PATTERN RECOGNITION RECEPTORS AND INFLAMMASOME ACTIVATION IN THE BLOOD-BRAIN BARRIER: NEW PLAYERS IN NEUROINFLAMMATION

PH.D. THESIS

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SZEGED

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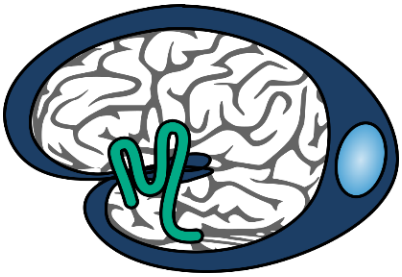

PATTERN RECOGNITION RECEPTORS AND INFLAMMASOME ACTIVATION IN THE BLOOD-BRAIN BARRIER: NEW PLAYERS IN NEUROINFLAMMATION

Ph.D. Thesis

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Szeged

2018

"Research is to see what everybody has seen
and think what nobody has thought."

Albert Szent-Györgyi

LIST OF PUBLICATIONS

The Ph.D. thesis is based on the following *in extenso* publications:

I. **Péter Nagyőszi*, Ádám Nyúl-Tóth*, Csilla Fazakas, Imola Wilhelm, Mihály Kozma, Judit Molnár, János Haskó, István A. Krizbai.** Regulation of NOD-like receptors and inflammasome activation in cerebral endothelial cells. *JOURNAL OF NEUROCHEMISTRY*, 2015; 135: 551-564. Impact factor: 3.842; **Quartile Score: Q1**
***shared first authorship**

II. **Ádám Nyúl-Tóth, Mihály Kozma, Péter Nagyőszi, Krisztina Nagy, Csilla Fazakas, János Haskó, Kinga Molnár, Attila E. Farkas, Attila G. Végh, György Váró, Péter Galajda, Imola Wilhelm, István A. Krizbai.** Expression of pattern recognition receptors and activation of the non-canonical inflammasome pathway in brain pericytes. *BRAIN BEHAVIOR AND IMMUNITY,* 2017; 64: 220-231. Impact factor: 5.964; **Quartile Score: D1**

III. **Imola Wilhelm, Ádám Nyúl-Tóth, Mihály Kozma, Attila E. Farkas, István A. Krizbai.** Role of pattern recognition receptors of the neurovascular unit in inflamm-aging. *AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY*, 2017; 313: H1000-H1012. Impact factor: 3.348; **Quartile Score: Q1**

Further publications related to the field but not included in the Ph.D. thesis:

IV. **Ádám Nyúl-Tóth, Maria Suciu, Judit Molnár, Csilla Fazakas, János Haskó, Hildegard Herman, Attila E. Farkas, József Kaszaki, Anca Hermenean, Imola Wilhelm, István A. Krizbai.** Differences in the molecular structure of the blood-brain barrier in the cerebral cortex and white matter: an in silico, in vitro, and ex vivo study. *AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY*, 2016; 310: H1702-H1714. Impact factor: 3.348; **Quartile Score: Q1**

V. **Monique Culturato Padilha Mendonça, Edilene Siqueira Soares, Marcelo Bispo de Jesus, Helder José Ceragioli, Ângela Giovana Batista, Ádám Nyúl-Tóth, Judit Molnár, Imola Wilhelm, Mário Roberto Maróstica Jr., István Krizbai, and Maria Alice da Cruz-Höfling.** PEGylation of Reduced Graphene Oxide Induces Toxicity in Cells of the Blood-Brain Barrier: An in Vitro and in Vivo Study. *MOLECULAR PHARMACEUTICS*, 2016; 13:3913-3924. Impact factor: 4.44; **Quartile Score: D1**

VI. **István A. Krizbai, Ádám Nyúl-Tóth, Hans-Christian Bauer, Attila E. Farkas, Andreas Traweger, János Haskó, Hannelore Bauer, Imola Wilhelm.** Pharmaceutical Targeting of the Brain. *CURRENT PHARMACEUTICAL DESIGN*, 2016; 22:5442-5462. Impact factor: 2.611; **Quartile Score: Q1**

VII. **Imola Wilhelm, Ádám Nyúl-Tóth, Maria Suciu, Anca Hermenean, and István A. Krizbai.** Heterogeneity of the blood-brain barrier, *TISSUE BARRIERS,* 2016; 4:e1143544. Impact factor: -; **Quartile Score: Q1**

VIII. **Judit Molnár, Csilla Fazakas, János Haskó, Orsolya Sipos, Krisztina Nagy, Ádám Nyúl-Tóth, Attila E. Farkas, Attila G. Végh, György Váró, Péter Galajda, István A. Krizbai, Imola Wilhelm.** Transmigration characteristics of breast cancer and melanoma cells through the brain endothelium: role of Rac and PI3K, *CELL ADHESION AND MIGRATION*, 2016; 10:269-81. Impact factor: 3.872; **Quartile Score: Q2**

IX. **István A. Krizbai, Csilla Fazakas, János Haskó, Judit Molnár, Ádám Nyúl-Tóth, Attila E. Farkas, Imola Wilhelm.** Molecular structure and function of biological barriers, *ACTA BIOLOGICA SZEGEDIENSIS*, 2015; 59:39-50. Impact factor: - **Quartile Score: Q3**

X. **János Haskó, Csilla Fazakas, Judit Molnár, Ádám Nyúl-Tóth, Hildegard Herman, Anca Hermenean, Imola Wilhelm, Yuri Persidsky, and István A. Krizbai.** CB2 Receptor Activation Inhibits Melanoma Cell Transmigration through

the Blood-Brain Barrier, *INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*, 2014; 15:8063-8074. Impact factor: 2.862; **Quartile Score: Q3**

XI. **Imola Wilhelm, Csilla Fazakas, Judit Molnár, János Haskó, Attila G. Végh, László Cervenak, Péter Nagyőszi, Ádám Nyúl-Tóth, Attila E. Farkas, Hannelore Bauer, Gilles J. Guillemin, Hans-Christian Bauer, György Váró, István A. Krizbai.** Role of Rho/ROCK signaling in the interaction of melanoma cells with the blood-brain barrier, *PIGMENT CELL AND MELANOMA RESEARCH*, 2014; 27:113-123. Impact factor: 4.619; **Quartile Score: D1**

1. TABLE OF CONTENT

2. LIST OF ABBREVIATIONS

AIM2	absent in melanoma 2
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
AUF1	AU-rich elements /poly(U)-binding/degradation factor 1
BBB	blood-brain barrier
BCA	bicinchoninic acid
CARD	caspase activation and recruitment domain
CASP	caspase
CCL	C-C motif ligand
CEC	cerebral endothelial cell
CLR	C-type lectin receptor
CM	culture medium
CNS	central nervous system
CSF	cerebrospinal fluid
CXCL	C-X-C motif ligand
DAMP	damage-associated molecular pattern
DAP	(meso-)diaminopimelic acid
DOC	deoxycholic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
HBVP	human brain vascular pericyte
H_2O_2	hydrogen-peroxide
hCMEC/D3	human cerebral microvascular endothelial cell line D3
HuR	human antigen R
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	IFN-γ-induced protein-10

LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MEK	MAPK/ERK kinase
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1α
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NVU	neurovascular unit
OMV	outer membrane vesicle
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PDGFRβ	platelet-derived growth factor receptor β
PDTC	pyrrolidine dithiocarbamate
pERK	phospho-extracellular signal-regulated kinase
PRR	pattern recognition receptor
RANTES	regulated on activation, normal T cell expressed and secreted
RIPA	radioimmunoprecipitation assay
RLR	retinoid acid-inducible gene-1 (RIG-1)-like receptor
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SRC3	steroid receptor co-activator 3
TBS-T	Tris-buffered saline with 1 mL/L Tween-20
TCA	trichloroacetic acid
TIA1	T cell intracellular antigen 1
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
TTP	tristetraprolin
VCAM-1	vascular cell adhesion molecule-1
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

3. INTRODUCTION

3.1. The blood-brain barrier and the neurovascular unit

The central nervous system (CNS) — composed of the brain and the spinal cord — is a complex neural network, which uses a combination of electrical and chemical signals that only works properly in a very precisely regulated milieu. To create and maintain this environment, special barriers are formed in the CNS. These cellular interfaces separate and interconnect the neural interstitium or cerebrospinal fluid (CSF) and the blood, controlling material flux and cellular traffic into and out of the brain. They regulate uptake of essential nutrients and remove unnecessary or potentially harmful components from the CNS. Consequently, they are considered as physical, metabolic and immunologic barriers, supplemented with transport functions (Abbott and Friedman, 2012). These barriers are the blood-CSF barrier in the choroid plexus, the arachnoid barrier and the blood-brain barrier (BBB) (Wilhelm *et al.*, 2016). Out of these, the BBB has the most extensive surface area, which is approximately thousand times larger than the surface of the blood-CSF barrier (de Boer and Gaillard, 2006). The BBB is a highly selective barrier formed by cerebral endothelial cells (CECs) at the level of brain capillaries, arterioles and venules (Figure 1). Capillaries supply blood to close proximity of neurons reducing the diffusion distance between blood vessels and neurons to approx. 8–25 μm. Based on all these, the BBB has a key role in maintaining CNS homeostasis (Abbott and Friedman, 2012). Another cell type of the BBB is the pericyte located in the duplication of the basement membrane. Pericytes have important developmental and regulatory roles. CECs and pericytes are covered by endfeet of astrocytes, which form the glia limitans associated with the basement membrane (Krizbai and Deli, 2003). CECs, astrocytes and pericytes are in a close functional relationship with microglia and neurons, together forming the neurovascular unit (NVU) whose main function is neurovascular coupling (regulation of local blood flow in response to neural activity) and formation of the BBB.

CECs are connected by intercellular junctions, which are precisely regulated by diverse signaling molecules. Permeability of the endothelial barrier is largely determined by the integrity of the tight and adherent junctions (TJs and AJs) between CECs. TJs of CECs are composed of transmembrane proteins like occludin, claudins (mainly claudin-5, but claudin-1,

-3, -12 are also present), junctional adhesion molecules, *etc.*; and plaque proteins including zonula occludens proteins (ZO-1, ZO-2) and other associated molecules. TJs are supported by AJs, which are mainly formed by cadherin-catenin complexes (Figure 1) (Wilhelm and Krizbai, 2014).

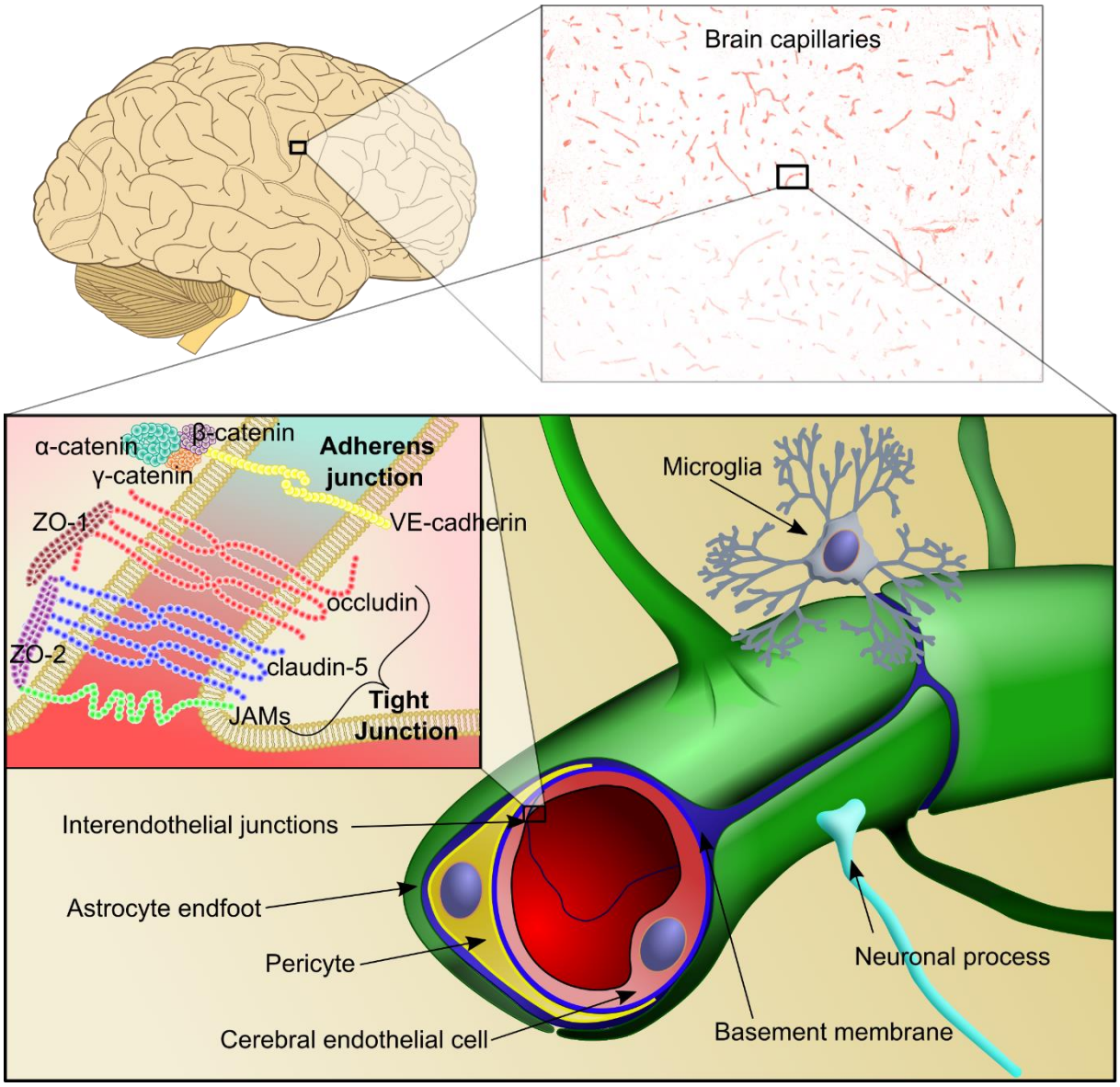


Figure 1. Structure of the BBB and of interendothelial junctions.

