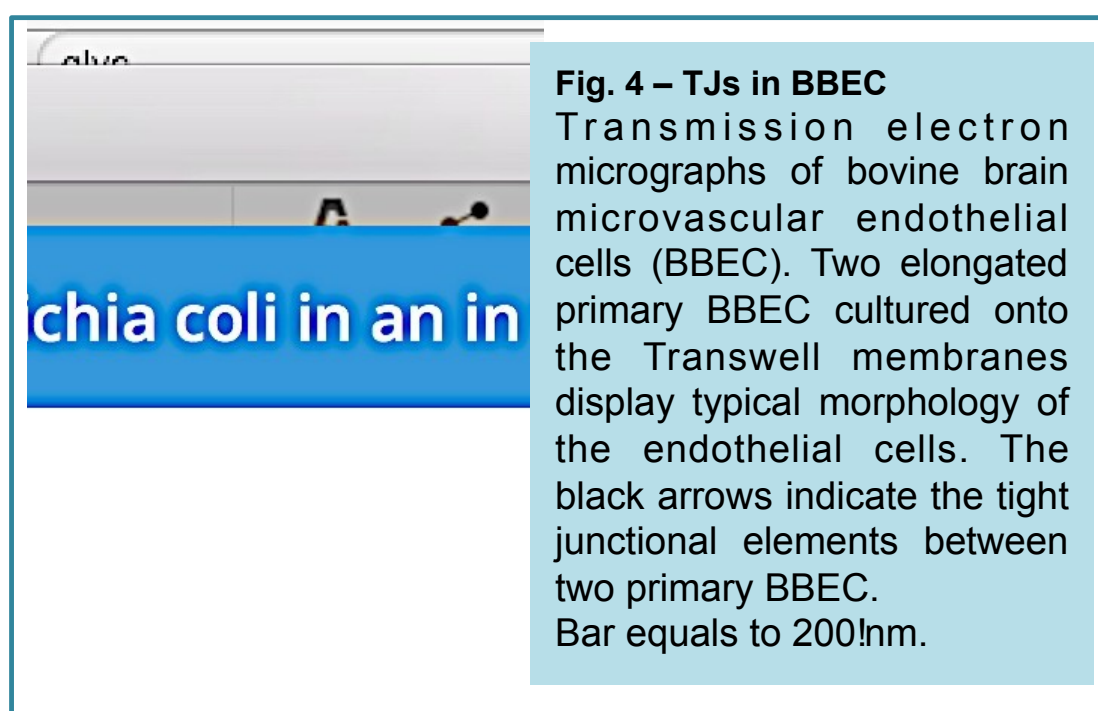
Statistical analysis

Statistical significance between two groups was analysed by Student's test. One-way analysis of variance (anova), followed by Tukey's *post hoc* test, was used to compare the means for the multiple groups. The *P*-value < 0.05 was considered statistically significant.

RESULTS

***E. coli* K1 infection determines changes of TEER and permeability to sodium fluorescein in BBEC/BRPC co-cultures**

TEM of BBEC growing on Transwell filter in the *in vitro* BBB model is shown in Fig. 4. TJ sealing adjoining BBEC are evident.



The BBB model was validated by measuring TEER and sodium fluorescein flux across BBEC in monolayer and in co-culture with BRPC (Table 1). Co-culture showed high values of TEER and very low permeability to sodium fluorescein compared with BBEC mono-culture. Incubation for 60 min with *E. coli* K1 (10^7 cfu per well) caused a significant TEER reduction (about 3.2-fold) and an increase in permeability (about 2.3-fold) in comparison to BBEC/BRPC control co-cultures.

		TEER ($\omega \times \text{cm}^2$)	Pe (10^{-6} cm/s)
mono-culture	BBEC	85 $\pm$ 21.4	8.1 $\pm$ 0.6
mono-culture	BBEC + <i>E. coli</i>	60 $\pm$ 16.6	6.6 $\pm$ 0.8
co-culture	BBEC/BRPC	250 $\pm$ 51.1 [†]	3.2 $\pm$ 0.15 [†]

Table 1 – Evaluation of the barrier integrity
 Evaluation of the barrier integrity. TEER and permeability to sodium fluorescein (Pe) determination in microvascular endothelial cells in mono- and in co-culture, in the absence or presence of *E. coli* K1 strain. BBEC (40,000 cells/cm²) were cultured in monolayers in Ham's F-10 medium containing 10% FBS or were grown on the top surface of the Transwell insert (6-well type, 3.0-mm pore size) in which BRPC (40,000 cells/cm²) were first plated on the outside of the membrane, in 50% DMEM plus 50% F-10 HAM's containing 10% FBS. After 3 days, the cells were incubated in the absence or presence of *E. coli* (10⁷ CFU/well) for 60 min and measurements of TEER and cell permeability on BBEC were performed. Values were expressed as $\omega \times \text{cm}^2$ and were calculated by the formula: [the average resistance of experimental wells - the average resistance of blank cells] $\times$ 0.33 (the area of the transwell membrane). For sodium fluorescein determination, flux across cell-free inserts was measured and transendothelial permeability coefficient (Pe) was calculated. Values (means $\pm$ SEM) are from three independent experiments (n=3). Statistically significant differences of TEER and Pe, measured in BBEC in co-culture as compared to mono-culture are indicated by [†] ([†]p < 0.01) and values from *E. coli*-stimulated BBEC as compared to control non-stimulated BBEC are indicated by asterisks (**p < 0.01).

***E. coli* K1 stimulates phospholipase A₂ activities, PGE₂ production and VEGF release**

BBEC in mono- and co-culture stimulated with *E. coli* K1, showed a strong PLA₂ activity (about 2.2-fold in mono- and 2.6-fold in co-cultures) compared with the unstimulated BBEC, whereas in BRPC it was weakly stimulated. The presence of 50 μM AACOCF₃ (PLA₂ activity dual blocker) or 2.5 μM BEL (iPLA₂ inhibitor) for 120 min (pre-incubation of 60 min followed by incubation for 60 min in the presence of bacteria) in control BBEC mono-cultures, reduced enzyme activity of 28% and 19%, respectively; in *E. coli* K1 treated BBEC mono-cultures, AACOCF₃ and BEL reduced PLA₂ activity by 62% and 55%, respectively. In control BRPC mono-cultures, AACOCF₃ and BEL caused a decrease by 32% and 24% respectively, and in *E. coli* stimulated BRPC mono-cultures, the inhibitors reduced PLA₂ activity by 36% and 28% respectively.

	PLA ₂ activity (pmol min ⁻¹ mg ⁻¹)	Control cells	Cells + <i>E. coli</i>
mono-culture	BBEC	19.9 ± 1.5	44.7 ± 3.2*
	BBEC + AACOCF ₃	14.4 ± 1.1†	17.1 ± 1.5†
	BBEC+BEL	16.1 ± 1.3	20.0 ± 1.7†
	BRPC	13.5 ± 1.1	17.1 ± 1.3
	BRPC + AACOCF ₃	9.2 ± 0.8†	11.0 ± 1.2†
	BRPC + BEL	10.3 ± 1.2†	12.3 ± 0.9†
co-culture	BBEC	27.4 ± 2.5	71.2 ± 6.2*
	BBEC + AACOCF ₃	19.8 ± 1.3†	23.1 ± 2.1†
	BBEC+BEL	22.1 ± 2.2†	31.2 ± 2.4†
	BRPC	12.2 ± 1.1	16.1 ± 1.3
	BRPC + AACOCF ₃	7.7 ± 0.9	10.3 ± 0.9†
	BRPC + BEL	8.0 ± 1.2	10.2 ± 1.3†

Table 2 – PLA₂ activity in BBEC and BRPC in mono- and co-culture stimulated and not-stimulated by *E. coli* K1
BBEC were cultured in monolayers or on the top Transwell insert in which BRPC were first plated on the outside of the membrane. All incubations were performed at 37°C in absence or presence of *E. coli* (10⁷ cfu per well) for 60 min with or without 50 mM AACOCF₃ or 2.5 mM BEL. Values (means ± SEM) are from three independent experiments (*n* = 3). ANOVA and the Tukey post-test were used to compare enzyme activities measurements in the 24 different experimental conditions (*P* < 0.05). Enzyme activities measured in *E. coli*-stimulated BBEC in comparison with control un-stimulated BBEC, are indicated by asterisk (*), and in presence of inhibitors in comparison with the values in absence of inhibitors are indicated by dagger (†).

In control BBEC/BRPC co-culture, AACOCF₃ and BEL reduced PLA₂ enzymatic activity by 28% and 20%, respectively. After *E. coli* K1 treatment in presence of the inhibitors, the reduction was stronger (68% and 56%, respectively). Moreover, the two inhibitors decreased PLA₂ activity in control and in *E. coli* treated BRPC by about 36%. The incubation with BEL allowed us to discriminate between the cPLA₂ and iPLA₂ activity contribution.

PGE₂ in supernatants of BBEC or BRPC in mono- and in co-culture were measured. Table 3 shows an increase in BBEC monocultures infected with *E. coli* K1 (2.5 fold) compared with the respective control whereas bacterial incubation in presence of AACOCF₃ or BEL decreased PGE₂ production by 63% and 60%, respectively. The contribution in PGE₂ production from *E. coli*-treated BRPC monocultures was negligible.

	PGE ₂ (pg ml ⁻¹)	Control cells	Cells + <i>E. coli</i>
	BBEC	88 ± 7.8	220 ± 21.1*
	BBEC + AACOCF ₃	73 ± 6.1 ^a	82 ± 7.9 ^a
	BBEC+BEL	78 ± 6.8 ^a	88 ± 8.1 ^a
mono-culture	BRPC	70 ± 6.9	73 ± 6.9 ^b
	BRPC + AACOCF ₃	52 ± 4.8 ^a	56 ± 5.1 ^a
	BRPC + BEL	57 ± 5.1 ^a	59 ± 5.4 ^a
co-culture	BBEC/BRPC	203 ± 19.9 ^c	612 ± 46.8 ^{c*}
	BBEC/BRPC + AACOCF ₃	162 ± 15.8 ^a	182 ± 18.8 ^a
	BBEC/BRPC + AACOCF ₃	171 ± 16.5 ^a	192 ± 21.1 ^a

Table 3 - Prostaglandin (PGE₂) production in BBEC and BRPC in mono- and co-culture stimulated and non-stimulated by *E. coli* K1

a. The statistically significant differences in PGE₂ production of cultures incubated with PLA₂ inhibitors in comparison with the respective in absence of inhibitors.

b. The statistically significant differences in PGE₂ release in untreated BRPC mono-cultures in comparison with untreated BBEC mono-cultures.

c. The statistically significant differences, between not- and stimulated co-cultures versus the respective mono-cultures.

BBEC were cultured in monolayers or on the top surface of the Transwell insert in which BRPC were first plated on the outside of the polycarbonate membrane. Cell culture supernatants from mono- and co-cultures in absence (control) and presence of *E. coli*, with or w/o 50 mM AACOCF₃ or 2.5 mM BEL, were assayed for PGE₂ production. Inhibitors were added to the culture medium 60 min before *E. coli* addition. Values (means ± SEM) are from three independent experiments (n = 3). ANOVA and the Tukey post-test were used to compare PGE₂ production in the 18 different experimental conditions (P < 0.05). Stimulated cells versus control cultures (not stimulated by bacteria), are indicated by asterisk (*).

E. coli K1 treatment of BBEC/CRPC co-cultures increased PGE₂ production by 3.0 fold in comparison with the respective untreated co-cultures. Furthermore, in the supernatants of *E. coli*-stimulated co-cultures, incubated in presence of PLA₂ inhibitors AACOCF₃ or BEL, PGE₂ levels decreased by about 70% and 68%, respectively.

BBEC monocultures produced low amounts of VEGF in the conditioned medium. Incubation of BBEC monocultures with *E. coli* K1 led to a 3.1 fold increase in the release of VEGF (Table 4). Conversely, untreated BRPC monocultures expressed VEGF at basal levels 1.6-fold higher than untreated BBEC monocultures and the presence of *E. coli* did not changed the secretion. *E. coli* treatment of co-cultures induced an 3.2 fold increase in comparison with control untreated co-cultures. Incubation of *E. coli*-treated BBEC monocultures with AACOCF₃, BEL or with COX-2-specific inhibitor NS-398 reduced *E. coli*-induced VEGF release by 70%, 67% and 71% respectively. Incubation of untreated co-cultures with the three inhibitors reduced VEGF release by

about 33%, while in *E. coli*-treated co-cultures AACOCF3, BEL and NS-398 reduced VEGF release by 77%, 75% and 79%, respectively. The effect of the inhibitors on VEGF release in untreated and *E. coli*-treated BRPC monocultures was about 22% and 21% respectively.