Besides continuous TJs, CECs have other special properties contributing to the barrier function as well: expression of specific transporter systems, very low pinocytotic and transcytotic activity, and presence of a high number of mitochondria suggesting high energy demand of these cells (Daneman and Prat, 2015; Wilhelm and Krizbai, 2014).

BBB functionality is relatively stable under physiological circumstances. However, different diseases can alter barrier properties, which means elevated para- and transcellular transport through the barrier. These changes are accompanied by inflammatory processes in the brain (Banks, 2015).

3.2. Neuroinflammation

Neuroinflammation can accompany a wide range of pathological conditions including traumatic brain injury, infections, ischemia, brain tumors, neurodegenerative disorders and also aging (Labzin *et al.*, 2018; Michaud *et al.*, 2013; Ransohoff *et al.*, 2015; Suthar and Sankhyan, 2018; Tohidpour *et al.*, 2017). It is a complex inflammatory response of the nervous tissue to various harmful effects to protect and restore the structural and functional integrity of the CNS. However, neuroinflammation acts as a double-edged sword: moderate inflammatory responses can have a protective role in the CNS, which favor the recovery of injured neurons, whereas an excessive or long lasting inflammation might exacerbate the impairment of tissue homeostasis (Kielian, 2016). To maintain neuronal functionality and plasticity, it is important to keep the beneficial nature of neuroinflammation (Tohidpour *et al.*, 2017).

The BBB restricts the traffic of peripheral immune cells under physiological conditions, thus they can mainly enter the CNS under pathologic conditions (Banks, 2015; Ransohoff and Brown, 2012). In the normal brain parenchyma, there are no cells capable of exiting the CNS and entering a lymph node for antigen presentation. These main differences represent the cellular basis of immune privilege of the CNS (Galea *et al.*, 2007; Ransohoff *et al.*, 2015). Microglia, astrocytes or infiltrating cells can release a variety of proinflammatory cytokines, chemokines, reactive oxygen and nitrogen species, which regulate neuroinflammatory processes (Bellezza *et al.*, 2018). Furthermore, neurons and regulatory T cells may also secrete anti-inflammatory cytokines and neuropeptides, which can help protect the nervous system from excessive neuroinflammation (Bellezza *et al.*, 2018; Kempuraj *et al.*, 2017). Notably, all cells of the CNS — including cells of the BBB — appear to have the capacity to contribute to the inflammatory process.

3.2.1. Role of the BBB in neuroinflammation

Neuroinflammation and several other pathological conditions (*e.g.*, diabetes, liver diseases) may increase BBB permeability (Banks, 2015; Daneman and Prat, 2015). Elevated permeability contributes to the recruitment of peripheral inflammatory cells to the brain, to an increased transport of cytokines and to other inflammatory reactions (Tohidpour *et al.*, 2017). These might cause disturbed homeostasis of the CNS with severe consequences like impaired clearance of metabolites, brain edema, demyelination, neuronal damage and subsequent death which can ultimately lead to cognitive damage (Hernandez-Romero *et al.*, 2012; Takeda *et al.*, 2013; Takeda *et al.*, 2014). Neuronal damage induced by BBB breakdown may also trigger neuroinflammation inducing a vicious circle.

In the last decades, bidirectional communication between the CNS and the peripheral immune system was revealed (Engelhardt *et al.*, 2017). In this scenario, the BBB is not only a passive part of the neuroinflammatory processes. Besides immune cells and resident cells with well-characterized immune functions, cellular components of the BBB like CECs and brain vascular pericytes can be involved in inflammatory responses (Lénárt *et al.*, 2016; Navarro *et al.*, 2016a). In these cells innate immune mechanisms are launched during the initial phase of neuroinflammation. This further supports the key role of the BBB in neuroinflammation.

3.2.2. Brain endothelial cells as a "neuro-immune interface"

As the most important constituents of the BBB, CECs substantially contribute to the protection of the brain against the variable and potentially harmful environment of the blood, and contribute to the homeostasis of the brain (Abbott, 2013). They are the first cells of the NVU in contact with circulating pathogens, activated immune cells and cytokines. CECs have receptors for different immune-related signals, which can alter barrier function. For example, LPS, TNF-α and leukaemia inhibitory factor can increase BBB permeability by acting on CECs (Banks, 2015). BBB also prevents unregulated access of interleukins (ILs) and chemokines to the CNS. However, a certain amount of cytokines and chemokines (such as IL-1α, IL-1β, IL-6, IL-15, TNF-α or CCL2) can be transported across CECs and act directly on their CNS receptors. Brain-to-blood passage of several cytokines has also been noted (Banks, 2015). Furthermore,

CECs themselves are able to secrete immunoactive substances like GM-CSF and IL-6 (Dohgu *et al.*, 2011), IL-1α, TNF-α, and IL-10 (Verma *et al.*, 2006). Therefore, the cerebral endothelium is an important regulator of the neuro-immune axis by separating and modulating the interaction of the immune and central nervous systems (Banks, 2015). Structural and functional integrity of CECs is a major factor affecting the course of neuroinflammation.

3.2.3. Pericytes as potential modulators of neuroinflammation

Pericytes are contractile cells embedded in the duplication of the basement membrane of cerebral microvessels. They are involved in controlling key neurovascular functions, *i.e.* cerebral blood flow and permeability of the BBB. Pericytes induce the expression of BBB-associated proteins in CECs during brain development. Furthermore, they can contribute to the clearance of cellular debris by phagocytosis (Sweeney *et al.*, 2016).

Pericytes can be targets of inflammatory stimuli and might modulate inflammation by releasing inflammatory factors as well. LPS-treated brain pericytes upregulate expression of adhesion molecules like ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) resulting in increased adhesion of leukocytes (Guijarro-Muñoz *et al.*, 2014). In addition, TNF-α and IL-1β downregulate pericyte markers and also enhance the expression of adhesion molecules in cerebral pericytes (Persidsky *et al.*, 2016). Moreover, pericytes can respond to immune challenge by releasing cytokines and chemokines, including IL-6, -8, CXCL1-3 (C-X-C motif ligand 1-3) and CCL2-4 (C-C motif ligand 2-4) in human (Guijarro-Muñoz *et al.*, 2014) and IL-1α, -3, -9, -10, -12, -13, TNF-α, IFN-γ, G-CSF (granulocyte colony stimulating factor), GM-CSF (granulocyte-macrophage colony stimulating factor), eotaxin, CCL-3 and -4 in mouse cell cultures (Kovac *et al.*, 2011). Brain pericytes respond to TNF-α by secreting IL-6 and MIP-1α (macrophage inflammatory protein-1α) and by upregulating IL-1β and iNOS (inducible nitric oxide synthase) mRNA expression (Matsumoto *et al.*, 2014). IFN-γ treatment increases MHC (major histocompatibility complex)-I and MHC-II expression conferring antigen presenting capacity to pericytes (Balabanov *et al.*, 1999). IFN-γ also decreases α-actin expression and increases PDGFRβ (platelet-derived growth factor receptor β) phosphorylation and internalization (Jansson *et al.*, 2016). Reduction in the expression of pericyte markers and enhanced expression of antigen presenting and phagocytic

capacities have been suggested to confer microglia-like properties to cerebral pericytes (Rustenhoven *et al.*, 2016). Based on these findings, pericytes can considerably influence neuroinflammation.

3.3. Pattern recognition receptors and inflammasome activation

3.3.1. Structure and function of pattern recognition receptors

During central or peripheral inflammation, innate immune mechanisms are the first to be activated. The innate immune system senses and processes potential danger signals and alarms the whole system to prepare further immune reactions. One of the most important steps of the triggering of the innate immune process is activation of pattern recognition receptors (PRRs) (Tohidpour *et al.*, 2017).

PRRs are receptors of the innate immune system able to recognize potential danger signals like conserved molecular patterns of infectious agents (*i.e.* pathogen-associated molecular patterns – PAMPs) or cell components released during tissue damage (*i.e.* damage-associated molecular patterns – DAMPs). PRRs consist of at least four major families: members of the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs) are bound to membranes, while nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) have a cytoplasmic localization (Medzhitov, 2007; Takeuchi and Akira, 2010). TLRs and NLRs — which are the most characterized PRRs — became the focus of interest in the past decade in the field of neuroinflammation.

To date, 13 members of the TLR family have been identified in mammals. Mice express all TLRs (TLR1-TLR13), while humans express only TLR1-10. Activation of TLRs triggers a series of signaling pathways, leading to the induction of genes, which are involved in antimicrobial host defence. TLRs can recognize bacterial and fungal cell wall components like lipopolysaccharide (LPS) of Gram-negative bacteria, which is recognized by TLR4; zymosan detected by TLR2 in concert with TLR6; or triacyl lipopeptide, which is the ligand of TLR1 and TLR2. Other bacterial and viral components like dsRNA can be ligands of TLR3; TLR5 identifies flagellin (FliC); ssRNA is recognised by TLR7 or TLR8, while the ligand of TLR9 is

CpG DNA (summarized in Table 1). Upon ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of Toll/interleukin-1 receptor (TIR) domain-containing adaptor molecules to the TIR domain of the TLR. The specificity of the signaling process depends on the differential recruitment of adaptor proteins by different TLRs. Recruitment of adaptors initiates a cascade of signaling routes and ultimately leads to activation of transcription factors such as nuclear-factor kappa B (NF-κB) and members of the interferon regulatory factor (IRF) family. Furthermore, these adaptor proteins also activate mitogen-activated protein kinases (MAPKs), such as c-jun NH2- terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) 1/2 which activate AP-1 (activating protein 1). These transcription factors can induce the gene expression of inflammatory molecules such as inflammatory cytokines, adhesion molecules and different immune receptors like NLRs and TLRs as well (Figure 2) (for review see: Kawai and Akira, 2006).

Table 1. TLRs and their ligands.

Receptor	Species	Most important ligand
TLR1	human, murine	triacyl lipopeptide
TLR2	human, murine	triacyl lipopeptide, zymosan
TLR3	human, murine	dsRNA
TLR4	human, murine	LPS
TLR5	human, murine	flagellin
TLR6	human, murine	zymosan
TLR7	human, murine	ssRNA
TLR8	human, murine	ssRNA
TLR9	human, murine	CpG DNA
TLR10	human, murine	-
TLR11	murine	-
TLR12	murine	-
TLR13	murine	23S ribosomal RNA

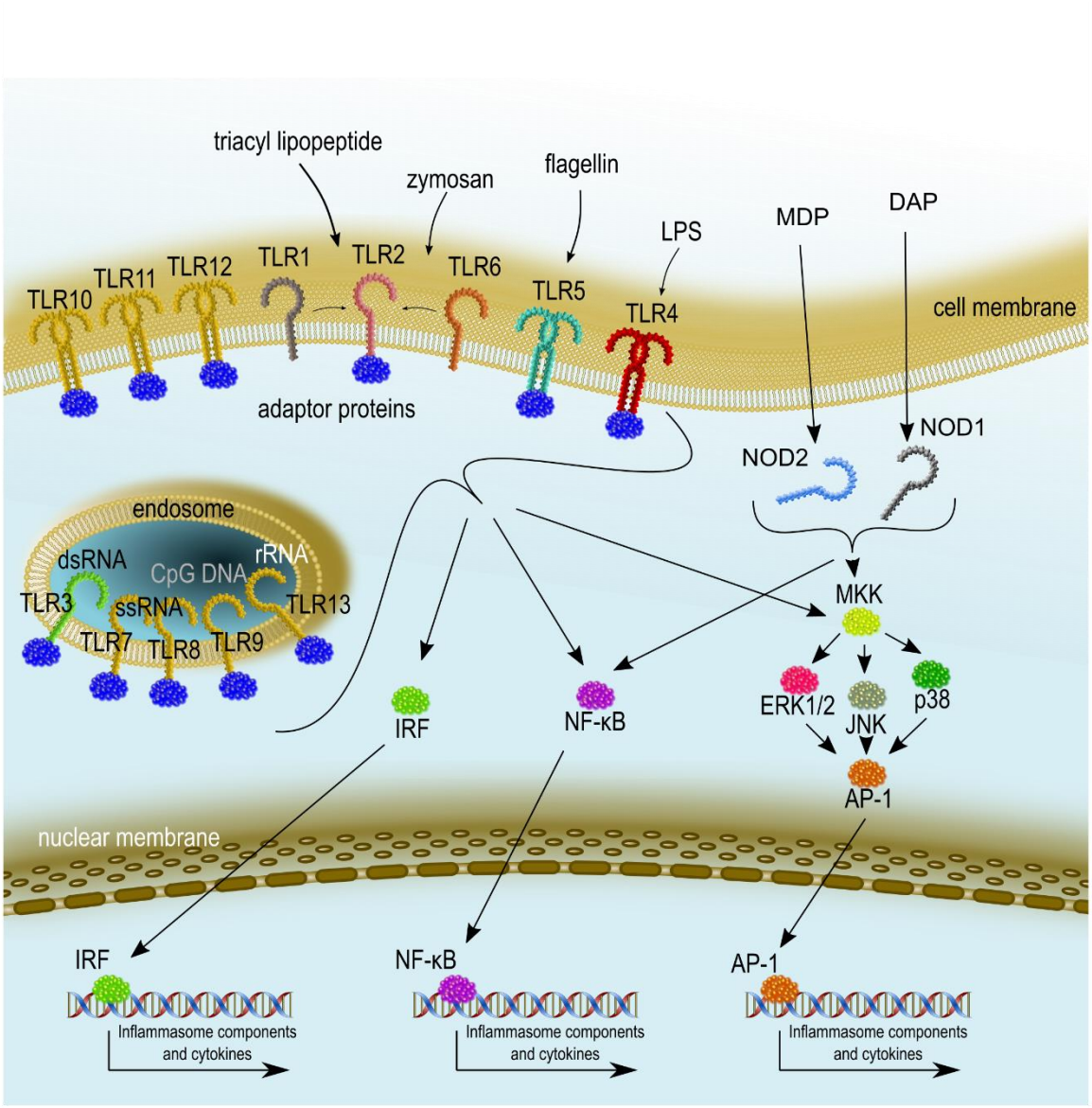


Figure 2. Signaling pathways activated by TLRs and NLRs.

The NLR family consists of intracellular receptors classified into four subfamilies based on the type of the N-terminal domain (the NLRA, NLRB, NLRC and NLRP subfamilies) and an additional subfamily, with only one member, NLRX1, the N-terminal domain of which has no strong homology to the same domain of the other subsets (Ting *et al.*, 2008). NLRs can be activated by a wide range of microbial and danger signals, except for NLRA which has no described specific ligand so far. It functions as a transactivator of MHC class II gene expression. NLRB or NAIP has an important role in the recognition of bacterial flagellin as a co-receptor of NLRC4 (Kim *et al.*, 2016). NOD1 and NOD2 (members of the NLRC family) are sensors of

distinct peptidoglycan fragments, such as meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively (Figure 2). NLRC4 and NAIP can detect bacterial flagellin (FliC), whereas NLRP1 can be activated by anthrax lethal toxin and MDP as well. The broadest range of stimuli acts on NLRP3, including RNA, adenosine triphosphate (ATP), MDP, bacterial toxins, hyaluronan, nigericin and even silica crystals or aluminium salts which eventually lead to K^+ efflux (for review see: Benko *et al.*, 2008; Opitz *et al.*, 2009; Shaw *et al.*, 2010). NLRP6 can be activated by taurine and histamine; NLRP7 by pathogen-derived acylated lipopeptides, NLRP9 by dsRNA and NLRP12 by *Yersinia pestis* infection (Table 2) (for review, see Place and Kanneganti, 2017; Vladimer *et al.*, 2012).

Table 2. NLRs and their activators.

Receptor	Species	Ligand or activator
NLRA	human, murine	IFN-γ
NLRB	human, murine	flagellin
NOD1	human, murine	DAP
NOD2	human, murine	MDP
NOD3	human	?
NLRC4	human, murine	flagellin
NLRC5	human	IFN-γ
NLRP1	human, murine	anthrax lethal toxin, MDP
NLRP2	human, murine	ATP
NLRP3	human, murine	RNA, ATP, MDP, hyaluronan, nigericin, silica crystals
NLRP4	human	?
NLRP5	human	?
NLRP6	murine	taurine, histamine
NLRP7	human	Lipopeptides
NLRP8	human	?
NLRP9	murine	dsRNA
NLRP10	human	?
NLRP11	human	?
NLRP12	murine	*Yersinia pestis*
NLRP13	human	?
NLRP14	human	?
NLRX	human	?

Ligand recognition and activation of several members of the NLR family (NLRP1, NLRP3, NLRC4 and possibly NLRP2, NLRP6, NLRP7 and NLRP12) and other proteins like absent in melanoma 2 (AIM2) and pyrin can initiate the formation of multiprotein complexes called inflammasomes, to activate inflammatory caspases, resulting in the production of active cytokines (IL-1β, IL-18) and pyroptotic cell death (Broz and Dixit, 2016). Besides NLRs, inflammasomes consist of an adaptor molecule (apoptosis associated speck-like protein containing a CARD (caspase activation and recruitment domain) – ASC) and caspase-1 or caspase-5. Inflammasome assembly is a well-controlled and very effective system to transform infection or tissue damage into a robust inflammation. It requires two signals (at least in case of NLRP3 inflammasome assembly), a priming and an activator signal (Figure 3). The priming signal [*e.g.* LPS, tumour necrosis factor-α (TNF-α) or interferon-γ (IFN-γ)] induces transcription of inflammasome components and deubiquitination of NLRP3 (Juliana *et al.*, 2012). The activator signal (*e.g.* MDP, ATP or nigericin) triggers the assembly of the inflammasome. Besides the canonical pathway, inflammasome activation may also occur through a non-canonical pathway in response to intracellular LPS (*e.g.* phagocytized Gram-negative bacteria or uptake of outer membrane vesicles (OMVs) released by bacteria). This pathway initiates activation of caspase-4/5 in human or caspase-11 in mouse cells resulting in activation of the NLRP3 inflammasome and pyroptosis (Figure 3) (Broz and Dixit, 2016).

Activation of inflammasomes and IL synthesis is under the control of a complex network of signaling molecules, the most important being NF-κB and MAPKs. In addition, many RNA-binding proteins (RBPs) can regulate protein synthesis of ILs by translational arrest or destabilizing RNA. The most studied of these proteins are the AU-rich elements /poly(U)-binding/degradation factor 1 (AUF1), TIA1 (T cell intracellular antigen 1), TTP (tristetraprolin), HuR (human antigen R) and SRC3 (steroid receptor coactivator 3) (Anderson, 2010).

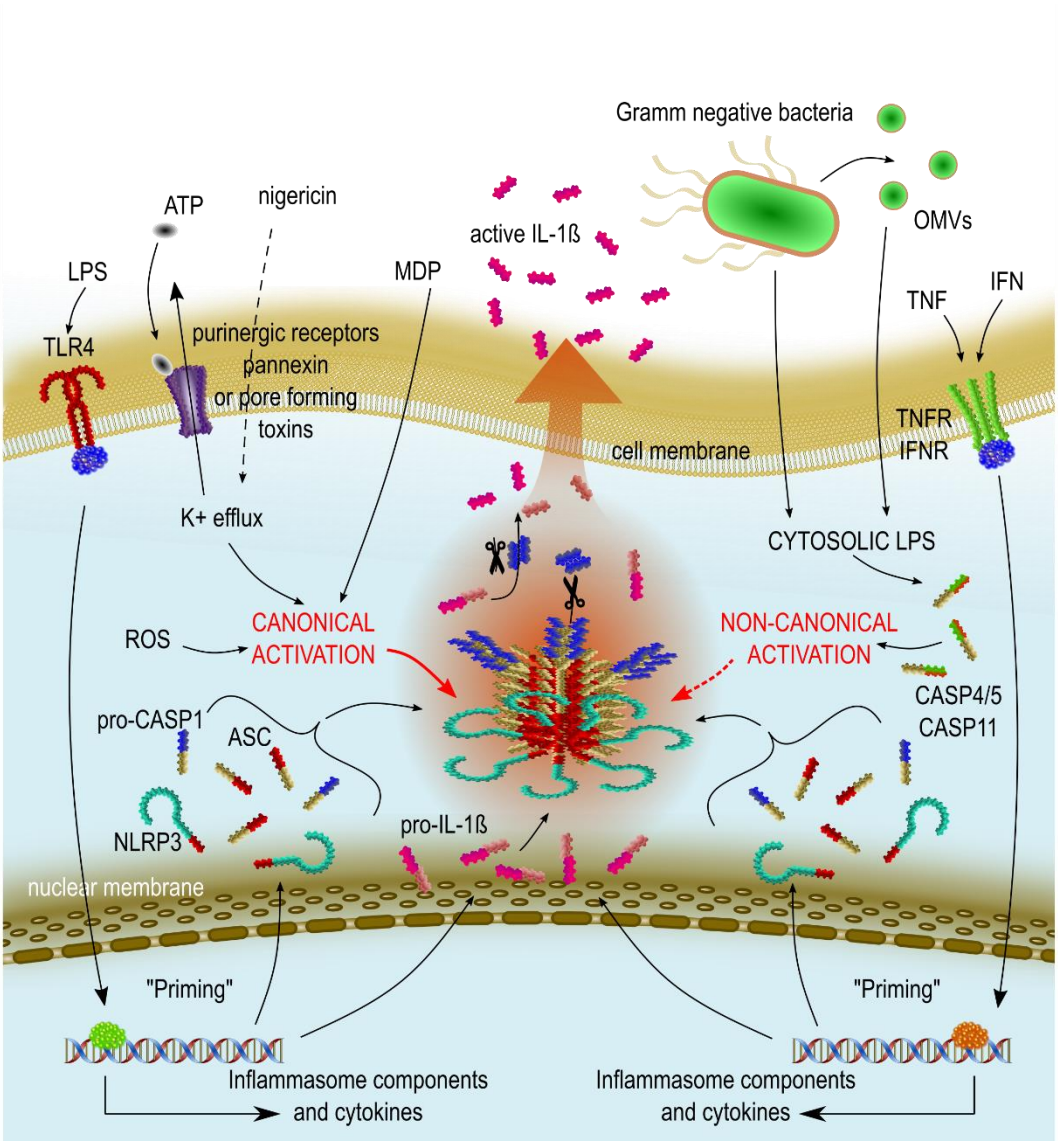


Figure 3. Canonical and non-canonical inflammasome activation.

3.3.2. PRRs in the cells of the BBB

Increasing evidence suggests that besides immune cells, cells of the NVU can also express and activate PRRs and inflammasomes. Recent research has revealed that astrocytes and neurons express several TLRs (Lee *et al.*, 2013) and NLRs, and they are able to activate inflammasomes in various neurological diseases (Lénárt *et al.*, 2016). However, there is only few data about the expression and activity of PRRs and inflammasomes in CECs and brain pericytes and their role in neuroinflammation.

Sensing of PAMPs and DAMPs by CECs might depend on PRRs. Several members of the TLR family have already been shown to be expressed in CECs (Nagyoszi *et al.*, 2010). They have a crucial role in inflammatory permeability increase of the BBB and leukocyte recruitment into the CNS. For example, TLR4-dependent induction of ICAM-1 expression in CECs and leukocyte recruitment into the CNS have been proved (Zhou *et al.*, 2009). Furthermore, activation of TLR4 or TLR2/6 leads to increase in BBB permeability (Nagyoszi *et al.*, 2010; Veszelka *et al.*, 2007). Besides TLR4, TLR2 is the main sensor of bacterial infections in CECs, while TLR3 responds to dsRNA with cytokine release (Fischer *et al.*, 2009). In addition, oxidative stress upregulates the expression of TLR2, TLR3, TLR4 and TLR6 *in vitro* (Nagyoszi *et al.*, 2010). However, only two members of the NLR family (namely NLRP3 and NLRA) have been previously identified in CECs during infection (Girvin *et al.*, 2002; Reimer *et al.*, 2010) and no evidence of inflammasome-assembly has been shown in these cells.