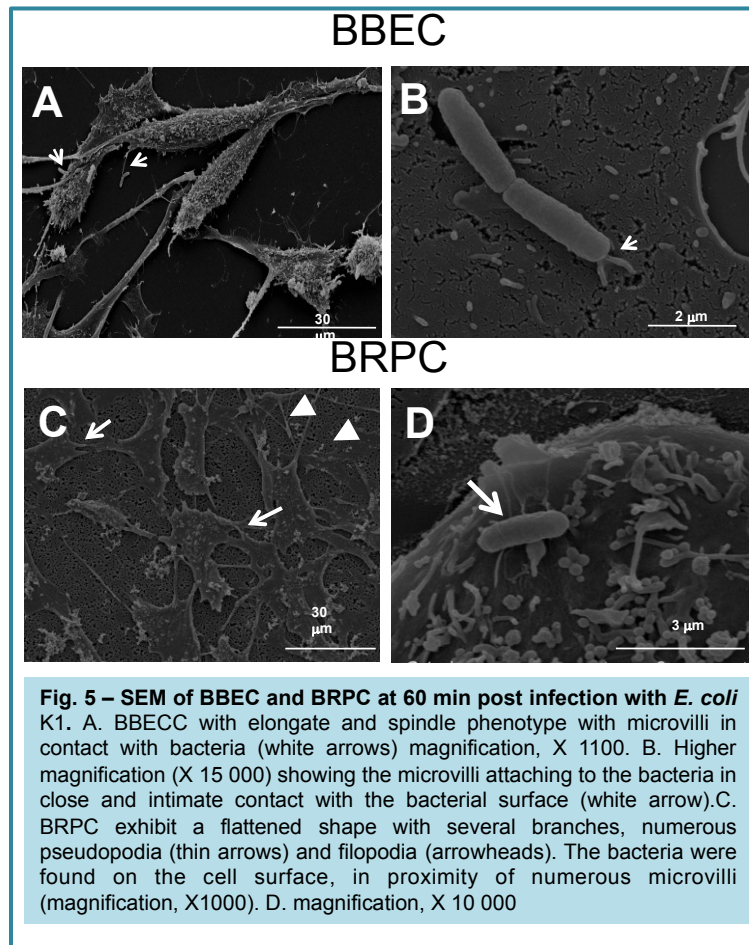
	VEGFA (pg ml ⁻¹)	Control cells	Cells + <i>E. coli</i>
mono-culture	BBEC	22.3 ± 1.9	70.4 ± 7.5*
	BBEC + AACOCF3	13.5 ± 1.4 ^a	21.1 ± 2.3a
	BBEC+BEL	16.3 ± 1.8 ^a	23.2 ± 3.4a
	BBEC + NS-398	15.5 ± 1.7 ^a	20.4 ± 2.3a
	BRPC	35.7 ± 3.2 ^b	33.8 ± 2.9b
	BRPC + AACOCF3	27.3 ± 2.3 ^a	26.1 ± 2.5a
	BRPC + BEL	29.0 ± 3.1 ^a	27.2 ± 3.1a
	BRPC + NS-398	26.8 ± 2.3 ^a	26.8 ± 3.2
co-culture	BBEC/BRPC	80.7 ± 6.7 ^c	260.5 ± 22.1*
	BBEC/BRPC + AACOCF3	54.2 ± 4.7 ^a	60.4 ± 5.3 ^a
	BBEC/BRPC + BEL	56.4 ± 4.2 ^a	65.1 ± 5.6 ^a
	BBEC/BRPC + NS-398	50.8 ± 4.3 ^a	55.2 ± 4.7 ^a

Table 4 - VEGFA determination in BBEC and BRPC in mono- and co-culture stimulated and non-stimulated by *E. coli* K1

a. Statistically significant differences in VEGF release of cultures incubated with inhibitors in comparison with the respective in absence of inhibitors. **b.** The statistically significant differences in VEGF release in untreated BRPC mono-cultures in comparison with untreated BBEC mono-cultures. **c.** The statistically significant differences in VEGF release in co-cultures in comparison with the mono-cultures. BBEC and BRPC were cultured in monolayers or on Transwell. Aliquots of medium from mono- and co-cultures in absence and in presence of *E. coli* were incubated with bovine VEGF-A antibody. Values (means ± SEM) are from three independent experiments ($n = 3$). ANOVA and the Tukey post-test were used to compare VEGF release in the 24 different experimental conditions ($P < 0.05$). Statistically significant differences of *E. coli*-stimulated mono- and co-cultures in comparison with not stimulated (cultures are indicated by asterisk (*)).

***E. coli* adhere to BBEC and BRPC but only in BBEC the invasion occurs**

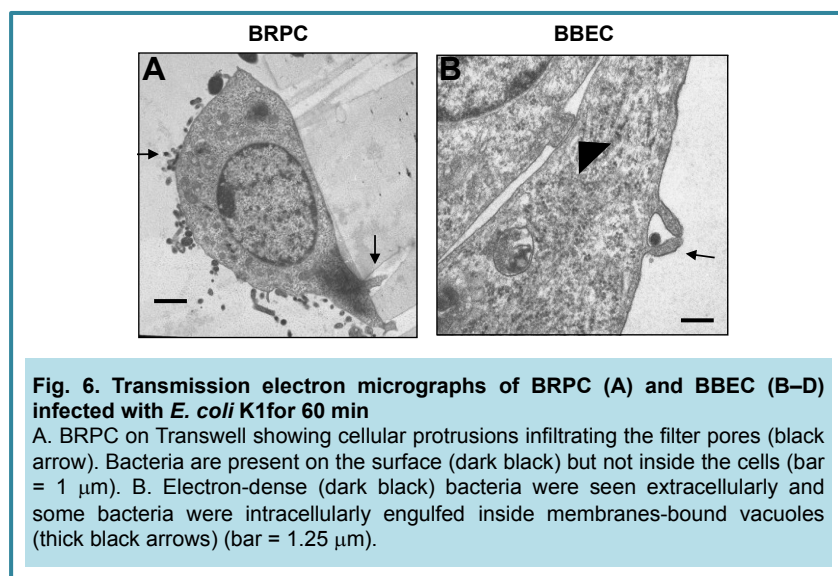
SEM images of BBEC, (Fig. 5, A and B) or BRPC (Fig. 5, C and D), after incubation with *E. coli* K1, show the cells with an elongated and spindle phenotype, with numerous microvilli distributed on most of the cell surface (Fig. 5 A). The images at higher magnification show the bacteria attached to the apical surface of the BBEC in contact with microvilli (Fig. 5 B).



BRPC are branched and flat with short microvilli on the surface area (Fig. 5C). Few bacteria were found attached to the smooth part of the BRPC surface without any sign of specific membrane interaction (Fig. 5 D).

TEM/SEM

Figure 6 A shows BRPC with numerous bacteria on the cell surface where they remain and no endocytosed bacteria were found. In *E. coli*–BBEC interaction, electron-dense bacteria both intra- and extracellular were seen and also BBEC formed microvilli-like protrusions that surrounded and endocytosed the bacteria (Fig. 6 B).

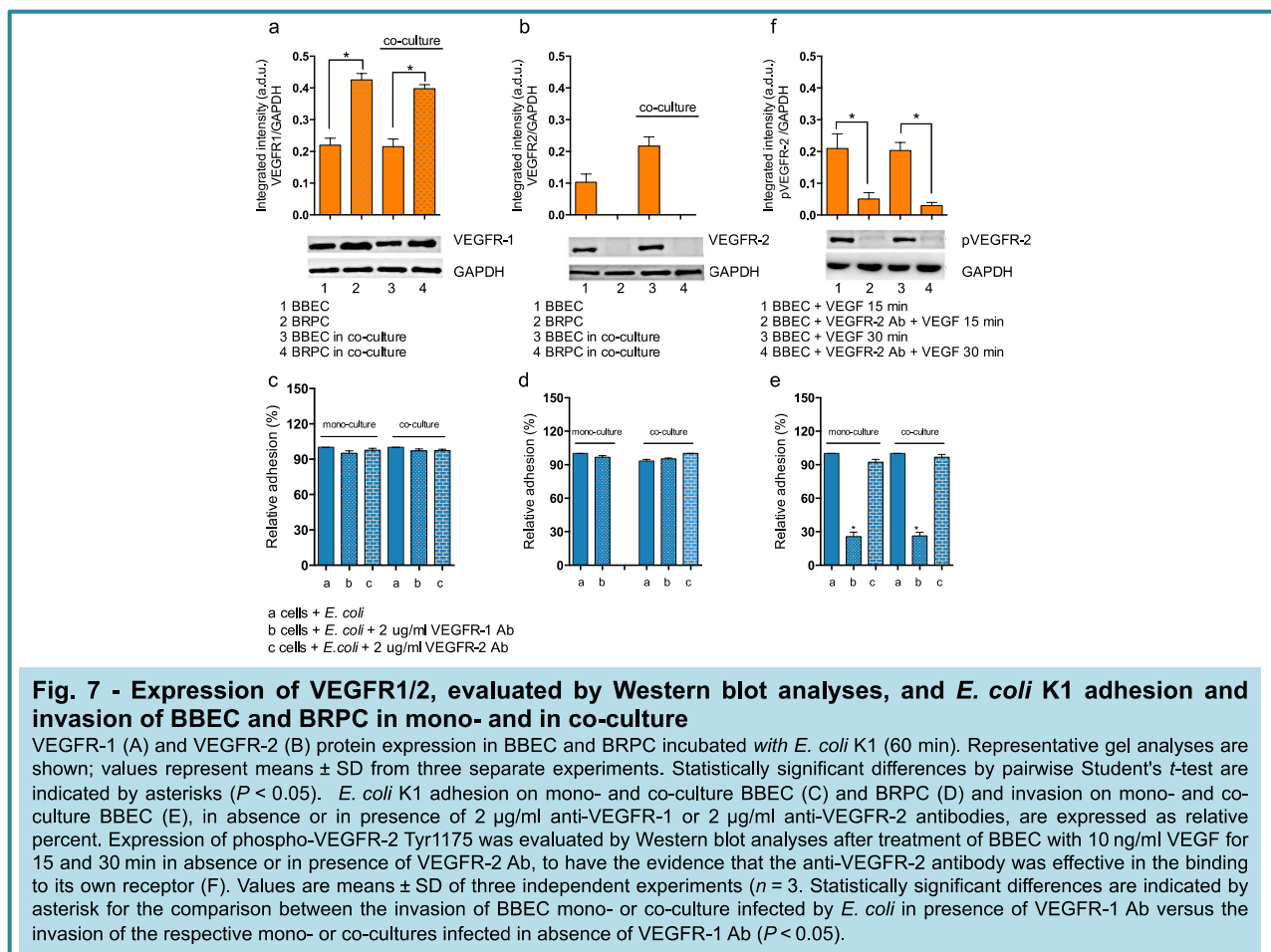


VEGFR-1 is involved in *E. coli* adhesion and invasion of BBEC

Western blot analysis showed that VEGFR-1 expression in BRPC mono- and co-cultures was 2.0 fold and 2.2 fold higher than that of BBEC in mono- and in co-cultures, respectively (Fig. 7 A). VEGFR-2 expression did not change in BBEC grown in contact with BRPC in respect to BBEC monoculture. Interestingly, VEGFR-2 protein was not detected in BRPC (Fig. 7 B).

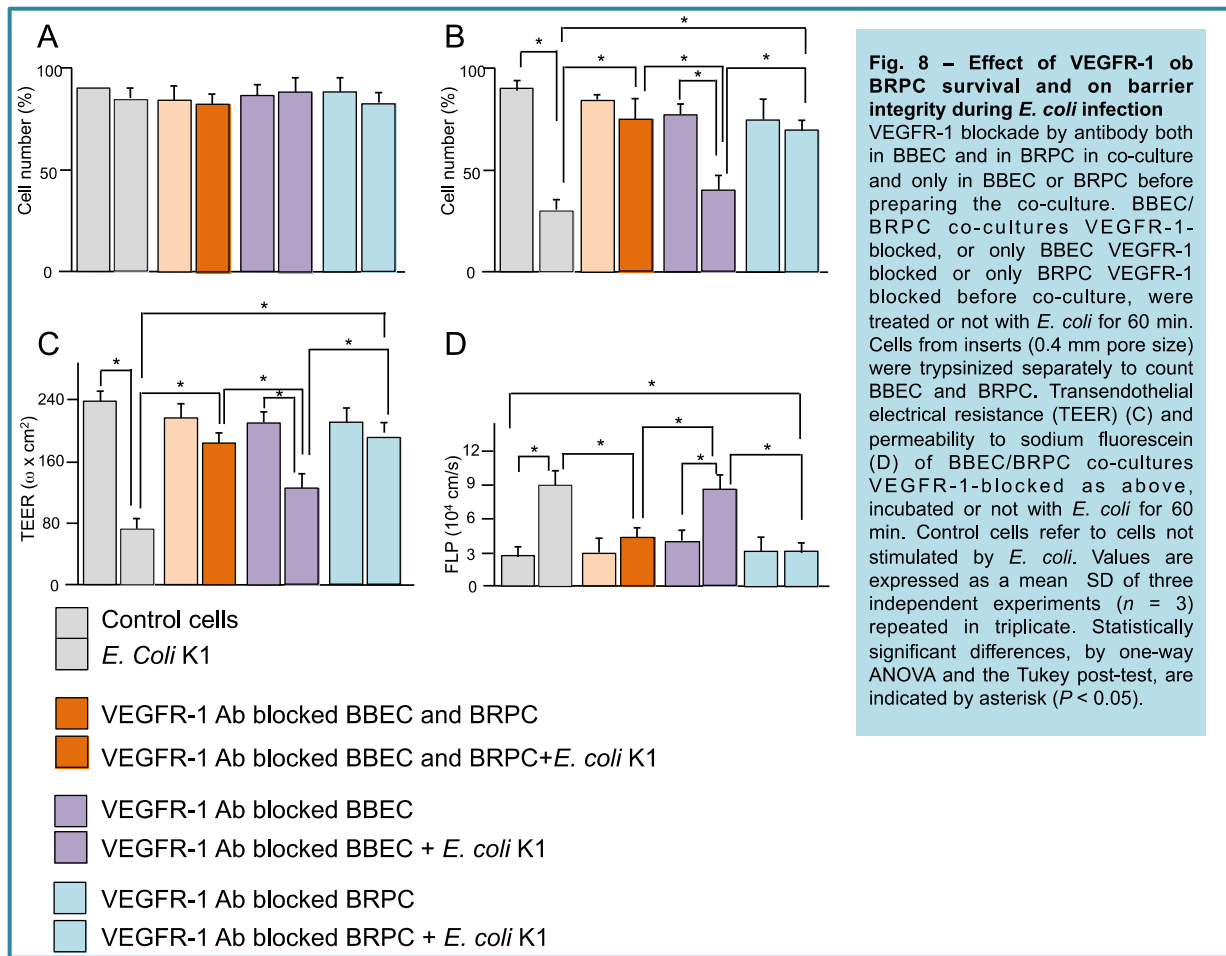
In order to understand the involvement of VEGF receptors in *E. coli* K1 adhesion and invasion, assays were performed in presence of VEGFR-1 or VEGFR-2 antibodies (Ab). Fig. 7 C show that VEGFR-1 Ab and VEGFR-2 Ab have no effect on *E. coli* adhesion to BBEC both in mono- and in co-cultures, as well as on *E. coli* adhesion after addition to BRPC in mono- and in co-cultures (Fig. 7 D). VEGFR-1 Ab significantly reduced *E. coli* invasion in BBEC by 30% and by 48% in mono- and in co-cultures, respectively (Fig. 7 E), while VEGFR-2 did not cause any change in the *E. coli* invasion of BBEC. These results indicate a positive correlation between the block of VEGFR-1 and the block of *E. coli* K1. Treatment of BBEC with 10 ng/ml VEGF for 15 and 30 min in absence or in presence of VEGFR-2 Ab showed an increase of phosphorylation of VEGFR-2 at Tyr1175, as assessed by Western blot, while treatment with 10 ng/ml of VEGF in presence of 2 μ g/ml VEGFR-2 antibody inhibited VEGFR-2 phosphorylation (Fig. 7 F). Experiments were also performed by treating BBEC with 10 ng/ml⁻¹ VEGF for 15 and 30 min in absence or in presence of

VEGFR-1 Ab. Phosphorylation of VEGFR-1 at Tyr1333 was observed in BBEC treated for 15 and 30 min with VEGF.



VEGFR-1 negatively regulates BRPC survival and its blockade protects the barrier integrity

To clarify if the prevention of the pericyte death and monolayer permeability can be attributed to VEGFR-1, experiments blocking the receptor by its specific antibody were performed on co-cultures or single cell type monocultures. After incubation of BBEC/BRPC co-cultures with *E. coli* K1, TEER decreased by 64% and permeability to sodium fluorescein increased by 3.2 fold in comparison with non-infected co-cultures (Fig. 8 C and D).



In co-culture, simultaneous block of VEGFR-1 in presence of *E. coli* K1 was able to preserve BRPC viability by almost 3 fold in comparison with infection in the absence of VEGFR-1 Ab (Fig. 8 B). Moreover, in presence of *E. coli* K1 and receptor blockade, TEER values increased by 2.3 fold and permeability decreased by 2.2 fold in comparison with infected co-cultures in absence of receptor blockade. Furthermore, only 10% of TEER reduction and a 20% of increase in permeability were found in comparison with non-infected control cells in co-culture (Fig. 8 C and D).

When only the BBEC were treated with VEGFR-1 Ab and *E. coli* K1, 45% of BRPC adhering to the co-culture insert was found (Fig. 8 B), while BBEC number was unchanged (Fig. 8 A). Moreover, a significant decrease in TEER values (about 56%) and a significant increase in permeability (approximately 2.8 fold) in *E. coli* infected in comparison with non-infected co-cultures were found. Furthermore, a 38% of TEER decrease and a 2 fold permeability increase were

found in comparison with *E. coli* K1 infected co-cultures in which both BBEC and BRPC were blocked by VEGFR-1 Ab (Fig. 8 C and D). These results demonstrated that VEGFR-1 Ab was able to partially reduce *E. coli* K1 internalization (about 50%, as shown in Fig. 7) but not to completely avoid the activation of signalling pathway leading to VEGF release, detachment of BRPC and loss of barrier properties.

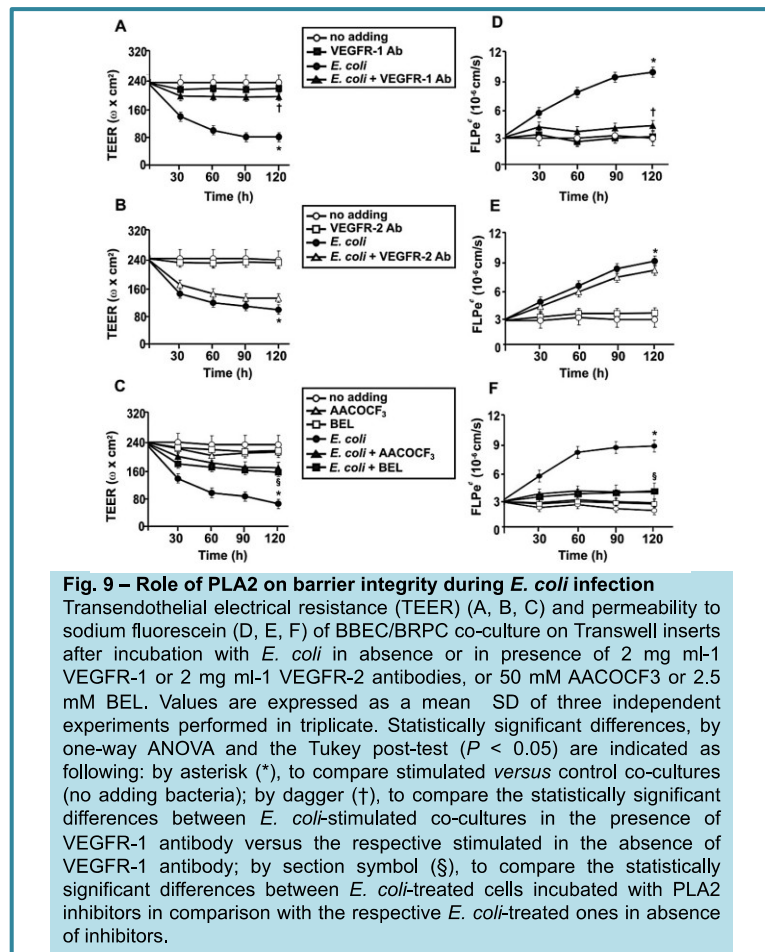
Blockade of only BRPC in presence of *E. coli* K1 did not change BRPC number in co-culture (Fig. 8 B), whose value was very similar to that of BRPC in absence of infection. Moreover, the values of TEER and permeability in this experimental condition were similar to non-infected control cells in co-culture. Furthermore, TEER increased by 1.7 fold and by 2.4 fold and permeability decreased by 60% and 64% in comparison with *E. coli* K1 infected co-cultures in which only BBEC were blocked by VEGFR-1 Ab and in comparison with *E. coli* infected co-cultures in which no blocking was induced, respectively (Fig. 8 C and D). Probably, in absence of VEGFR-1 Ab on BBEC, *E. coli* K1 is able to invade BBEC without limits and to activate the signalling pathway leading to VEGF production in large amounts. The VEGF released by BBEC cannot bind VEGFR-1 on BRPC, because blocked by the specific antibody, it therefore does not result in the detachment of pericytes. In this experimental condition, about 90% of BRPC adhered to co-culture and the values of TEER and permeability were very similar to those of the non-infected co-cultures.

These results demonstrate that the prevention of pericyte loss and monolayer permeability can be attributed to VEGF blockade.

To further confirm our results, experiments on BBEC/BRPC co-cultures incubated with *E. coli* K1 for 30, 60, 90 and 120 min, in absence or in presence of VEGFR-1 or VEGFR-2 Abs were performed.

As shown in Fig. 9, TEER values gradually decreased after 30 min *E. coli* K1 treatment (37% reduction) to reach 60% following incubation for 60 and 120 min (panels A, B and C). The presence of VEGFR-1 Ab, but not VEGFR-2 Ab, restored TEER to values very similar to the controls at all

incubation time (panel A). No significant change were observed in the TEER values after incubation of the co-cultures with VEGFR-1 Ab and VEGFR-2 Ab in absence of *E. coli* K1 (panels A and B). When co-cultures were infected with *E. coli* in the presence of AACOCF₃ or BEL, TEER was restored by about 35% at 60 and 120 min (panel C). Regarding permeability studies, fluorescent intensity in the receiver chamber had already increased of about 2 fold after *E. coli* K1 incubation for 30 min (Fig. 9 D–F) and the fluorescence was more intense at each designated time point than untreated co-cultures. Incubation in the presence of VEGFR-1 Ab, but not VEGFR-2 Ab, attenuated of about 2.5-fold the diffusion of fluorescein sodium at each studied time (panels D and E). Moreover, when the cells were infected with *E. coli* K1 for 30, 60, 90 and 120 min in the presence of AACOCF₃ or BEL, permeability was restored by about 44% in comparison with cells *E. coli* K1 in absence of PLA₂ inhibitors (panel F). The incubation of non-infected cells with AACOCF₃ or BEL did not affect significantly TEER or permeability.



DISCUSSION

Neonatal *E. coli* meningitis is associated with significant morbidity and mortality. In order to reach the brain, circulating bacteria need to cross the BBB, consisting of brain microvascular EC that interact dynamically with other cells nearby, PC, astroglia, perivascular microglia and neurones (Ballabh *et al.*, 2004).

PC communicate with EC releasing soluble factors responsible of the upregulation of BBB functions (Hori *et al.*, 2004; Dohgu *et al.*, 2005; 2011; Takata *et al.*, 2007; Nakagawa *et al.*, 2009). Despite the fact that they share a common basement membrane with EC, they were not studied in a co-culture BBB model to test the molecular mechanism of bacterial invasion. Lipopolysaccharide-induced sepsis in mice leads to detachment of brain PC from the basal lamina (Nishioku *et al.*, 2009), suggesting that brain PC plays a crucial role in BBB integrity. In addition, the genetic animal models of progressive PC loss with age have shown that BBB integrity is determined by the amount of PC coverage of cerebral microvessels (Bell *et al.*, 2010). Therefore, loss of PC in brain microvasculature is often linked to BBB dysfunction.

E. coli K1 exhibits brain tropism (Presadarao *et al.*, 1999) and it has been demonstrated that VEGFR-1 is involved in *E. coli* K1 invasion of human brain EC via recruitment of the PI3K/Akt pathway (Zhao *et al.*, 2010).

By using TEM and SEM, we showed that *E. coli* K1 is able to adhere and enter BBEC, but not BRPC, by a vesicle-mediated mechanism and is able to induce PLA₂ activation and PGE₂ synthesis, these latter contributing to BBB disruption. In fact, anti-inflammatory drugs are administered for palliative care and treatment during Gram-negative bacterial infection (Kim *et al.*, 2009; Hulscher *et al.*, 2010) and the elimination of PGE₂ across the BBB is inhibited by either intracerebral or intravenous administration of antibiotics and by intracerebral administration of non-steroidal anti-inflammatory drugs as well (Akanuma *et al.*, 2011).

BRPC secrete higher levels of VEGFR in basal conditions compared to BBEC, suggesting a

stabilizing role for BBEC. Treatment with AACOCF3 and BEL, as well as COX-2 inhibitor NS-392, reduces *E. coli* K1 induced VEGFA secretion of BBEC/BRPC co-cultures, indicating the involvement of PLA₂, AA production and its metabolization in eicosanoids in the production of VEGFA, in agreement with other studies (Pai *et al.*, 2001). Other studies further confirmed the role of PLA₂ activation in secretion of VEGF (Bamba *et al.*, 2000; Ottino *et al.*, 2004; Barnett *et al.*, 2010). Increase of BBB permeability could be related to PC coverage ablation due to PGs and VEGF biological effects on BBB.