Although more and more data suggest that pericytes might also be important mediators of neuroinflammation, expression profile of PRRs, possible inflammasome activation and secretion of active IL-1β have not been described in these cells. In brain pericytes, only TLR4, NOD1 and NOD2 have been shown to be expressed (Guijarro-Muñoz *et al.*, 2014; Navarro *et al.*, 2016b).

4. AIMS OF THE STUDY

There is increasing evidence that CECs and brain pericytes are both involved in inflammatory processes as active participants of the neuro-immune axis. PRRs are major elements of this process and are indispensable for the activation of innate immune mechanisms. **Therefore, the first aim of this study was to identify the PRRs — including members of TLR and NLR family — expressed in CECs and brain pericytes and to investigate their regulation by different stress factors and signaling pathways.**

Activation of some NLRs by specific priming and activation signals leads to inflammasome assembly resulting in active IL-1β secretion. **The second aim of the study was to test whether CECs and brain pericytes are able to activate inflammasomes via canonical or non-canonical pathways, inducing caspase activation-dependent active IL release.**

Expression of PRRs and cytokines and inflammasome activation are under very strict regulation. **The third aim was to understand the transcriptional and translational regulation of IL-1β and to describe the signaling pathways modulating inflammasome assembly in CECs and brain pericytes.**

Brain pericytes have phagocyte-like attributions, which may lead to proinflammatory cytokine secretion through activation of the non-canonical inflammasome pathway. **Our fourth aim was to characterize the cytokine response and inflammasome activation of brain pericytes induced by *E. coli* infection or OMVs released by the bacteria.**

5. MATERIALS AND METHODS

5.1. Reagents

All reagents, if not otherwise stated, were purchased from Sigma-Aldrich. The following chemicals were used: ATP, hydrogen-peroxide (H_2O_2), TNF-α, IL-1β, IFN-γ, LPS from *Escherichia coli (E. coli)* strain 0111:B4, U0126, PDTC (pyrrolidine dithiocarbamate). MDP was from Bachem, Lipofectamine® 2000 from Thermo Fisher Scientific, flagellin/FLiC from Novus Biologicals, while nigericin and carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) were from Tocris.

5.2. Cell culture and treatments

For the experiments, a human cerebral endothelial cell line (hCMEC/D3) (Weksler *et al.*, 2005) and human brain vascular pericytes (HBVP cells: pericytes isolated from human brain and cryopreserved at first passage after purification, ScienCell) were used. CECs were grown on rat tail collagen-coated plates in EBM-2 medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) and 25 g/L fetal bovine serum (FBS, Sigma) (Wilhelm *et al.*, 2008). Pericytes were grown in poly-L-lysine-coated plates in PM (pericyte medium, ScienCell) supplemented with 50 g/L FBS (Sigma). Confluent monolayers of CECs (passages 30–50) and pericytes (passages 3–7) were treated in serum-free culture medium with 600 mmol/L H_2O_2, 10 ng/mL IL-1β, 100 ng/mL IFN-γ, 10 ng/mL TNF-α, 1 μg/mL LPS, 100 mg/mL MDP or LPS + MDP (1 + 100 μg/mL respectively) for 24 hours or for the indicated time intervals. For inflammasome activation, CECs were treated with 1 μg/mL LPS or 100 μg/mL MDP alone for 24 hours, or in a combination of 6 hours LPS treatment followed by 18 hours MDP treatment (LPS→MDP). To study the signaling pathways involved in inflammasome expression and activation, CECs were treated with 100 μmol/L PDTC, 10 μmol/L U0126 or 20 μmol/L Z-VAD-FMK alone, or in different combinations for 24 hours. Pericytes were also treated with 5 mM ATP or 10 ng/mL FLiC (for the indicated time intervals). For intracellular LPS administration to pericytes, Lipofectamine® 2000 was used at a concentration of 5 μL/well (in a 6-well plate) preincubated for 15 minutes with 2 μg/mL LPS (as indicated by the manufacturer). EZ4U assay (Biomedica

Medizinprodukte GmbH) was used to detect the viability changes of the cells. Phase contrast imaging of pericytes was performed with a Nikon Eclipse TE2000U microscope connected to a digital camera (Spot RT KE, Diagnostic Instruments).

Pericytes were also encountered with 10^7 CFU *E. coli* (strain: RP437) (Parkinson, 1978) for the indicated time intervals. This strain was previously transformed with the pMPMA2-GFPmut2 plasmid for green fluorescent protein (GFP) expression. Bacteria were grown overnight in 3 mL lysogeny broth (LB) medium supplemented with antibiotics (50 μg/mL streptomycin and/or 50 μg/mL ampicillin) at 30 °C in plastic tubes in an incubator shaker (200 rpm) (Nagy *et al.*, 2015). Next morning, cultures were diluted and cells at a concentration of OD600 (optical density measured at 600 nm) between 0.25 and 0.30 were centrifuged (3000 rpm, for 10 minutes, on room temperature, two times) and resuspended in Leibovitz's L15 culture medium.

Primary porcine brain endothelial cells (used for immunofluorescence staining) were isolated and cultured as previously described (Nyúl-Tóth *et al.*, 2016).

5.3. Isolation and characterization of outer membrane vesicles (OMVs)

OMVs were isolated from 100 mL culture media of overnight growing bacterial colonies. Samples were centrifuged at 10,000 g at 4 °C for 15 minutes to settle bacterial cells and the supernatant was filtered with a 0.45 μm and subsequently a 0.2 μm pore size syringe filter. Samples were then ultracentrifuged in a Sorvall™ WX+ ultracentrifuge series centrifuge (Thermo Fisher Scientific) at 10,000 g at 4 °C for 15 minutes. Supernatant was discarded and the pellet was resolved in PBS. The OMV content of the samples was examined with an Asylum MFP-3D-type atomic force microscope (Asylum Research). Samples were dried onto freshly cleaved mica surfaces and images were acquired in alternate contact mode using OMCL-AC240TS probes. Final pictures were plane fitted and are presented as height and amplitude images. BCA method (Santa Cruz Biotechnology) was used to determine the protein content of the samples.

5.4. End-point and real-time reverse transcription PCR (RT-qPCR)

The RNA content of cells was isolated using TRIzol reagent or Ambion RNAqueous kit (Thermo Fisher Scientific) following the manufacturer's instructions. RNA was treated with DNase in order to avoid amplification of genomic DNA. RNA was transcribed into cDNA with Superscript III cDNA Synthesis Kit or Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Maxima SYBR Green, Luminaris Color HiGreen (both from Thermo Fisher Scientific) or Bio-Rad's iTaq^{TM} Universal SYBR Green supermixes were used for the amplification which was performed in a Bio-Rad iQ5 instrument under the following conditions: 40 cycles of 95 °C for 15 seconds, 56–63 °C for 30 seconds and 72 °C for 30 seconds. Forward and reverse primer pairs used for the amplification of human TLRs, NLRs, inflammasome components, cytokines and RNA-binding protein mRNAs are listed in Table 3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control of the amplification. The ΔΔCt method was used for quantification, calculated from the threshold cycle values, which were determined using the software of the instrument.

5.5. Sample preparation and immunoprecipitation for western blot

Cell culture media were collected and the cells were lysed in ice cold RIPA buffer (20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 mL/L Triton X-100, 10 g/L sodium deoxycholate, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 1 g/L SDS, 1 mmol/L EDTA and 1 mmol/L Pefabloc). After 30 minutes incubation on ice, cell lysates were centrifuged at 9500 g for 30 minutes at 4 °C and the soluble fraction was used afterwards. Protein content of cell culture media samples was precipitated with either the methanol-chloroform or the TCA (trichloroacetic acid)-DOC (deoxycholic acid) method, as described previously (Peterson, 1977). Briefly, samples were treated with 1 mM EDTA, vortexed and centrifuged at 2500 g at 4 °C for 1 minute to settle cellular debris. For the methanol-chloroform method, an equal volume of methanol and 1/8 volume chloroform was added to the supernatant, mixed and centrifuged at 16,000 g for 5 minutes at 4 °C. Supernatant was discarded, protein interphase was washed with ice-cold methanol and pelleted with further centrifugation. The pellet was air-dried then resolved in ice-cold 2-times concentrated Laemmli-sample buffer.

During TCA-DOC protein precipitation, samples were treated with 100 g/L DOC, vortexed and incubated on ice for 30 minutes. TCA was added to the samples at a final concentration of 110 mL/L, mixed and centrifuged at 10,500 g for 15 minutes at 4 °C. The pellet was washed twice with ice-cold acetone. Pellet was air-dried and resolved in ice-cold RIPA cell lysis buffer. Cell lysates were centrifuged at 9500 g for 15 minutes at 4 °C. Protein concentration of RIPA soluble fractions was determined by the BCA method (Santa Cruz Biotechnology).

For immunoprecipitation, concentrations were equilibrated with RIPA buffer, then samples were treated with anti-NOD2 or anti-NLRP3 antibodies at a final concentration of 0.01 g/L, gently mixed and rotated for 2 hours at 4 °C. Protein A conjugated sepharose beads (GE Healthcare) were washed in ice-cold Tris-buffered saline four times and used to precipitate protein-antibody complexes overnight at 4 °C. Beads were washed four additional times and resolved in Laemmli sample buffer. After centrifugation soluble fractions were used for western blot. All samples were heated with Laemmli buffer at 95 °C for 5 minutes prior to electrophoresis.

5.6. Western blot

Protein samples were electrophoresed with standard denaturing SDS-PAGE procedures and blotted on polyvinylidene difluoride or nitrocellulose (Bio-Rad) membranes. The nonspecific binding capacity of the membranes was blocked with 30 g/L bovine serum albumin or 50 g/L non-fat milk in TBS-T (Tris-buffered saline with 1 mL/L Tween-20). After blocking, membranes were incubated with primary antibodies in TBS-T using the following dilutions: anti-human IL-1β 1:1000 (Santa Cruz Biotechnology; used in Appendix I.), anti-human IL-1β 1:400 (R&D Systems; used in Appendix II.), anti-caspase-1 1:500, anti-NOD2 1:200, anti-NLRP3 1:200 (all three from Santa Cruz Biotechnology), anti-pERK1/2 1:500 (Cell Signaling Technology) or anti-β-actin 1:4000 (Sigma-Aldrich). Blots were washed in TBS-T three times for 10 minutes, and incubated with the secondary antibody in TBS-T, as follows: HRP-conjugated anti-mouse IgG 1:5000 (GE Healthcare), HRP-conjugated anti-goat IgG 1:5000 (Santa Cruz Biotechnology) or HRP-conjugated anti-rabbit IgG 1:10,000 in case of IL-1β, 1:3000 in case of NOD2 and NLRP3, 1:5000 in case of pERK1/2 (all three secondary antibodies from Jackson Immunoresearch). After three further 10 minutes washing steps, immunoreaction

Table 3. Members of the NLR and TLR families (according to the nomenclature approved by the Human Genome Organization Gene Nomenclature Committee), inflammasome components, caspase-cleaved interleukins, RNA-binding proteins and the primers used to investigate their expression by RT-PCR and RT-qPCR.