In our model system, permeability increased within 30 min, suggesting that other factors may be involved in the increased permeability induced by VEGF. It is known that VEGF plays a key role in impairment of function of BBB (Murata *et al.*, 1995; Hofman *et al.*, 2000; 2001; Witmer *et al.*, 2003), and activates pathways that were also activated during *E. coli* K1 invasion of microvascular endothelial cells (Zhao *et al.*, 2010): VEGF induces tyrosine kinase receptor phosphorylation, internalization and cleavage of VE-cadherin, which can cause disruption of adherens junctions coupled with increase of vascular permeability (Dejana *et al.*, 2008).

After *E. coli* K1 treatment of co-cultures with VEGFR-1 blocked in both cellular type or in co-cultures in which only BRPC, and not BBEC, were treated with VEGFR-1 Ab, the values of TEER and permeability to sodium fluorescein were very similar to those of non-infected co-cultures.

Moreover, the important role played by PLA_{2s} in maintaining barrier properties was demonstrated performing experiments in which we observed a rescue of almost 40% in TEER and permeability after *E. coli* K1 infection of co-cultures in presence of AACOCF3 or BEL in comparison with co-cultures infected with *E. coli* K1 in absence of PLA₂ inhibitors.

Zhao *et al.* (2010) already showed the involvement of VEGFR-1 during *E. coli* K1 invasion of brain endothelial, while Salmeri *et al.* (2019) demonstrated that c- and iPLA₂ activities and cPLA₂ phosphorylation were stimulated in microvascular endothelial cells after *E. coli* incubation and that PI3K and ERK 1/2 inhibitors reversed cPLA₂ phosphorylation (Salmeri *et al.*, 2012). Arachidonic acid released after *E. coli* K1 infection could become the substrate of cyclooxygenases for the

production of PGs, whose could exert a proangiogenic influence by inducing VEGF secretion by endothelial cells. VEGF released by endothelial cells could target VEGFR-1 on the membrane of adjacent pericytes and determine their leak, acting as a negative regulator. The VEGF negative role on pericyte function was already shown in the C310T1/2 pericyte line, (Greenberg *et al.*, 2008). It has also been showed that systemic administration of VEGF ablates pericytes from the mature retinal vasculature through the VEGFR-1 mediated signalling pathway, leading to increased vascular (Cao *et al.*, 2010).

Results of this study show the defensive role played by the pericytes during a bacterial attack. The association of an antibiotic therapy with a drug able to block the VEGFR-1 on PC could represent a novel strategy against neonatal bacterial meningitis because would mean slowing down PC loss, thus protecting the anatomical integrity of the microvessels and BBB overall.

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BACKGROUND: second study

As already discussed, TJs seal BMECs each other and depend on the presence of proteins such as Claudins, Occludin, β -catenin and cytoplasmic ZO proteins to create a continuous sheet covering the inner surface of microcapillaries (Abbott *et al.*, 2010). When TJs expression is reduced, BBB permeability increases (Dejana *et al.*, 2009). BMPCs are located on abluminal surface of the endothelium and orchestrate many neurovascular functions, such as BBB formation and regulation of capillary blood flow (Winkler *et al.*, 2011). Moreover, pericytes possess features of multipotent cells and therefore and great potential for therapy have been addressed on them (Dore-Duffy, 2008). Despite pivotal roles exerted by BMPCs, less is known about the mechanisms by which vasoactive molecules, such as adenosine, regulate their physiological function.

Adenosine derives from the catabolism of extracellular adenosine triphosphate (ATP).

Role and metabolism of adenosine

In physiological conditions, adenosine exerts beneficial anti-inflammatory effects. Conversely, chronic elevation of adenosine levels is present during pathological conditions characterized by inflammation (Borea *et al.*, 2017). After an insult to the cellular membrane, the cell release high concentrations of ATP, that is first converted into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by CD39 extracellular enzyme; then, CD73 extracellular enzyme will convert AMP to adenosine (Borea *et al.*, 2017).

Adenosine receptors

Adenosine binds its receptors (ARs), which are purinergic G protein-coupled receptors. There are four different subtypes of ARs, A₁, A_{2A}, A_{2B} and A₃. A₁ and A₃ receptors induce an inhibition of adenylyl cyclase through G_{i/o} protein family, thus reducing intracellular levels of cAMP whereas A_{2A} and A_{2B} activate cAMP synthesis via G_s. Moreover, A₁ and A_{2A} have high affinity whereas A₃ and A_{2B} have low affinity for adenosine (Do-Geun *et al.*, 2015). It has been demonstrated that ARs and extracellular enzymes are expressed in brain endothelial cells in mice and humans and that CD73 expression is very low and not detectable in vivo (Do-Geun *et al.*, 2015). It has also been demonstrated that the activation of ARs with AR agonists increased BBB permeability by reducing TJ protein expression (Bynoe *et al.*, 2015; Carman *et al.*, 2011) indicating that these agonists are able to control the entry of different molecules into the CNS. Recent studies demonstrated that the permeability process, induced by A_{2A}-AR activation, is mediated by Rho signalling pathway (Denk-Lobnig *et al.*, 2017).

An important role in infection disease progression is played by adenosine A_{2B} receptors, as demonstrated by studies conducted on a murine model infected by *C. difficile* (Warren *et al.*, 2012) and on HeLa cells after chlamydial infection (Pettengill *et al.*, 2012). Souza *et al.* demonstrated the involvement of both A_{2A} and A_{2B} adenosine receptors in increased MMP-9 secretion in *S. aureus* infected macrophages (Souza *et al.*, 2009). Based on these studies, we focused our attention on determining whether *Haemophilus influenzae* (Hi) can modulate the BBB permeability through A_{2A} and A_{2B} -ARs to enter BMECs.

HAEMOPHILUS INFLUENZAE MENINGITIS

Haemophilus influenzae

Hi is an important human-restricted Gram-negative pathogen, which normally resides in the upper respiratory tract of humans and can cause severe localized (otitis media, sinusitis) and systemic (bacteremia, meningitis, pneumonia, septic arthritis, epiglottitis) infections in susceptible individuals (Stefani *et al.*, 2008). Some strains of Hi have a polysaccharide capsule representing the major virulence factor and antigen of this bacterial species. On the basis of its antigenic properties, six serotypes of encapsulated Hi are distinguishable (a, b, c, d, e, and f), and there are also non-encapsulated or non-typeable Hi (NTHi). Encapsulated strains exhibit a higher ability to cause invasive disease because the capsule prevents complement-mediated bacteriolysis in the absence of opsonizing antibodies (Prajapati *et al.*, 2017).

Haemophilus influenzae, serotype b

Of the six serotypes, serotype b (Hib) was a common cause of childhood meningitis before the Hib conjugate vaccine was introduced (Slack, 2006).

Despite the widespread use of vaccines, several populations remain vulnerable to Hib disease even with vaccination (Zarei *et al.*, 2016). Moreover, Hib vaccination does not confer protection against other serotypes of Hi. Other serological types of Hi, besides Hib, cause significant morbidity and mortality and their prevalence appears to be increasing in the Hib vaccine era (Zarei *et al.*, 2016).

Haemophilus influenzae, serotype a

Non-Hib diseases are becoming an emergency worldwide; likely Hib, of Hi serotype a (Hia) capsule represents an important virulence factor contributing to development of invasive disease (Ulanova *et al.*, 2009). It is unknown if the emergence of invasive Hia disease is due to the serotype

replacement after the wide use of Hib vaccine for child immunization (Ulanova *et al.*, 2014; Ladhani *et al.*, 2010). Given that Hia disease did not received adequate surveillance worldwide, it is now recognized as a pathogen responsible of serious diseases comparable to Hib in severity and case-mortality rates (Lima *et al.*, 2010; Gona *et al.*, 2014; Jin *et al.*, 2007) and deserving of interest to understand the evolving nature of the disease both to control the development of infection and for prevention. So far, no *in vitro* studies are conducted to clarify the mechanisms by which Hia, which colonizes the upper respiratory tract, is able to cross BBB, leading to meningitis.

AIM OF THE STUDY

Despite the progresses of antimicrobial treatments, bacterial meningitis remain infection of the CNS with high morbidity and mortality, (Kim, 2008). It is known that the bacteria can leave the principal organ of infection by using the blood flux and, as circulating bacteria, reach and cross BBB leading to meningitis (Kim, 2003).

By using an *in vitro* model of BBB, made of hBMECs and hBMPCs in co-culture, we have hypothesized that Hia-infected endothelial cells release high amount of adenosine, which binds A_{2B}–ARs on hBMECs and hBMPCs, triggering VEGF release, responsible for BBB breakdown.

MATERIAL AND METHODS

All reagents and antibodies were purchased from Sigma (St. Louis, MO) or E. Merck (Darmstadt, Germany) unless otherwise indicated. Antibodies against α actin, NG2, Von Willebrand factor, VE-Cadherin, CREB, phosphorylated CREB, Rho-A and phosphorylated Rho-A were from Santa Cruz Biotechnology Inc. (CA).

Cell cultures

Primary human brain microvascular endothelial cells (hBMECs) and human brain microvascular pericytes (hBMPCs) were purchased from Innoprot and fed with basal medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1% endothelial cell growth supplement (ECGS).

Construction of *in vitro* BBB model

Transwell inserts (Transwells, Corning, Corning, NY) were coated on the upper and bottom side with 2 mg/ml solution of rat tail collagen before being placed in complete medium (half and half DMEM/HAM's). To construct an *in vitro* model of BBB, BRPC (2×10^4 cells cm^2) were first plated on the outside of the Transwell inserts membrane and placed upside down in the well culture plate to allow adhesion. After adhesion, the Transwells were inverted and BBEC (2×10^4 cells cm^2) were seeded on the top surface of the insert. Under these conditions, the two type of cells on direct contact established an *in vitro* BBB model within 3 days.

***H. influenzae* preparation and infection**

Hia strain (NCTC 8466, type a) was grown in chocolate agar (Difco, Sparks, MD) for 24 h at 37°C in an atmosphere enriched with CO₂ before to be harvested, suspended in 0.5 ml of PBS (Gibco, Invitrogen, Carlsbad, CA) and serially diluted to the needed concentration.

After reaching confluence, FBS-containing medium was removed from hBMECs and hBMPCs in monoculture and in co-culture, and serum-free medium was added 4h before infection with Hia (10⁷ CFU/well). After infection with bacteria, the co-cultures were washed three times with PBS, and hBMECs and hBMPCs were collected separately by trypsinization.

Electron microscopy

For Scanning Electron Microscopy (SEM) preparations, cells grown in co-culture on filters were fixed with 1.5% glutaraldehyde overnight, following by an additional fix in 1% OsO₄ (1 h). Then, cells on the membrane were dehydrated in graded ethanol, critical point dried, sputtered with 5 nm gold layer using an Emscope SM 300 (Emscope Laboratories, Ashford, UK) and observed using a Hitachi S-4000 (Hitachi High-Technologies America, Schaumburg, IL) field emission scanning electron microscope.

For Transmission Electron Microscopy (TEM), after dehydrating in a graded series of acetone, cells were embedded in Durcupan ACM (Fluka Chemika-Biochemika, Buchs, Switzerland). Ultrathin sections were cut using a Reichert Ultracut E microtome and double stained with uranyl acetate and lead citrate. Observations were carried out using a Hitachi H-7000 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Immunoblotting

The lysates of hBMECs and hBMPCs, grown in co-culture, control (uninfected) and incubated with Hia for 6h, were prepared for Western blotting. Membranes were incubated overnight at 4°C with primary monoclonal antibodies against β -actin, NG2, von Willebrand factor, VE-cadherin, CREB,

p-CREB, Rho-A and p-Rho-A (dilution, 1:1000) and then with a secondary antibody (dilution, 1:2000) for 1h at room temperature.

Fluorescence microscopy

To investigate changes in VE-cadherin in hBMECs in co-culture with hBMPCs, after 6h infection with Hia, hBMPCs, grown on one side of the filter, were scraped to leave only hBMECs. The filters with hBMECs were fixed by adding 4% paraformaldehyde, and processed for immunocytochemistry using anti-VE-cadherin antibody (dilution, 1:100) and, as a secondary antibody, green fluorescence-labeled fluorescein isothiocyanate (dilution, 1:1,000). Hoechst® 33342 nucleic acid stain, was used to highlight the nuclei. The distribution of immunocomplexes was observed by an epifluorescent microscopy using appropriate filters.

Evaluation of the barrier integrity

Millicell electrical resistance system (Millipore) was used to measure TEER. Values were expressed as $\Omega \times \text{cm}^2$ and were calculated by the following formula: (average resistance of experimental wells x average resistance of blank wells) x 0.33 (area of the Transwell membrane). To address the role of ARs in BBB dysfunction, experiments were carried out by incubating the co-cultures, in presence or in absence of Hia infection, with 1 μM 8-[4-[4-(4-Chloro-phenyl) piperazine-1-sulfonyl) phenyl]]-1-propylxanthine (PSB 603), a specific A_{2B} adenosine receptor antagonist or with 1 μM 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4] triazolo[1,5-c]pyrimidin-5-amine (SCH58261), a specific A_{2A} adenosine receptor antagonist or with 1 μM 8-cyclopentyl-1,3- dipropylxanthine (DPCPX), a specific A_1 adenosine receptor antagonist. In parallel, co-cultures were treated with 1 μM 2-Chloro-N6-cyclopentyladenosine (CCPA), a specific A_1 adenosine receptor agonist or with 1 μM 2-(2-carboxyethyl)- phenethyl-amino-5'-N-ethylcarbox-amidoadenosine (CGS 21680), a specific A_{2A} adenosine receptor agonist or with 1 μM

5'-(N-ethylcarboxamido)-adenosine (NECA), a generic A₂ adenosine receptor agonist in absence of Hia infection.

Moreover, TEER was measured after blocking of VEGFR-1 and VEGFR-2 by their specific antibodies (Ab) in both hBMECs and in hBMPCs in co-culture. For these simultaneous blockade experiments, co-cultures were treated with 2 µg/ml VEGFR1 or 2 µg/ml VEGFR2 Abs for 60 min before 6h treatment with Hia.

Bacterial invasion assay

Incubations of bacteria (10⁷ CFU/well) with hBMECs/hBMPCs co-culture were performed at 37°C for 1, 3, 6 and 9h to allow invasion to occur. At the end of the incubation times, hBMECs were removed from the filter and the number of intracellular bacteria was determined after incubation with gentamicin (100 µg/ml) for 1 h at 37°C to kill all extracellular bacteria. Cells were then lysed with 0.5% Triton X-100 and lysates were seeded on chocolate agar plates to be numbered the day after. The results were expressed as percent invasion [100 x (number of intracellular bacteria recovered)/(number of bacteria inoculated)].

Cell viability

The number and viability of hBMECs/hBMPCs co-cultures after Hia infection for 1-3-6 and 9h was determined by trypan blue dye. Cells from inserts were trypsinized separately, cell suspensions were mixed with a 0.4% trypan blue solution, and the number of live cells was determined using a hemocytometer.

ENTPDase enzymatic activity assay

ENTPDase/5-nucleotidase enzymatic activity was determined by the release of inorganic phosphate (Pi). Briefly, 1 × 10⁵ hBMEC or hBMPC in monoculture, or hBMEC/hBMPC co-cultures, infected

or non infected, were washed 3 times with phosphate-free buffer and pre-incubated in 100 μ L buffer in a 96-well microplate at 37°C for 30 min. To distinguish between CD37 and CD73 activity, 200 μ M ARL67156, a CD39-specific inhibitor, or 100 μ M methylene adenosine5'-diphosphate (APCP), a CD73-specific inhibitor, was added. The release of Pi was determined by using the Malachite Green Phosphate Detection Kit (R&D Systems) according to the manufacturer's protocol.

cAMP Detection Assay

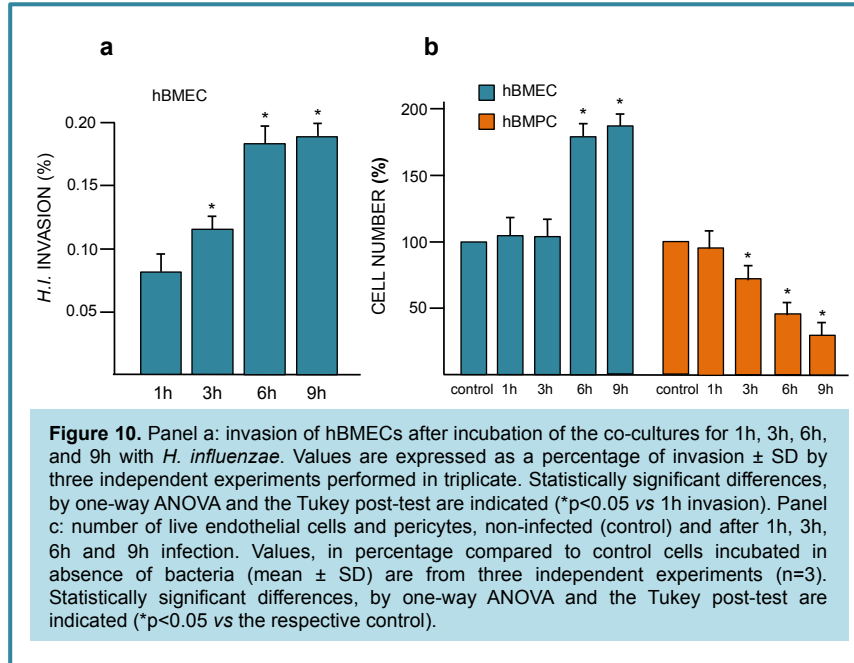
Total cAMP concentration was evaluated by using cAMP Biotrak Enzyme Immunoassay (Amersham Biosciences, Piscataway, NJ, USA). Control cells, non-infected, were incubated with forskolin (10 μ mol/L). Mono- and co-cultures were infected 6h with Hia, in the absence or presence of SCH58261 (1 μ M) or PSB603 (1 μ M). After incubation, the two cell types were trypsinized separately from inserts and lysed for cAMP determination, following the manufacturer's protocols.

Statistical analysis

Statistical significance between two groups was analyzed by Student's test. One-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was used to compare the mean for the multiple groups. *P* values < of 0.05 were considered statistically significant.

RESULTS

Bacterial invasion modified cell number

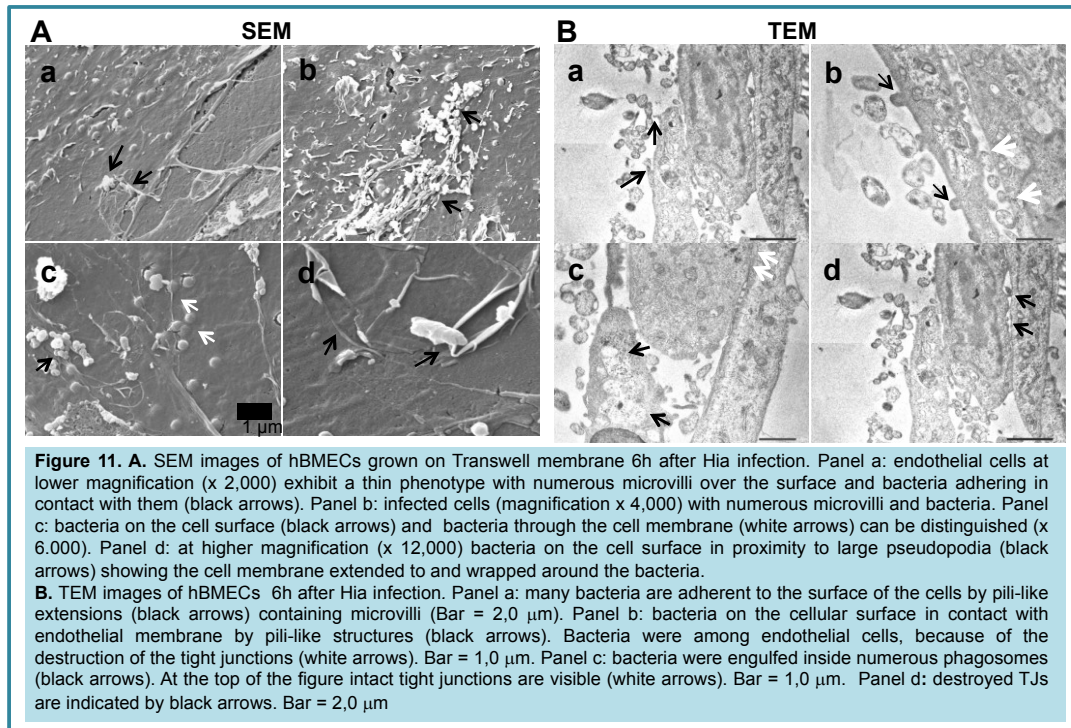


Invasion after 3h and 6h of infection increased 1.5 and 2.2 fold, respectively, compared to invasion after 1h (Fig. 10, panel a), whereas the percentage of infection after 9h was similar to that of 6h, indicating that the majority of bacteria were able to enter cells after 6h of incubation. So, we have chosen 6h incubation for all the infection experiments. Trypan blue exclusion test showed that after 1h infection, cell viability was not affected (Fig. 10, panel b). After 3h, 6h and 9h of infection, hBMPCs number decreased 1.3-, 2.1- and 3.3 fold, respectively, compared to non-infected cells (control), whereas the number of endothelial cells after 6h and 9h infection increased 1.8- and 1.9 fold, in comparison with control cells. Probably, the increased endothelial cell number is related to the detachment of pericytes which would no longer be able to control endothelial proliferation.

H. influenzae is able to enter hBMECs

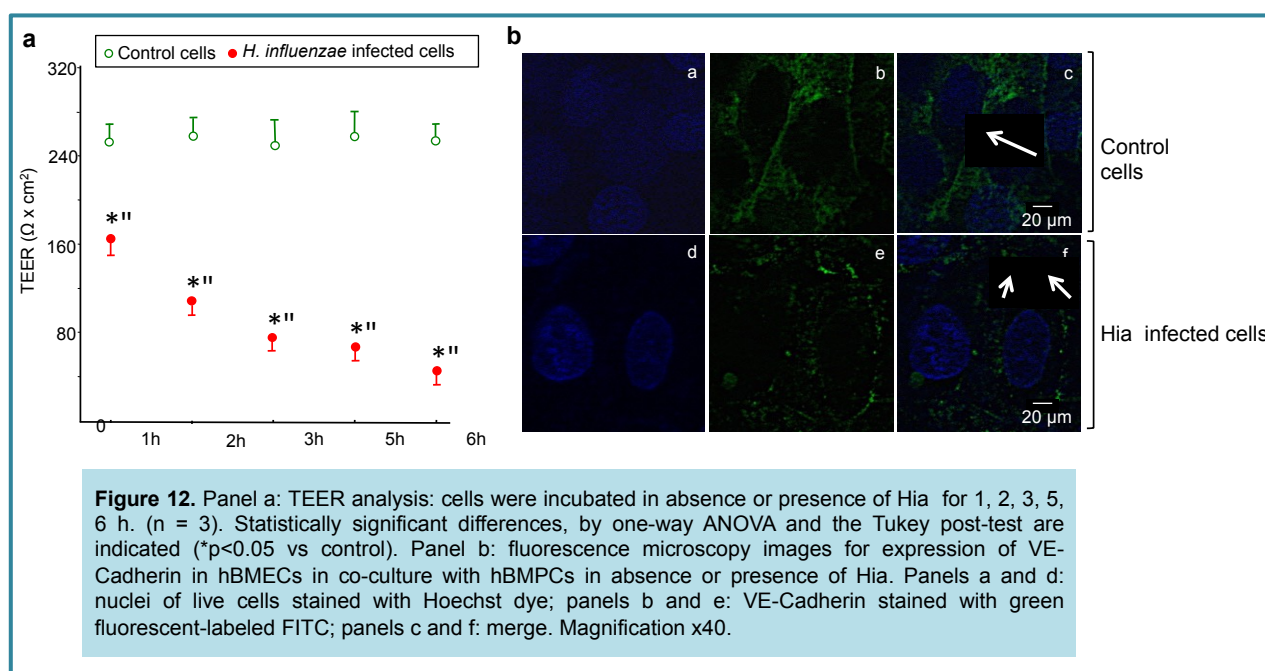
Fig. 11, panel A, shows SEM images of hBMECs grown on Transwell filters at 6h post-infection

with Hia. In panels a and b, the endothelial cells at lower magnification show a thin phenotype with numerous microvilli on the surface and bacteria adhering in contact with them (black arrows). In panel c, bacteria are visible on the cell surface (black arrows) and through the cell membrane, suggesting that Hia were able to adhere and enter endothelial cells. In panel d, bacteria in contact with large pseudopodia are visible. In 11, panel B, TEM images of hBMECs infected 6h with Hia are shown. Panels a and b show numerous bacteria in contact with the surface of the cells by pili-like extensions containing microvilli and bacteria engulfed inside phagosomes. Many bacteria are present in the space between the endothelial cells because of the disruption of TJs.