Name	Forward 5'-3'	Reverse 5'-3'	Amplicon (bp)
TLR1	GCCTTGTCTATACACCAAGT	CCAATTGTTGCAGAGACTTC	310
TLR2	TCTCCCATTTCCGTCTTTTT	GGTCTTGGTGTTCATTATCTTC	125
TLR3	TAAACTGAACCATGCACTCT	TATGACGAAAGGCACCTATC	101
TLR4	CCGCTTCCTGGTCTTATCAT	TCTGCTGCAACTCATTTCAT	141
TLR5	ACGGACTTGACAACCTCCAA	AGTGGATGAGGTTCGCTGTA	291
TLR6	CCCAAGGAGAAAAGCAAAC	TTCACCATCATCCAAGTAAAT	156
TLR7	CAGAGCTGAGATATTTGGACT	TTGGTAAGTATCTGTTATCACCT	308
TLR8	CGGCAGAGTTATGCAAATAGT	GTAAGAGCACTAGCATTATCA	341
TLR9	AGGAGCTGTCTGCCATTTGA	AGCCAGTTGCAGTTCACCAG	483
TLR10	CTCCCAACTTTGTCCAGAAT	TGGTGGGAATGCAATAGAAT	132
NOD1	GCGCTGCATCTACGAGACAC	GATGCGTGAGGCCTTTGCAT	398
NOD2	TGCCACGGTGAAAGCGAATG	CGAGACTGAGCAGACACCGT	144
NLRC3	TCAACGTGTTGCACTGCCTG	GCGACAGTCCTTCCCACTCA	308
NLRC4	CCAGTCCCCTCACCATAGAAG	ACCCAAGCTGTCAGTCAGACC	119
NLRC5	AATCACTGCCCGAGGCATCA	CCTGGCCTGAAGCATCCTGA	244
NLRP1	GGATCAGGGATCAGGCTGCA	CTCGGGCTATCAGCTGCTCT	193
NLRP2	GCAACGATTACGCCCCGAG	GCGCCGAAGACACCATCTTG	150
NLRP3	AGTGGGATTCGAAACACGTGCA	GTGCCGTGTTCACTGCCTGGTAT	381
NLRP4	AGGGAGACCTTGGAGCTTCGA	CTCAGTCCAGGGAATCTGCTTGAG	306
NLRP5	CTGGACACGGCTGGCTGTGG	TGCCGGTTGCAGGAAAGGGC	395
NLRP6	GACGCTGCTCTCCGTGTC	GTGAAGCGCTTGGTGATCTT	114
NLRP7	AGCTGACACACCTGTGCTTGGC	AGCAGCTCCAGAGGCGTAGGT	285
NLRP8	GGACGACGCGCTTGGGATGT	GCCACGGTCTTGGGCTGTCT	341
NLRP9	TCGTCTACTGGCGGGAGCTT	CTGGGAGAGGCTAGTGCCGT	251
NLRP10	AGAGGGGAGTTGGAGGGCCTGA	GGGCTCTCTGAGCTGGGCTTG	269
NLRP11	AGCACGAAGCGCTGACTCACC	CAAGGCCTCCGTCACCACTCTT	312
NLRP12	CGGCACCAACCCACATCTGG	GGTCTGGAGCTTGCACGTGG	283
NLRP13	GGATGCACGCATGGAACAGCA	GACAGCTGGCTGCCGACAAG	351
NLRP14	TTGGCGGAATGGGCACCTCA	AGTCTGGGCCGACCAGTTGAT	382
NAIP1	TGCGTCCTTCAGGAACTGGC	CTCCCACAGCTGATTCCCGG	398
CIITA	CTTCGCTCGCTGCATCCCTG	GTCCACATCGCCAGCGTCTC	208
NLRX1	TCCGAGACCTGTTGCTGCAT	AAGTCTCGCAAGACCTGGCG	349
CASP1	TCTTGGAGACATCCCACAATGGGC	TGCGCTCTACCATCTGGCTGC	140
CASP4	CAAGAGAAGCAACGTATGGCA	AGGCAGATGGTCAAACTCTGTA	267
CASP5	TCTGTTTGCAAGATCCACGA	GTTCTATGGTGGGCATCTGG	223
ASC	GCCGGGGATCCTGGAGCCAT	GCGCAGCACGTTAGCGGTGA	240
AIM2	CATCTGCAGCCATCAGAA	CGCTTCTGAAACCCTTCTCT	127
IL-1β	AGCTCGCCAGTGAAATGATG	GCCCTTGCTGTAGTGGTGGT	164
IL-18	CTTCATTGACCAAGGAAATCGGCCT	AGCCATACCTCTAGGCTGGCTATCT	124
IL-33	ATCAGGTGACGGTGTTGATGG	CCTGGTCTGGCAGTGGTTTT	133
IL-37	CAGCCTCTGCGGAGAAAGGAAGT	GTTTCTCCTTCTTCAGCTGAAGG	120
AUF1	GATCAAGGGGTTTTGGCTTT	GTTGTCCATGGGGAGCTCTA	245
TIA1	GCAACCCGAAAGCCTCCC	TAGCATTTCACAACATGA	302
HuR	ATGAAGACCACATGGCCGAAGACT	AGTTCACAAAGCCATAGCCCAAGC	189
TTP	CATGGCCAACCGTTACACC	AGCCAGCGGTGCGAAGCC	259
SRC3	TCTCAACCCACTTCCTTCAG	GGTCTGCCAAGCCATAGG	174
GAPDH	GTGAAGGTCGGTGTCAACG	GTGAAGACGCCAGTAGACTC	298

was visualized using Bio-Rad Clarity Chemiluminescent Substrate in a ChemiDoc MP imaging system (Bio-Rad). Image lab software version 5.2 (Bio-Rad) was used for the quantification of the blots by densitometry.

5.7. IL-1β ELISA (enzyme-linked immunosorbent assay) and cytokine array

For quantification of IL-1β secreted by CECs and pericytes, we used a human IL-1β solid phase sandwich ELISA (R&D Systems) following the manufacturer's recommendations. Cell culture media were collected, treated with 1 mmol/L EDTA and centrifuged at 2500 g at 4 °C for 1 minute to settle cellular debris. Supernatant was used as a sample for the assay.

For the parallel determination of the relative levels of selected human cytokines and chemokines synthesised by brain pericytes, we used the Proteome Profiler^{TM} Array (R&D Systems). After co-culturing the primed pericytes with *E. coli* bacteria for 4 hours, culture media were discarded and cell lysates were used for the assay following the manufacturer's instructions. Spots were visualized with the ChemiDoc MP System.

5.8. Immunofluorescence studies

Pericytes and CECs (primary porcine brain endothelial cells) were grown on coverslips and after priming they were encountered with *E. coli* bacteria for 4 hours or LPS→MDP for 24 hours, respectively. Samples were washed extensively, then pericytes were fixed with PFA at 4 °C, while CECs were fixed with methanol at –20 °C. After four washing steps with PBS, samples were permeabilized with 0.5 % TritonX-100 in PBS. The nonspecific binding capacity was blocked with 50 g/L normal goat serum in PBS, and anti-α-actin (1:200, Abcam), anti-PDGFRβ (1:100, Abcam) or anti-NLRP3 (1:100, Santa Cruz) and anti-claudin-5 (1:100, Invitrogen) antibodies were applied on the coverslips in 20 g/L normal goat serum in PBS overnight. After three washings in PBS, coverslips were incubated with Cy3-labeled anti-rabbit, anti-mouse or Alexa Fluor 488-labeled IgG secondary antibodies (Jackson ImmunoResearch), in 1:200 dilution. After three further washing steps, samples were mounted with FluoroMount-G media (SouthernBiotech). Nuclear staining was performed with Hoechst 33342 (0.66 μg/mL)

during the second washing step. Fluorescent signals were examined with an Olympus Fluoview FV1000 confocal laser scanning microscope.

5.9. Statistical analysis

Statistical tests were performed and heatmap was generated with open source R Statistical Software version 3.4.0 extended with "ggpolt2", "gplots" and "lattice" packages. Expression changes of investigated genes were clustered and visualized as a heatmap (-ΔΔCt or R – correlation coefficient) or on graphs (fold change). All data presented on graphs are means ± standard error of mean (SEM). We used analysis of variance followed by Fisher's least significant difference (LSD) or Bonferroni's post hoc tests for comparing the values of different samples within the experiments, except for data presented in Figure 7A where Mann-Whitney U-test was used and Figure 6D where Pearson correlation was used. Changes were considered statistically significant at $p < 0.05$. Each experiment was repeated at least three times.

6. RESULTS

6.1. Expression of TLRs, NLRs, inflammasome components and caspase-1 substrate ILs in CECs and pericytes

There is increasing evidence that Toll-like and NOD-like receptors are expressed in non-immune cell types as well. Expression of these receptors in CECs and brain pericytes is largely unexplored; therefore, we first evaluated the mRNA expression profile of the 22 known members of the human NLR family and 10 members of TLRs in human CECs and pericytes using RT-PCR. TLR expression in CECs was previously analysed (Nagyoszi *et al.*, 2010); therefore, here we focused on the expression of the NLRs and inflammasome components in these cells.

In CECs under control conditions, several members of the NOD-like receptor family were expressed, including NOD1, NOD2, NLRC4, NLRC5, NLRP1, NLRP3, NLRP5, NLRP9, NLRP10, NLRP12, NLRA and NLRX as well (Figure 4A). For primer functionality tests we used different cell types of human origin known to express the respective NLR receptor(s) (Figure 4A). In brain pericytes NOD1, NOD2, NLRC5, NLRP1-3, NLRP5, NLRP9, NLRP10 and NLRX are expressed in control conditions (Figure 4B), while NOD3, NLRC4, NLRP4, NLRP6-8, NLRP11-14, NAIP and NLRA mRNAs were not expressed. In brain pericytes, among the ten known TLRs, mRNA of TLR2, TLR4, TLR5, TLR6 and TLR10 could be detected (Figure 4B). There was one receptor, namely NLRP13, which we could not identify in our cells, nor did we find other cell types as positive control for its expression. According to literature data, this receptor has only been identified in human oocytes and its expression strongly decreased in embryos, leading to a very low expression level in day 5 embryos (Zhang *et al.*, 2008). These data strongly indicate that neither the CECs nor the pericytes express this receptor. Furthermore, we examined the presence of different inflammasome components and interleukins cleaved by caspases, main effectors of inflammasomes. We found that the adaptor protein ASC, caspase-1, -4, -5 inflammasome components were also expressed by CECs and brain pericytes, just like IL-1β, IL-18, IL-33, and IL-37. At the same time, AIM2 was only expressed in CECs, but not in pericytes (Figure 4A, C). To understand the relative expression

of these receptors in CECs and pericytes, in figure 4C we compared their mRNA levels in relation to GAPDH mRNA expression.

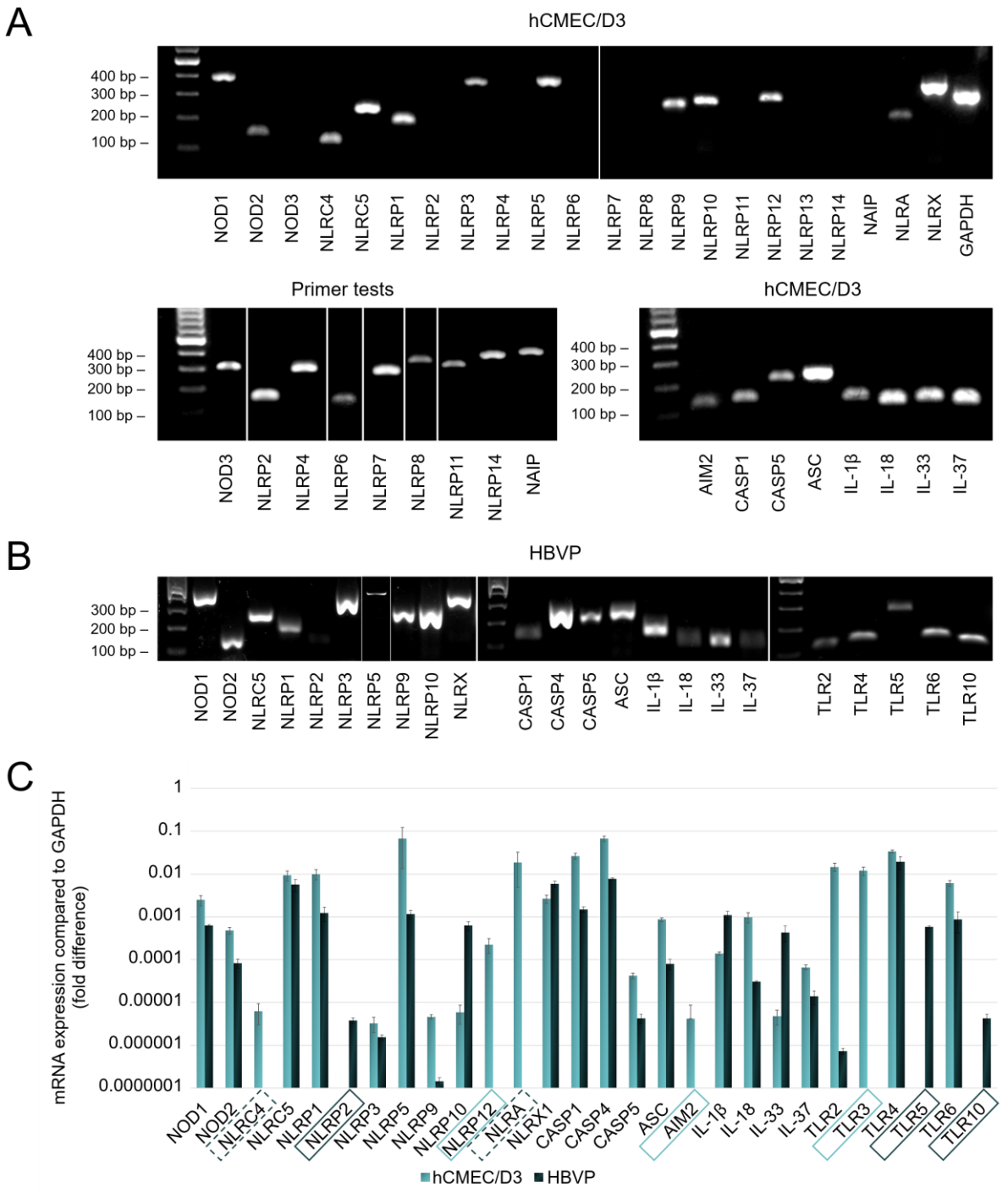


Figure 4. Expression of NLRs, inflammasome components, caspase-cleaved interleukins and TLRs in human CECs and brain vascular pericytes. Total RNA was isolated from untreated cells and examined by PCR for mRNA expression of different genes. Agarose gel images shown are representatives of at least five independent experiments. A) Expression of NLR mRNAs, inflammasome components and caspase-cleaved ILs in human

CECs and positive controls for primer functionality. B) Total RNA was isolated from untreated brain pericytes and examined by PCR for mRNA expression of indicated genes. C) RT-qPCR experiments were performed using total RNA isolated from untreated CECs (hCMEC/D3) or pericytes (HBVPs). Expression of PRRs and inflammasome components was compared to GAPDH. Relative amounts (calculated using the ΔCt values) ± standard error of mean (SEM) of three independent experiments are displayed. Dark grey frames indicate genes only detected in hCMEC/D3 cells (CECs), cyan frames indicate genes only expressed in HBVPs (cerebral pericytes), dashed frames indicate absence of respective mRNA in pericytes in control conditions with appearance upon stimulation (see later).