***H. influenzae* reduced TEER and VE-cadherin expression in hBMEC**

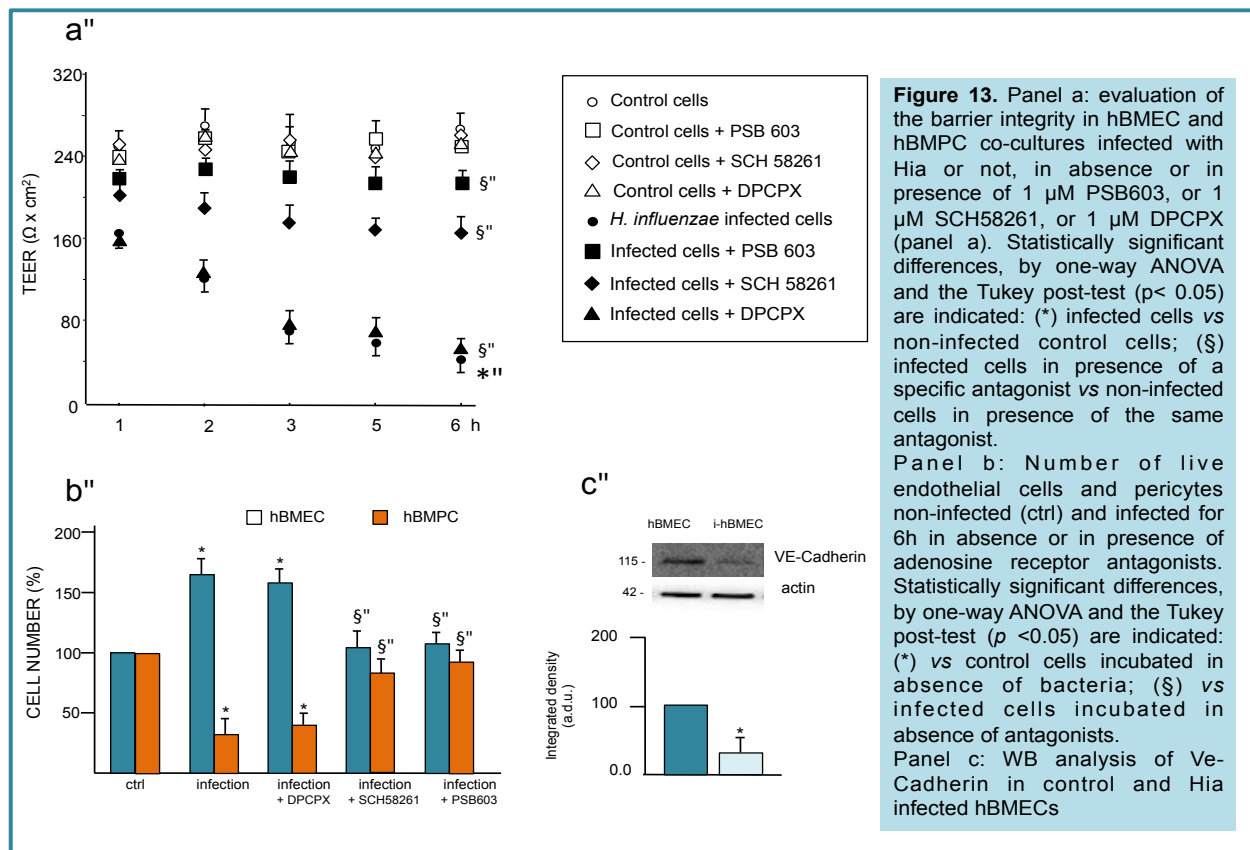
Incubation of co-cultures for 1-6h with Hia significantly reduced TEER values (about 6.2 fold after 6h) compared to non-infected control co-cultures at the same time of incubation (Fig. 12, panel a). These results were confirmed by the fluorescence-microscopy images, where the distribution of fluorescent VE-cadherin was reduced in Hia infected cells compared to control cells (Fig. 12, panel b).



Adenosine receptors were involved in *H. influenzae* infection

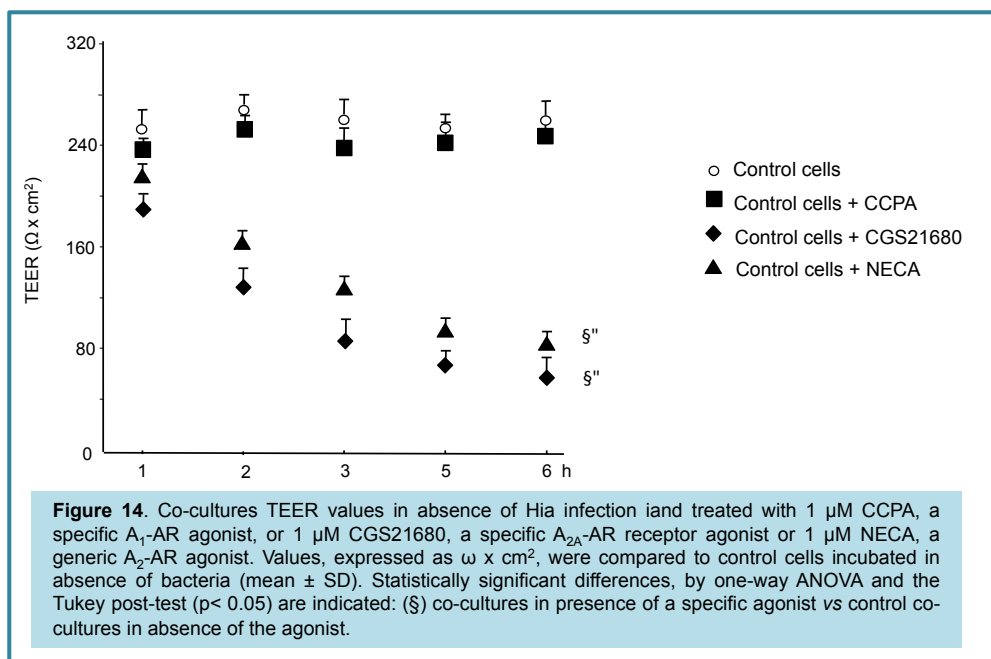
To deeper investigate the effect of adenosine, produced by infected endothelial cells, on close pericytes, TEER measurements after bacterial infection in presence of specific ARs antagonists was measured (Fig. 13). Bacterial infection significantly reduced TEER values (6.7 fold lower than control values after 6h, Fig. 13, panel a). Co-incubation of Hia with DPCPX, a specific A_1 adenosine receptor antagonist, did not change TEER values compared to 6h infected cells in absence of DPCPX. Conversely, cells co-incubated with Hia and SCH58261, a specific A_{2A} adenosine receptor antagonist, showed a significant increase of TEER values (4.1 fold) compared to 6h cells infected in absence of the antagonist. Even more surprisingly, a greater increase in TEER values was observed when Hia infection was performed together with PSB603 incubation, a specific A_{2B} adenosine receptor antagonist (5.4 fold increase), thus demonstrating that A_{2A} adenosine receptor and, more strongly, A_{2B} adenosine receptor play a pivotal role in BBB dysfunction during Hia infection. These results were confirmed cell count experiments after Hia infection in presence of the above mentioned antagonists (Fig. 13, panel b). Infection of the co-cultures with Hia for 6h reduced the pericyte number (3.3 fold) and increase endothelial cell number (1.7 fold). Infection in presence of DPCPX reduced pericyte number and increased

endothelial cell number likewise to infected cells without antagonist. When the infection was carried out in presence of SCH58261 or PSB603, the pericyte number increased 2.6 fold and 3.0 fold, respectively, compared with the number of infected pericytes in absence of antagonists. Under the same experimental conditions, endothelial cell number decreased 1.5 fold and 1.4 fold, in presence of SCH58261 and in presence of PSB603, respectively, compared with the number of infected endothelial cells in absence of antagonists. Thus, Hia is able to increase adenosine concentration inside endothelial cells, within which it is able to penetrate to cross BBB and reach the SNC. Western blot analysis showed a significant 4.0 fold reduction in the protein expression of VE-cadherin in infected hBMECs in comparison with control hBMECs (Fig. 13, panel c).



We investigated the role of the different ARs on integrity of BBB by incubating the co-culture with different adenosine receptor agonists, without bacterial infection. As shown in Fig. 14, treatment of co-culture for 6h with CCPA, a specific A_1 adenosine receptor agonist, did not changed TEER values, whit respect to control cells. Co-cultures incubated with CGS 21680, a specific A_{2A}

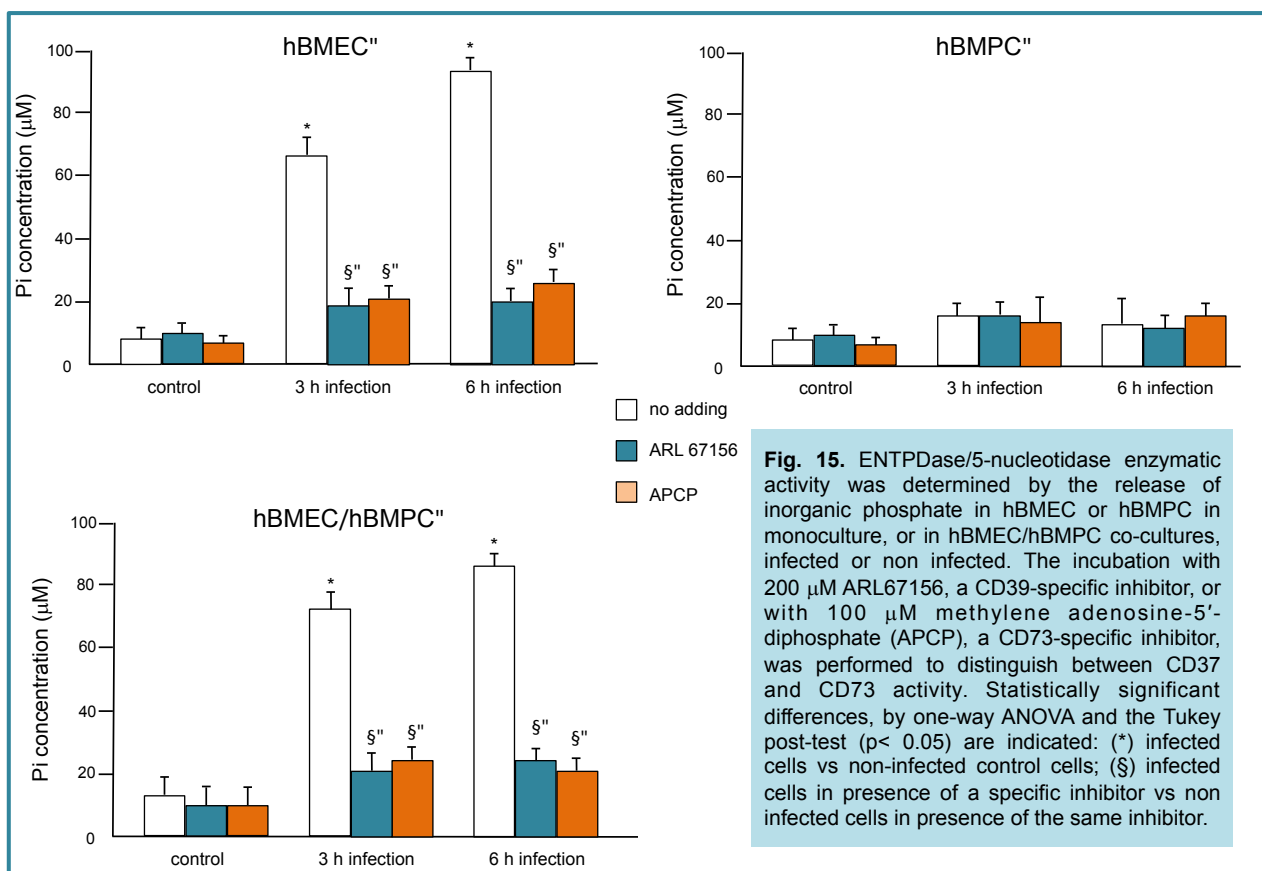
adenosine receptor agonist, or with NECA, a generic A_2 adenosine receptor agonist, showed a decrease of TEER values by 4.3- and 3.2 fold, respectively, in comparison with control cells. Thus, we could speculate that ARs may be able to control the integrity of BBB when stimulated with physiological adenosine concentrations. Conversely, high adenosine concentrations, released after a bacterial infection (in this experiments mimed by 6h treatment with adenosine A_2 receptor agonists), could lead to breakdown of the BBB.



Ectoenzyme activity

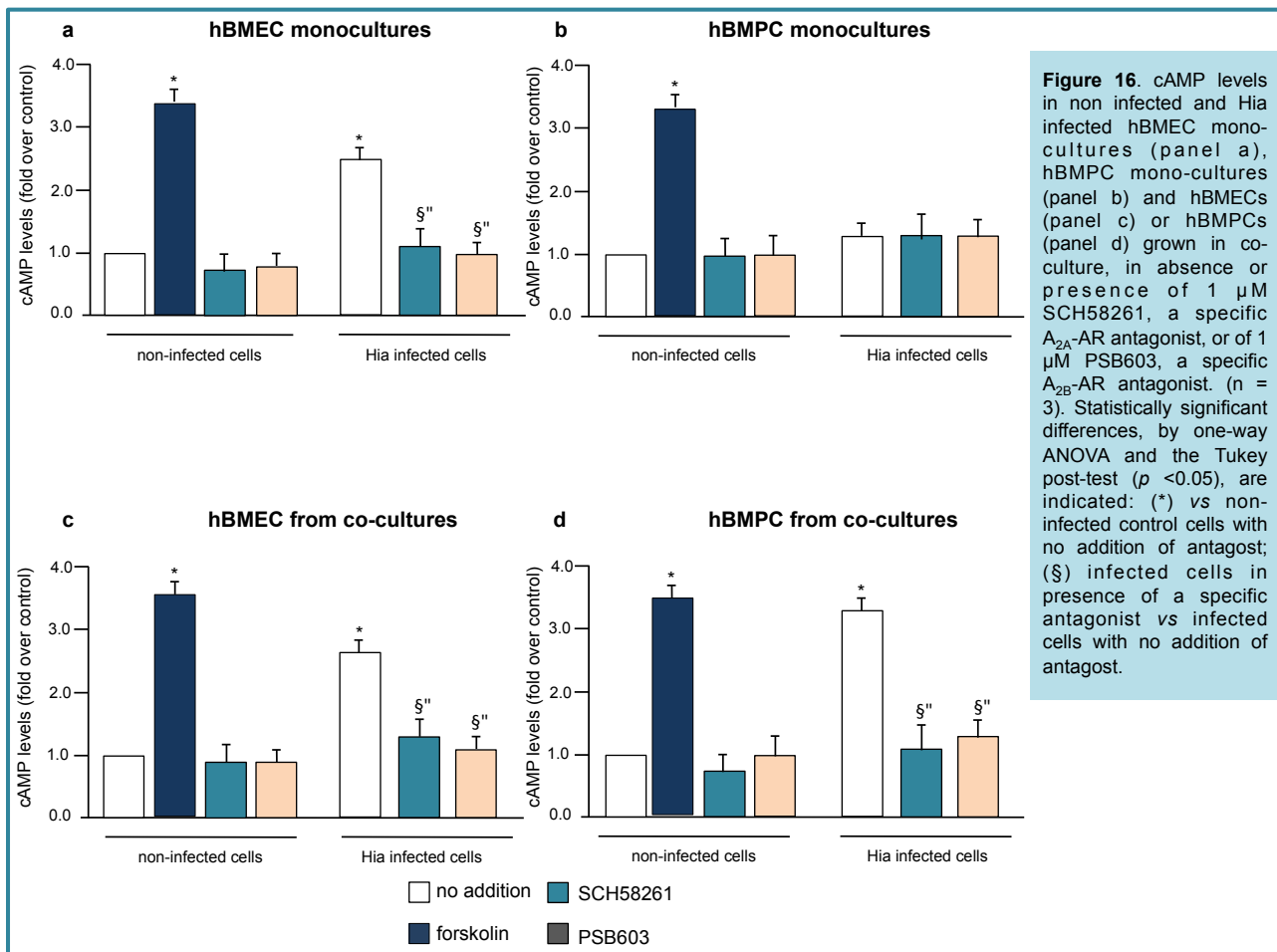
Ectoenzyme activity, determined by measuring Pi generated during ectoenzyme-mediated nucleotide dephosphorylation in hBMEC and hBMPC grown in mono- and in coculture, is shown in Fig. 18. After bacterial infection for 3 and 6h, the activity in hBMEC was significantly enhanced by 6.5 and 9.5 fold, respectively, indicating that bacterial infection was able to trigger adenosine synthesis. ENTPDase activity in infected endothelial cells significantly decreased in presence of 200 μ M ARL67156, a CD39-specific inhibitor, or 100 μ M methylene adenosine 5'-diphosphate (APCP), a CD73-specific inhibitor. In particular, ectoenzyme activity decreased 3.2 and 2.9 fold,

after bacterial infection for 3h, in presence of ARL67156 and APCP, respectively and, after 6h infection, the activity decreased 4.5 and 3.8 fold in presence of ARL67156 and APCP, respectively, compared to the activity in infected cells in absence of antagonists, at the same time of incubation. No difference was found in the enzyme activity after infection of hBMPC for 3 and 6h. The concentration of Pi released in the medium of infected co-cultures increase 4.8 and 5.6 fold after infection for 3h and 6h, respectively. Ectoenzyme activity decreased 3.7 and 3.0 fold, after bacterial infection for 3h, in presence of ARL67156 and APCP, respectively and, after 6h infection, the activity decreased 3.4 and 4.2 fold in presence of ARL67156 and APCP, respectively, compared to the activity in infected cells in absence of antagonists, at the same time of incubation. The data demonstrated that in hBMEC, bacterial infection significantly activates CD39 and CD73, enzymes which determine an increase in concentration of adenosine.



Bacterial infection increased cAMP production in co-cultures

Fig. 16 shows cAMP levels of hBMECs and hBMPCs, grown in mono- or in co-culture, after 6h infection with Hia in absence or in presence of specific ARs antagonists. Incubation with 10 μ mol/L forskolin (direct activator of adenylyl cyclase) significantly increased the amount of cAMP; similarly, 6h Hia infection significantly enhanced the production of cAMP in hBMEC monocultures (2.6 fold increase) in comparison with non-infected cells in absence of antagonists (Fig. 16, panel a). Infection performed in presence of SCH58261 or PSB603, decreased cAMP levels values very similar to non-infected cells without antagonists. In hBMPC monocultures (Fig. 16, panel b), forskolin significantly increased cAMP levels while the infection for 6h did not change cAMP levels, either in absence or in presence of SCH58261 or PSB603. In hBMECs grown in co-cultures, forskolin significantly increased cAMP levels (3.0 fold) with respect to non-infected cells (Fig. 16, panel c). Co-incubation of Hia with SCH58261 or PSB603, decreased cAMP levels by 2.3 fold and 2.7 fold, compared to infected cells without antagonists. Surprisingly, in hBMPCs grown in contact with hBMECs (Fig. 16, panel d), 6h infection induced an increase of cAMP very similar to forskolin treatment. Co-incubation of Hia with SCH58261 or PSB603, decreased cAMP levels to values similar to non-infected cells. These results suggest that the response of pericytes to bacterial infection is not due to contact with bacteria but depends on release of adenosine by endothelial cells, which are very close to pericytes. Activation of adenosine A_{2A} and A_{2B} receptors results in increased cAMP levels, activation of cytosolic proteins, physiologically involved in maintaining the contractile tone, leading to detachment of pericytes from endothelial cells.



H. influenzae stimulated VEGF release

As shown in Table 6, hBMEC-infected monoculture released 2.5 fold increase more VEGF with respect to non-infected cells; co-incubation of hBMECs in monoculture in presence of Hia and 1 μ M PSB603 or 1 μ M SCH58261 reduced VEGF release by 2.7- and 2.3, fold respectively, while the presence of 1 μ M DPCPX during the infection did not changed VEGF release compared to infected cells in absence of antagonists.

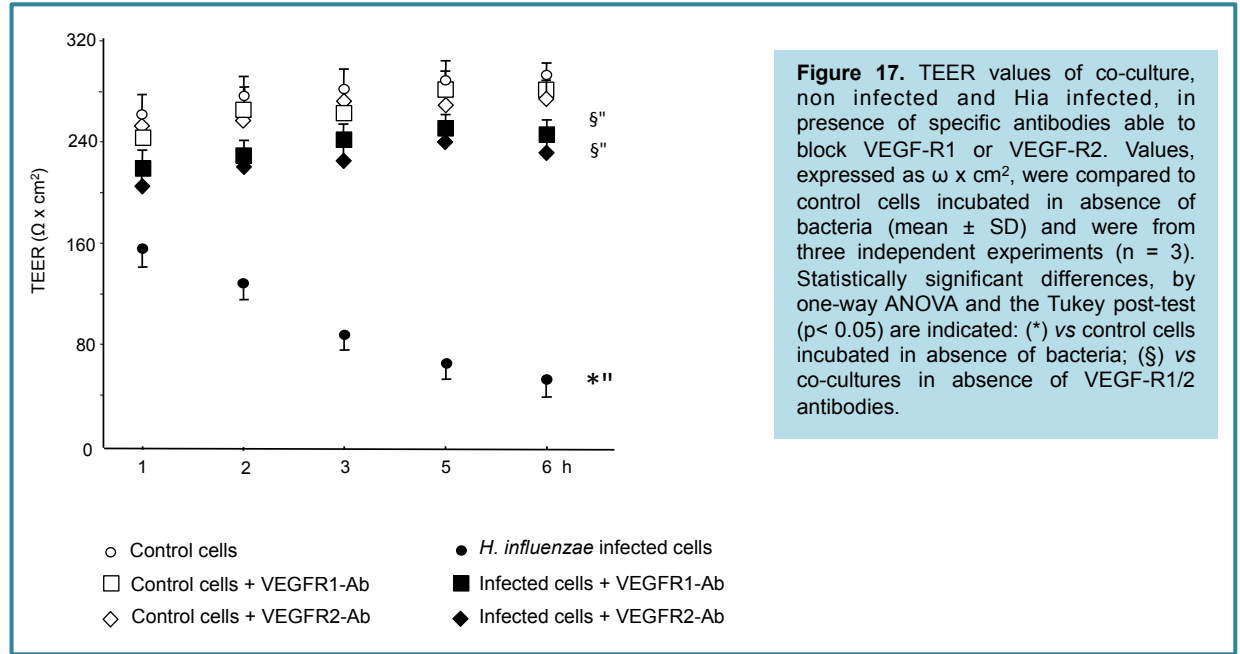
Furthermore, VEGF released by infected co-cultures increased 2.8 fold compared to non-infected cells. The presence of PSB603 or SCH58261, but not the presence of DPCPX, reduced bacterial-induced VEGF release by 4.3- and 3.3 fold, respectively, with respect to infected co-cultures without antagonists. Moreover, treatment of non-infected co-cultures with PSB603 or SCH58261, but not with DPCPX, reduced VEGF release by 1.4- and 1.6 fold, respectively, with respect to non-

infected co-cultures without antagonists. These results suggest that the adenosine binding to its receptors, mainly A_{2A}- and A_{2B}-AR, is involved in VEGF release both during the physiological cross talk between endothelial cells and pericytes and during bacterial infection.

VEGFA (pg ml ⁻¹)	Control cells	Cells + <i>H. influenzae</i>
hBMEC	33.7 ± 2.1	84.5 ± 7.3**
hBMEC+PSB603	29.2 ± 2.3*	30.5 ± 2.5*
hBMEC+SCH58261	34.1 ± 2.8*	36.9 ± 3.1*
hBMEC+DPCPX	28.6 ± 2.3*	77.5 ± 6.4*
hBMPC	50.9 ± 4.8**	55.6 ± 4.4
hBMPC+PSB603	48.7 ± 3.9*	53.8 ± 5.6*
hBMPC+SCH58261	47.3 ± 4.2*	44.0 ± 3.8*
hBMPC+DPCPX	51.2 ± 4.9*	57.2 ± 4.7*
hBMEC/hBMPC	157.3 ± 11.6§	439.9 ± 38.5***
hBMEC/hBMPC+PSB603	108.6 ± 9.7*	100.5 ± 11.4*
hBMEC/hBMPC+SCH58261	97.9 ± 8.6*	131.3 ± 12.5*
hBMEC/hBMPC+DPCPX	148.8 ± 10.1*	446.3 ± 40.8*