Expression levels of the majority of these genes in brain pericytes were comparable to that of CECs, except for NLRP5, NLRP9 and TLR2 which were more abundant in the endothelium, and NLRP10, the level of which was much higher in pericytes. Moreover, NLRP2, TLR5 and TLR10 were only expressed in brain microvascular pericytes and not in CECs, while NLRP12, AIM2 and TLR3 were only expressed in CECs and not in pericytes. In addition, NLRC4, NLRA and TLR9 were also not detected in pericytes in basal conditions (Figure 4C).

6.2. Regulation of the expression of PRRs and ILs in CECs and brain pericytes

As a next step, we aimed at understanding the transcriptional regulation (*i.e.* priming) of NLRs, inflammasome-associated proteins and TLRs in CECs and pericytes in response to different pathological conditions. The most important factors regulating PRR expression are oxidative stress, inflammatory cytokines and bacterial wall components, which often occur together. Therefore, we treated CECs and pericytes with relevant concentrations of H_2O_2, LPS, MDP, LPS + MDP, IFN-γ, TNF-α or IL-1β. Using the EZ4U assay, a slight decrease in the viability of the CECs in response to LPS→MDP was observed (80% ± 9% in case of LPS→MDP vs. 100% ± 6% in case of control, $p < 0.05$), while LPS or MDP alone did not significantly affect the viability of the cells (LPS: 90% ± 6%, MDP: 88% ± 8%). None of the treatments significantly downregulated viability of pericytes. On the other hand, we observed changes in the morphology of HBVPs, *i.e.* contraction and appearance of long processes in response to IL-1β, TNF-α and LPS + MDP (data not shown).

Using RT-qPCR, we analysed changes in the expression of 29 genes (found to be basally expressed in CECs and HBVPs) after treatment with H_2O_2, LPS, MDP, LPS + MDP, IFN-γ, TNF-α and IL-1β. Obtained -ΔΔCt values were organized into heat maps (Figure 5A, B, C). In

response to the different inflammatory stimuli, mainly elevation in the expression of the investigated genes was observed. IL-1β and TNF-α were the most potent to induce this elevation. TNF-α treatment markedly upregulated the expression of NOD2 in both CECs and pericytes but also of NLRP5 and NLRP10. Furthermore, it increased the expression of NLRP3, NLRA, AIM2 in CECs, and NLRC5 and TLR10 in pericytes. NLRC4 and TLR9, although not expressed in basal conditions in pericytes, could be detected after TNF-α treatment (Figure 5D). IL-1β caused a significant upregulation of the expression of NOD2, NLRP3, NLRP5 and NLRA in endothelial cells. It also induced marked upregulation in the gene expression of NOD2, IL-1β and TLR2, but also of NLRC5 and TLR10 in brain pericytes (Figure 5A, B, C). Expression of TLR9 was induced by IL-1β and IFN-γ, and this latter promoted expression of NLRA too in pericytes (Figure 5D). On the other hand, IL-1β did not induce expression of NLRA and NLRC4. Similarly, NLRA expression was not induced by TNF-α (not shown). IFN-γ upregulated several other genes, like NOD2, TLR2, caspase-1 and NLRC5 in pericytes and NLRP9 and NLRP12 in CECs.

Among inflammasome-forming NLRs, NLRP1 — which has a high basal expression in both pericytes and CECs — was not primed by either of the used stimuli. NLRP2 was upregulated by IFN-γ, NLRP3 was upregulated by IFN-γ and H_2O_2 in pericytes, while these factors did not induce NLRP3 expression in CECs. Investigations on NLR regulation showed that NOD2 and NLRP3 are among the most responsive inflammasome forming NLRs to stress factors in CECs (Figure 5A). These receptors can be activated by MDP; therefore, we investigated the expression of inflammasome components in response to MDP. In addition, we used stimulation with LPS – which is a priming stimulus for inflammasome activation – alone or with combination with MDP as well. MDP is the minimal bioactive peptidoglycan motif common to all bacteria, which has been shown to be recognized by NOD2, probably by NLRP3, but not by TLR2, TLR2/1 or TLR2/6 associations (Girardin *et al.*, 2003; Inohara *et al.*, 2003; Martinon *et al.*, 2004; Mo *et al.*, 2012). LPS is the major component of the outer membrane of Gram-negative bacteria, and binds the CD14/TLR4/MD2 receptor complex in many cell types.

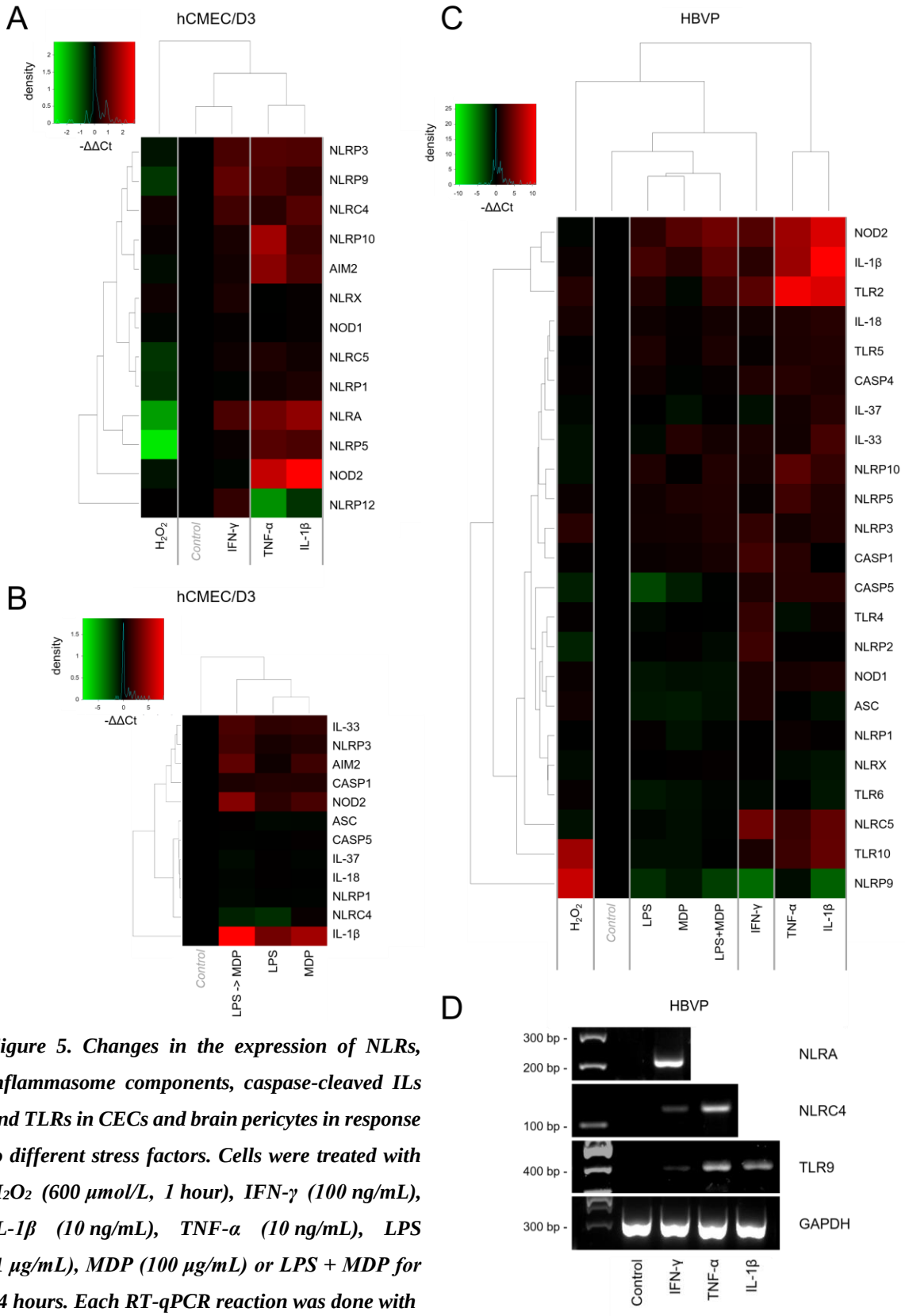


Figure 5. Changes in the expression of NLRs, inflammasome components, caspase-cleaved ILs and TLRs in CECs and brain pericytes in response to different stress factors. Cells were treated with H_2O_2 (600 µmol/L, 1 hour), IFN-γ (100 ng/mL), IL-1β (10 ng/mL), TNF-α (10 ng/mL), LPS (1 µg/mL), MDP (100 µg/mL) or LPS + MDP for 24 hours. Each RT-qPCR reaction was done with

at least three parallels. -ΔΔCt values of all genes investigated and found to be expressed in CECs or pericytes in basal conditions were clustered and visualized as heatmaps. –ΔΔCt values are represented from lowest (green) to highest (red) measured value. Normal distribution of data can be observed in the upper left panels. Fold change values of at least three independent experiments are displayed. A) Induction of NLR and AIM2 mRNA expression in CECs in response to different stress factors. B) Changes in the expression of inflammasome components in response to priming and activator stimuli in CECs. C) Changes in the expression of NLRs, inflammasome components, caspase-cleaved interleukins and TLRs in cerebral pericytes in response to different stress factors. D) Induction of NLRA, NLRC4 and TLR9 mRNA expression in HBVPs. Images are one representative of three independent experiments.

Comprehensive studies have shown that the NLRP3 inflammasome is tightly regulated by a transcriptional step, *i.e.*, TLR-engaging PAMPs (*e.g.*, TLR4-activating LPS) leading to an enhanced transcription of NLRP3 — this is the priming step prior to the activation of the inflammasome (reviewed in: Bauernfeind *et al.*, 2011). In our study, MDP treatment induced at least fivefold increase in NOD2 mRNA expression and an elevation in caspase-1 mRNA expression in both CECs and pericytes (Figure 5B, C). LPS alone upregulated significantly only the expression of caspase-1 as an inflammasome component in CECs. However, a combined treatment with MDP and LPS further upregulated the expression of NOD2 and NLRP3, whereas the expression NLRP1, ASC, caspase-5 and NLRC4 remained on a lower level or unchanged in CECs (Figure 5B, C).

Since the pro-forms of IL-1β, IL-18, IL-33 and IL-37 may be inflammasome substrates, we tested the effect of MDP and/or LPS on the mRNA expression of these ILs. IL-18 and IL-37 remained mainly unchanged in response to MDP and LPS treatment in both cell types. IL-33 increased approximately threefold in response to LPS or MDP treatments, and the combined treatment was even more effective in elevating IL-33 mRNA expression in CECs (Figure 5B, C). However, the most dramatic changes were observed in case of IL-1β. Both MDP and LPS increased its expression levels; however, the combined treatment resulted a more robust increase in CECs and brain pericytes too.