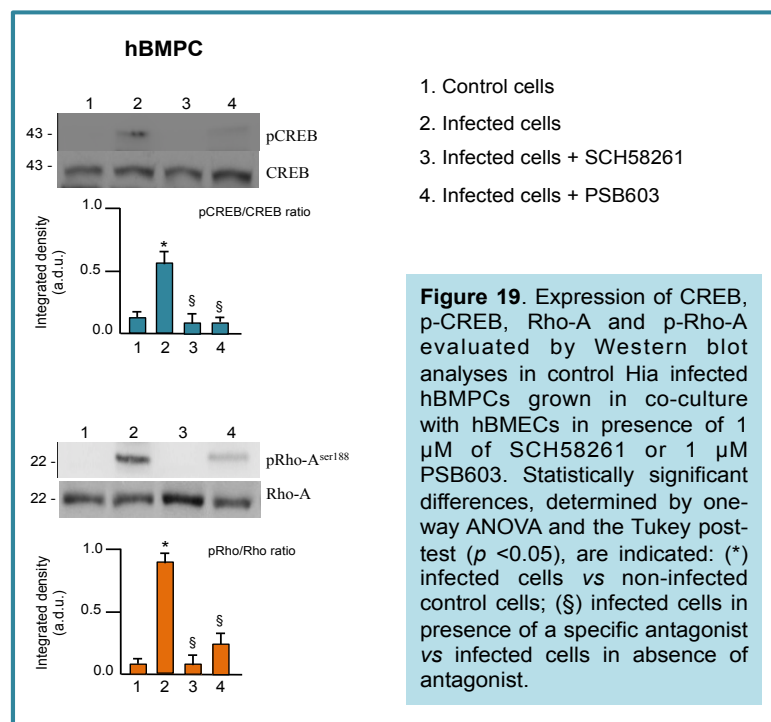
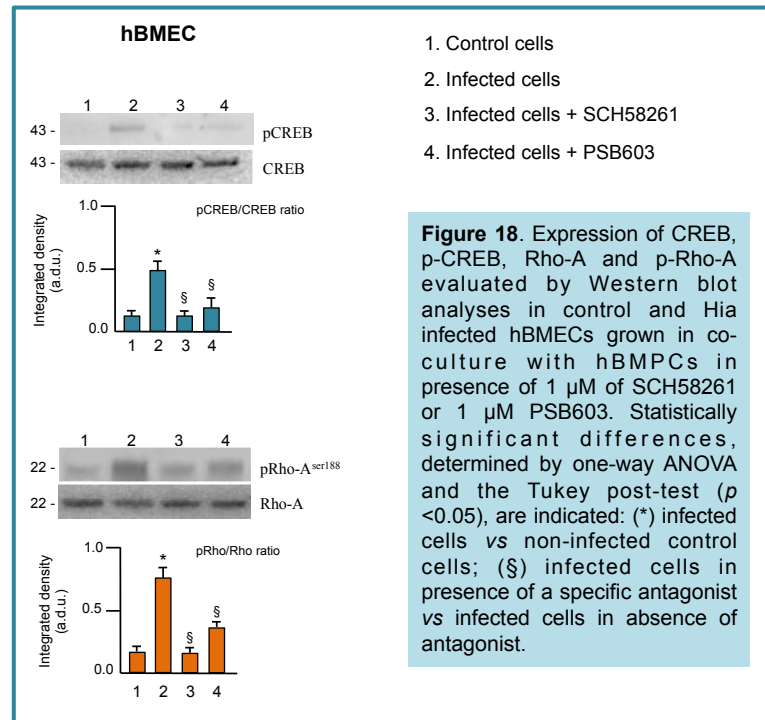
Table 6. VEGFA determination in hBMECs and hBMPCs in mono- and co-culture infected and non-infected by *H. influenzae*. Cell culture supernatants from mono- and co-cultures in absence (control) and presence of *H. influenzae* (10⁷ CFU/well), for 6h, with or w/o 1 µM PSB603, a specific A_{2B} adenosine receptor antagonist or with 1 µM SCH58261, a specific A_{2A} adenosine receptor antagonist or with 1 µM DPCPX, a specific A₁ adenosine receptor antagonist, were assayed for VEGF release. Inhibitors were added to the culture medium 60 min before *H. influenzae* addition. Values (means ± S.E.M.) are from three independent experiments (n = 3). Statistically significant differences by one-way ANOVA and the Tukey *post-test* (p<0.05) are indicated. (*) vs the same group w/o inhibitors; (**) vs control hBMEC; (***) vs control hBMEC / hBMPC co-cultures; (§) p<0.05 vs mono-cultures.

To deeper demonstrate the involvement of VEGF in BBB impairment generated by Hia infection, TEER analysis was performed in presence of specific antibodies blocking VEGF-R1 or VEGF-R2 (Fig. 17). Hia reduced TEER values, which were rescued by co-incubation with VEGF-R1 and VEGF-R2 Abs, thus demonstrating that VEGF, released after Hia infection, may be responsible for the endothelial cell proliferation and consequent BBB collapse.



CREB and Rho activation in co-cultures

It is known that the transcription factor CREB is involved in the expression of VEGF in microvascular endothelial cells after A_{2B} -AR stimulation (Acurio *et al.*, 2016; Du *et al.*, 2015). It is also known that Rho mediates BBB permeability by inducing morphological changes in actin-cytoskeletal reorganization (Do-Geun *et al.*, 2015). For these reason, we decided to address the effects of A_{2A} and A_{2B} -AR antagonists on CREB and Rho activation in hBMECs/hBMPCs co-culture after Hia infection. CREB and Rho-A protein expression did not changed in control and infected hBMECs in absence or in presence of antagonists. Phosphorylation of CREB and Rho-A significantly increased after Hia infection of hBMECs, with respect to non-infected cells, and the co-incubation with SCH58261 and PSB603 significantly abrogated the phosphorylation of CREB and Rho-A induced by infection (Fig. 18). Likewise, CREB and Rho-A protein expression did not changed in control and infected hBMPCs, in absence or in presence of antagonists. Phosphorylation of CREB and Rho-A significantly increased in infected hBMPC and was significantly reduced in response to A_{2A} - and A_{2B} -ARs antagonists (Fig. 19).



DISCUSSION

This study highlights the role of A₂A and A₂B adenosine receptors in maintaining the integrity of the blood brain barrier following Hia infection. The peculiar characteristic of endothelial cells of the brain microvessels is to possess TJs at their intercellular contacts (Wolburg et al., 2002; Weksler et al., 2005), responsible for their ability to block the passage of many agents. The in vitro model of BBB, used in this study and characterized by high TEER values, was performed by co-culturing human brain microvascular endothelial cells and pericytes; in these conditions, the two cell types have reproduced the physiological conditions present in BBB, thus repeating the highly effective barrier that separates the brain micro-environment from the circulating blood.

The interaction between the pathogenic microorganisms and the microvascular endothelial cells, responsible of the crossing of BBB, is characterized by several steps, the first of which is the link between host receptors and bacterial components (St Geme et al., 1996). Hia can interact with microvascular endothelial cells via OmpP2, which binds to the carboxy-terminal domains of laminin receptors (Orihuela et al., 2009) or by using pili or fibrils (St Geme et al., 1996). An important role, during the early phases of Hia adhesion, plays the platelet activating factor receptors, which facilitates the cross of bacteria into the CNS (Swords et al., 2001). The adhesion to these internalization receptors allows Hia to enter microvascular endothelial cells, thus determining the BBB collapse.

It has been shown that the breakdown of the barrier can be caused both by intracellular crossing of bacteria and their paracellular translocation; this latter mechanism is used by bacteria belonging to the *Haemophilus* family, which can cross the BBB and colonize the brain from the respiratory tract of infected patients (Dando et al., 2014).

In this study, we demonstrated that Hia cross the BBB with either intracellular and paracellular mechanism, as evidenced by TEM images. After contact between Hia and co-cultures

of endothelial cells and pericytes for 6h, the bacteria are visible both into endothelial cells and between the cells, demonstrating that both mechanisms are used by Hia to reach the brain .

Data obtained from invasion assays showed that a high percentage of bacteria were localized within the endothelial cells after 6h, and the confirmation of this result was provided by SEM and TEM images. At the same time of incubation, the cell count highlighted a reduction in the pericyte number and an increase in the endothelial cells number. The increased endothelial proliferation confirms the data from literature demonstrating the role of pericytes in the control of endothelial proliferation and in the stabilization of the microvessels (Trost et al., 2016). The consequence of this series of events is the BBB collapse, highlighted by a sharp reduction in both TEER values and VE-cadherin expression after infection by Hia for 6h.

Many factors, such as transforming growth factor β , angiopoietins, platelet-derived growth factor β , sphingosine-1-phosphate and Notch have a pivotal role in the intercellular communication between endothelial cells and pericytes (Dore-Duffy, 2008). It has been demonstrated the significant role of adenosine receptors in the maintaining of BBB permeability (Bynoe et al., 2015) and the presence of CD73 ecto-enzymes in hBMECs as well as the involvement of AR signaling in the modulation of BBB permeability following the binding of selective agonists (Do-Geun et al., 2015), thus promoting the entry of macromolecules into the CNS. Conversely, the reduction of the passage of macromolecules into the brain by AR antagonists makes the binding of the adenosine to a specific receptor in the BBB as a key that allows or blocks the passage of substances and bacteria by (Bynoe et al., 2015).

We speculated that the infection of brain endothelial cells can cause production of high concentration of adenosine which in turn could activate AR of adjacent pericytes, increasing BBB permeability. It has been demonstrated that the activation of A_{2A} AR causes relaxation of pericytes (Matsugi et al., 1997) and that the activation of AR in pericytes determines the opening of the K^+ -ATP channel and the increase of the capillary blood flow (Li et al., 2001).

By measuring the inorganic phosphate (Pi) generated during the ectoenzyme-mediated

nucleotide dephosphorylation in hBMEC grown in mono-culture and in hBMEC/hBMPC coculture, we indirectly demonstrated, that bacterial infection significantly activates CD39 and CD73 enzymes (ecto-enzymes responsible for generating extracellular adenosine), determining an increase in adenosine concentration. Differently, in hBMPC in mono-culture, the infection did not activate the ecto-enzymes. It has been already demonstrated that CD39 and CD73 are highly expressed on both primary human brain endothelial cells and human brain endothelial cell line HCMEC-D3 and that A_{2A} AR activation increases paracellular permeability in primary human brain endothelial cell monolayers (Do-Geun *et al.*, 2015).

Experiments, performed by infecting co-cultures in the presence of different AR antagonists demonstrated that the inhibition of A_{2A} and A_{2B} AR, but not A₁ AR, maintains unaltered the barrier characteristics. This was evidenced by high TEER values when infection by Hia was conducted in the presence of PSB 603, a specific A_{2B} adenosine receptor antagonist or with SCH58261, a specific A_{2A} adenosine receptor antagonist but not in the presence of DPCPX, a specific A₁ adenosine receptor antagonist. These results confirmed that A₁AR does not seem to be involved in BBB disfunction following Hia infection. Furthermore, PSB 603 and SCH58261 were able to prevent the pericytes loss following bacterial infection and consequently to prevent endothelial cells uncontrolled proliferation. In contrast, in the presence of DPCPX, the pericyte number remained very low after bacterial infection.

These results agree with those of other studies demonstrating that functional A₂ ARs are present on hBMPCs and hBMECs while the A₁ subclass is relatively inactive biologically, and highlighting the importance of cross-talk between the two cellular components in the maintaining of the integrity of the BBB (Takagi *et al.*, 1996).

To further confirm data obtained, experiments were conducted by incubating non-infected cells with AR agonists: TEER values were significantly reduced when co-cultures were incubated with CGS 21680, a specific A_{2A} adenosine receptor agonist, or with NECA, a generic A₂ adenosine receptor agonist, highlighting the role of A_{2A} and A_{2B} AR in maintaining the BBB characteristics.

Furthermore, in hBMECs, both in mono- and in co-culture, the cAMP levels were found high after Hia infection, confirming that A₂ AR was stimulated by Hia infection but not A₁ AR, because its stimulation does not increase cAMP levels. Furthermore, after infection for 6h, pericytes in co-culture with endothelial cells, but not pericytes in mono-culture, produced high cAMP concentrations, confirming that adenosine produced by endothelial cells can bind AR on pericytes. In infected hBMECs, both in mono- and in co-culture, and in hBMPCs in co-culture, cAMP concentration was reduced in the presence of A_{2A} and A_{2B} AR antagonists. This data suggests that hBMECs produced high adenosine concentrations after bacterial infection and that adenosine binds AR on pericytes, determining their detachment.

Our results also demonstrated that bacterial infection promotes the VEGF release in hBMPCs in co-culture and in hBMECs, both in mono- and in co-culture. Adenosine could be an important putative messenger responsible for the VEGF release by binding AR on endothelial cells. VEGF could be responsible for the BBB impairment after bacterial infection, because the blocking of VEGF receptors shields the BBB from the infection-induced breakdown (Anfuso et al., 2014; Lupo et al., 2014). These results were confirmed by data demonstrating that A_{2B} AR activation is involved in the induction of angiogenesis and VEGF production from BMECs and that A_{2A} AR stimulates VEGF gene expression in response to hypoxia-induced accumulation of adenosine in retinal vascular cells (Ryzhov et al., 2014).

In the model of BBB used in our experiments, we demonstrated that bacterial infection induced the activation of CREB and Rho-A and that A_{2A} and A_{2B} antagonists were able to inactivate them. Previous studies demonstrated the up-regulation of VEGF and cAMP-PKA-CREB signaling pathway in adenosine-induced angiogenesis in human endothelial cells (Yang et al., 2016; Nathan et al., 2016) and an A_{2A}-induced increase in Rho-A activity, cytoskeletal modification and BBB permeability (Do-Geun et al., 2015).

Our study, according to recent reports in literature, demonstrated that the moderate increase in adenosine concentration is able to inducing beneficial effects such as vasodilation and control of

the release of inflammatory molecules; chronic overproduction of adenosine, which can occur during various pathologies (Borea et al., 2017), can have adverse effects on the functionality of different organs and on BBB, which can undergo to the loss of its features. Among the pathologies involved in the over-production of adenosine, the bacterial infection also plays an important role. Bacterial meningitis is an important cause of child mortality in the clinical setting (Almeida et al., 2017; Hammitt et al., 2005; Ouchenir et al., 2017), and this study demonstrated the correlation between the signalling pathway, triggered by adenosine, and BBB breakdown after Hia infection, highlighting the modulatory role of A_{2A} and A_{2B} adenosine receptors.

The innovative aspect of this research is based on the demonstration of the existence of adenosine signalling in microvascular pericytes, responsible for TJ formation between endothelial cells and for the control of endothelial proliferation, thus inducing BBB properties. To date, the involvement of pericytes in Hia infection response has not been demonstrated.

Because the pericytes play a crucial role in microvascular stabilization, the reduction of the pericyte number during bacterial meningitis is a particularly important event responsible for the loss of the barrier functions. For their function, pericytes could be highly suitable for cell-based therapies in many infectious diseases, and the study of pathways leading to pericyte malfunction throws the bases for the development of new pharmaceutical targets.

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