Interestingly, H_2O_2 treatment significantly decreased the expression of NLRP5 in CECs. On the other hand, neither H_2O_2 nor the reactive oxygen species donor dimethoxy-naphthoquinone (DMNQ) (20 μmol/L, 24 hours; data not shown) affected significantly expression of NOD1, NOD2, NLRC4, NLRC5, NLRP1 or AIM2 in CECs. In contrast to CECs, oxidative stress radically upregulated NLRP9 and TLR10 in brain pericytes (Figure 5C).

6.3. Comparison of CECs and brain pericytes in respect of expression and regulation of PRRs and inflammasome components

In order to compare regulation of PPRs and inflammasome components in CECs and cerebral pericytes in a comprehensive manner, we expanded single gene analysis with a group-based comparison.

Heatmap has been widely accepted as one of the main visualization tools for high-throughput data, since Eisen *et al.* introduced it to group genes based on biological relevance (Eisen *et al.*, 1998). By comparing two data matrices (visualized by the featured heatmaps) in one framework as a correlation matrix adds a new dimension, *i.e.*, a correlation variable — depending on the similarity of one data to all others. We used this method to compare the expression pattern of PRRs and inflammasome components in CECs and brain pericytes. By examining the correlation heatmaps of commonly expressed genes in CECs and pericytes, we observed a high average correlation coefficient converging to 1 (Figure 6). This shows that changes in the expression of PRRs in response to inflammation and oxidative stress are very similar in CECs and brain pericytes. Only a few NLRs differed from the main pattern, *e.g.*, endothelial NLRP5 or NLRP9 in pericytes, which have a unique reactivity to oxidative stress (Figure 6A). In case of TLRs expressed in both cell types, correlation between CECs and pericytes was around 0.9 — which is again very close to 1 —, only TLR2 expression showed lower correlation probably because of its relatively low basal expression and strong reaction to IL-1β and TNF-α in brain pericytes (Figure 6C). The similarity and thus the correlation between brain pericytes and endothelial cells was highest in inflammasome and IL genes. Among these, in most cases the correlation coefficient was over 0.95, except for IL-1β, where the relatively lower correlation value can be explained by the enormous expressional elevation in response to LPS→MDP in CECs (Figure 6B). Finally, to sum our group-based comparison, the scatterplot constructed from our recent and previous experimental data clearly shows that — beside a few discrepancies indicated by arrows in Figure 6D — there is a very powerful correlation between brain pericytes and endothelial cells in the regulation of PRR and inflammasome-associated gene expression (Figure 6D).

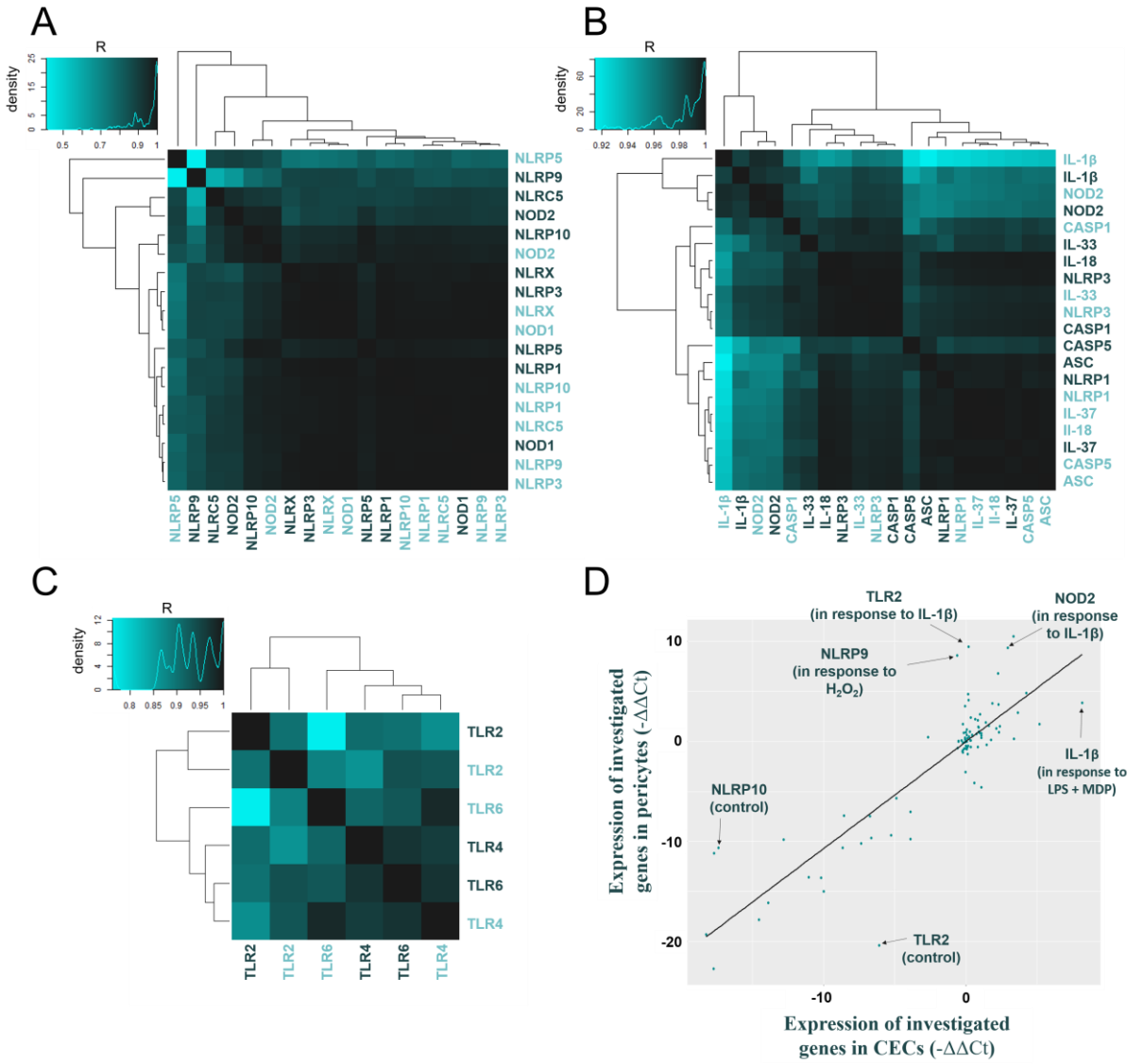


Figure 6. Close relationship between changes in the expression of PRRs and inflammasome-associated genes in CECs and brain pericytes. Expression data from figure 5 were used to calculate correlation between the gene expression patterns of the two cell types. Correlation coefficients are represented from lowest (cyan) to highest (dark grey) calculated value. Distribution of data can be observed in the upper left panels. Genes of endothelial cells are marked in cyan coloured letters and in dark those of brain pericytes. A) Hierarchical clustered correlation heatmap of commonly expressed NLRs expression changes in CECs and pericytes. B) Hierarchical clustered correlation heatmap of commonly expressed inflammasome-associated gene expression changes in CECs and pericytes. C) Hierarchical clustered correlation heatmap of commonly expressed TLR expression changes in CECs and pericytes. D) Dot plot showing correlation line of gene expression patterns of CECs and pericytes. „x" axis is endothelial gene expression data shown as –ΔΔCt, „y" axis is equivalent data from pericytes. R = 0.86, p < 0.05.

6.4. Canonical inflammasome activation in CECs

The two NLR inflammasome components which presented the most robust expression changes in response to LPS→MDP in CECs were NOD2 and NLRP3. Therefore, we have also assessed their changes on the protein level. We found that NOD2 and NLRP3 protein levels increased significantly in response to LPS priming followed by MDP activation stimuli (Figure 7A, B). It is well known that NLRP1 and NLRP3 inflammasomes mediate proteolytic cleavage of IL-1β, IL-18, IL-33 and IL-37. Treatment of CECs with MDP in combination with LPS caused a very high expression of IL-1β mRNA and upregulated the expression of several inflammasome components, including caspase-1, the activator of IL-1β, we wanted to investigate whether the LPS→MDP treatment resulted in elevated production of activated IL-1β by CECs. Our western blot experiments indicated that changes in IL-1β mRNA were paralleled by changes in protein expression as well: we could detect significant IL-1β pro-form level elevation in the LPS or MDP treated cells, but the most prominent increase was in response to the combined treatment (Figure 7C). Cleaved, active form of IL-1β could also be detected in the cell lysates. Expression of the active form increased only after MDP, but not LPS treatment. The combined treatment caused the most robust increase in the amount of active IL-1β (Figure 7C).

To confirm the release of IL-1β, we measured the concentration of IL-1β in the culture medium. Under control conditions, secreted IL-1β protein quantity was at the level of detectability in the culture medium. While MDP or LPS alone slightly increased the amount of secreted IL-1β, a combined treatment resulted in an almost 10-fold increase, reaching ~5 pg/mL (~5 pg secreted by 1.5 x 10^6 cells) (Figure 7D). Secretion of active IL-1β could be induced by LPS + ATP treatment as well (data not shown) and was mediated by caspases, as Z-VAD completely inhibited IL-1β release (Figure 7E).

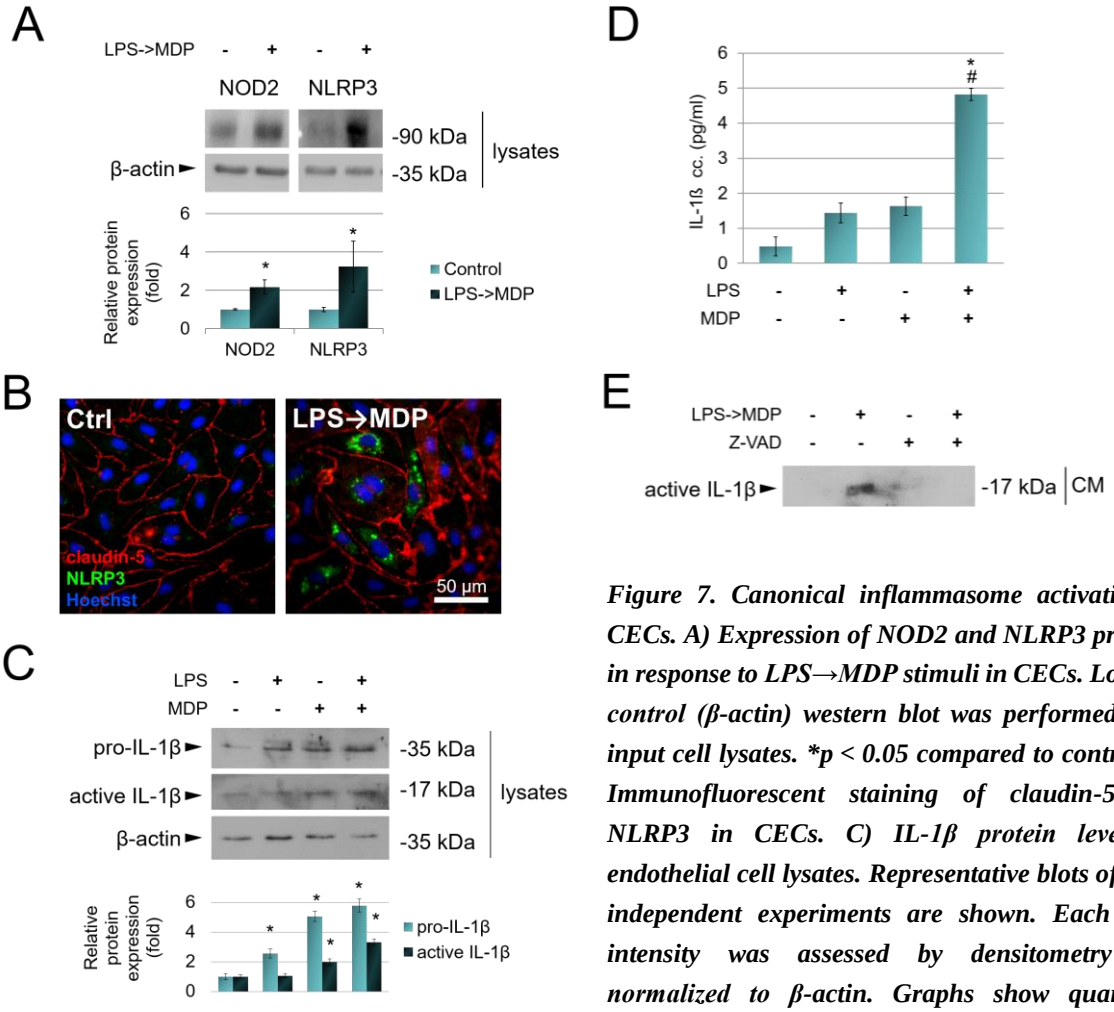


Figure 7. Canonical inflammasome activation in CECs. A) Expression of NOD2 and NLRP3 proteins in response to LPS→MDP stimuli in CECs. Loading control (β-actin) western blot was performed from input cell lysates. *p < 0.05 compared to control. B) Immunofluorescent staining of claudin-5 and NLRP3 in CECs. C) IL-1β protein levels in endothelial cell lysates. Representative blots of three independent experiments are shown. Each band intensity was assessed by densitometry and normalized to β-actin. Graphs show quantified results expressed as % of control (100%) as mean values ± standard error. *p < 0.05 compared to control. D) IL-1β protein levels in the culture medium of brain endothelial cells after LPS and/or MDP treatment. Protein concentration in the culture media was measured by solid phase sandwich ELISA. Graphs show quantified results in pg/mL as mean values ± standard error. *p < 0.05 compared to control; #p < 0.05 compared to LPS or MDP treatments. E) Inflammasome inhibition in CECs. Cells were treated with LPS (1 µg/mL), MDP (100 µg/mL) and Z-VAD-FMK (20 µmol/L) alone or in combination for 24 hours. IL-1β protein in the culture media was detected with western blot.

6.5. Signaling pathways involved in the expression of NLRP3 inflammasome components and IL-1β release in CECs

A complex signaling network regulates expression of inflammasome components in different cell types, the most important ones involving ERK1/2 and NF-κB activation. Indeed, LPS→MDP treatment induced a significant increase in ERK1/2 phosphorylation in CECs

which could be inhibited by the MAPK/ERK kinase inhibitor U0126 but not by the NF-κB inhibitor PDTC (Figure 8A). The LPS→MDP-induced increase in NLRP3 expression could be inhibited by both U0126 and PDTC, whereas expression changes of caspase-1 induced by MDP and LPS could be blocked by PDTC only. Interestingly, ASC levels were reduced by PDTC but were even increased by U0126 both in untreated endothelial cells and in LPS→MDP-treated cells (Figure 8B).

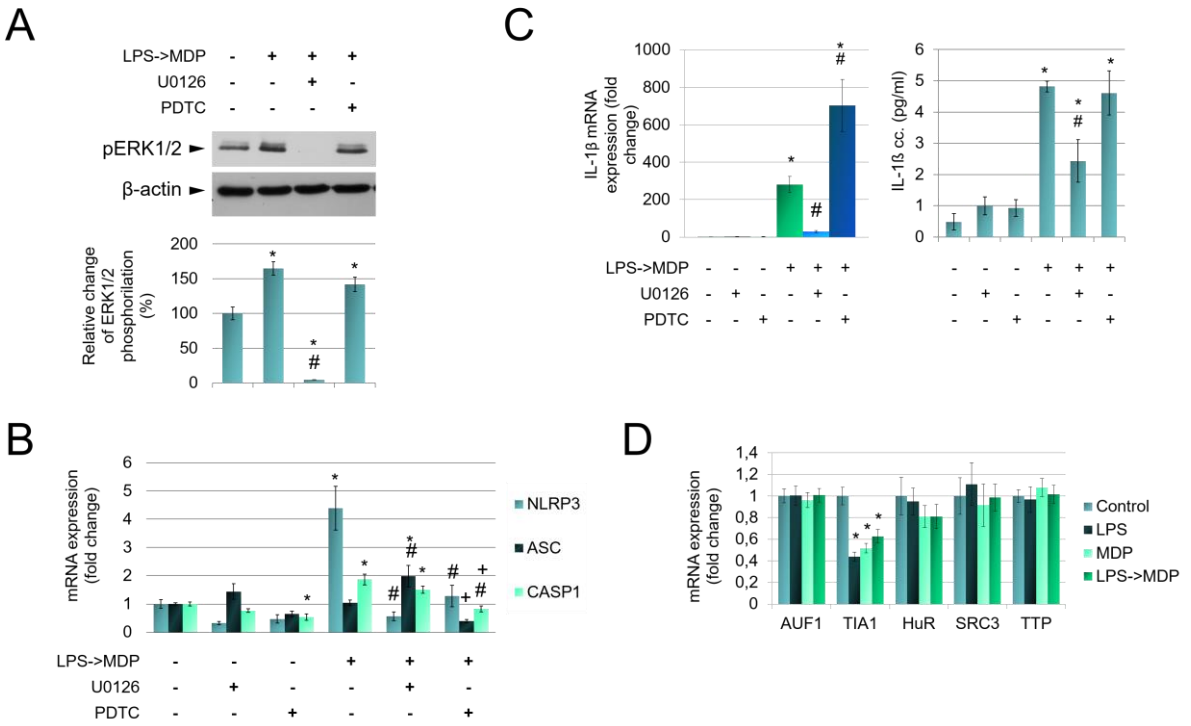


Figure 8. Signaling pathways involved in the regulation of the expression of NLRP3 inflammasome components and IL-1β and release of IL-1β. A) ERK1/2 phosphorylation level changes in response to LPS→MDP. Phosphoprotein level changes were detected with western blot. Representative blots of three independent experiments are shown. Each band intensity was assessed by densitometry and normalized to β-actin. Graphs show quantified results expressed as % of control (100%) as mean values ± standard error. *p < 0.05 compared to control; #p < 0.05 compared to LPS→MDP treatment. B) Gene expression changes of inflammasome components in response to LPS→MDP in CECs. Each PCR reaction was carried out with three parallels. Fold change values ± standard error of mean of three independent experiments are displayed. *p < 0.05 compared to control; #p < 0.05 compared to LPS→MDP treatment; +p < 0.05 compared to (LPS→MDP) + U0126. C) Signaling pathways involved in the regulation of IL-1β mRNA expression and secretion. On left panel fold change values ± standard error of mean (SEM) of at least three independent experiments are displayed. Protein concentration in culture media was measured by solid phase sandwich ELISA. Graphs on right panel show quantified results in pg/mL as mean values ± standard error. *p < 0.05 compared to control; #p < 0.05 compared to LPS→MDP treatment. D) RNA-binding protein expression in CECs. Each PCR reaction was carried out with three parallels. Fold change values ± standard error of mean (SEM) of three independent experiments are displayed. *p < 0.05 compared to control.

The LPS→MDP-induced more than 200-fold increase in IL-1β expression could be significantly but not completely inhibited by U0126. In contrast, PDTC treatment further increased IL-1β mRNA levels (Figure 8C). Changes in mRNA levels were reflected by active IL-1β levels in the culture medium as well. While we observed a 50% reduction of IL-1β levels in response to U0126 treatment, PDTC proved to be ineffective in decreasing IL-1β expression and secretion (Figure 8C).

Expression of inflammatory cytokines and chemokines can also be regulated by RNA-binding proteins in different types of immune cells. We investigated the five most known members of them in CECs. Only TIA1 mRNA quantity decreased to approximately half of the control level in response to LPS, MDP and the combined treatment, but none of the treatments had significant effects on the expression of AUF1, HuR, SRC3 or TTP (Figure 8D).

6.6. Absence of canonical inflammasome activation in cerebral pericytes

In order to detect possible inflammasome activation in cerebral pericytes — similarly to CECs — we tested secretion of active IL-1β in response to several combinations of priming and activation signals of NLRP1, NLRP2 and NLRP3 inflammasomes (LPS, MDP, LPS + MDP, LPS + ATP and TNF-α). Among these signals, TNF-α was the most potent in priming expression of pro-IL-1β protein in brain pericytes (Figure 9A), which is in accordance with the 100-times upregulation of IL-1β mRNA expression in response to TNF-α (Figure 5C). In addition, in line with the data presented in Figure 5C, only minor changes in the expression of pro-IL-1β protein were detected in response to LPS or LPS + MDP (Figure 9A). Despite marked upregulation of pro-IL-1β protein in TNF-α-treated cells, we could not detect secretion of active IL-1β in response to either of the treatments (Figure 9A).

Expression of caspase-1 mRNA and pro-caspase-1 protein, on the other hand, was potentiated by IFN-γ (Figure 5C, Figure 9B). Therefore, subsequently we challenged brain pericytes with the combination of IFN-γ and TNF-α. Priming of IL-1β was maximal after 2–4 hours on the mRNA and protein levels in response to IFN-γ and TNF-α (Figure 9C). However, no active IL-1β could be detected after IFN-γ and TNF-α treatment of HBVPs (Figure 9C). We then combined IFN-γ and TNF-α priming with MDP or ATP activation. The level of pro-IL-1β increased after a few hours, but no active IL-1β was formed in response to either of the stimuli

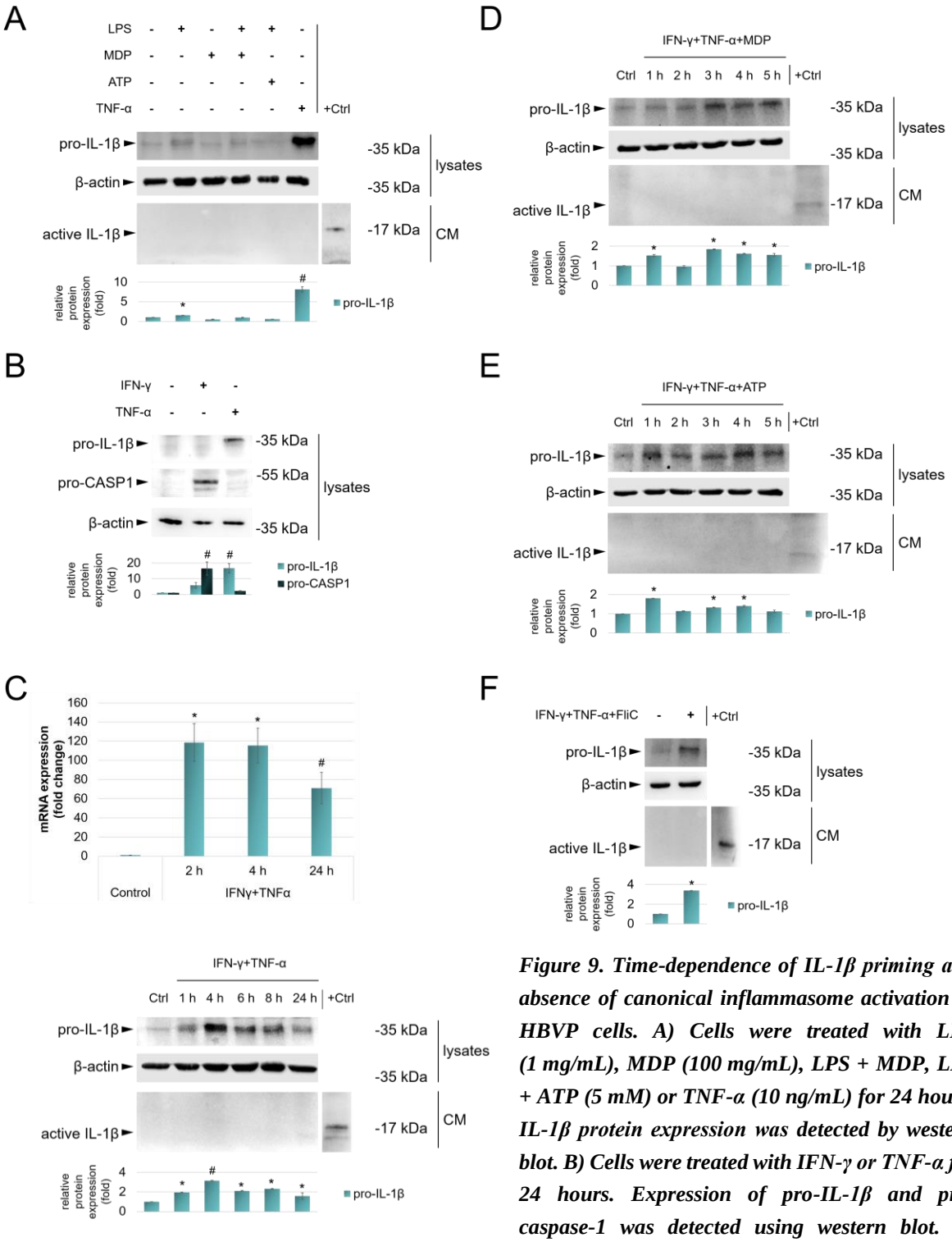


Figure 9. Time-dependence of IL-1β priming and absence of canonical inflammasome activation in HBVP cells. A) Cells were treated with LPS (1 mg/mL), MDP (100 mg/mL), LPS + MDP, LPS + ATP (5 mM) or TNF-α (10 ng/mL) for 24 hours. IL-1β protein expression was detected by western blot. B) Cells were treated with IFN-γ or TNF-α for 24 hours. Expression of pro-IL-1β and pro-caspase-1 was detected using western blot. C) Time-dependent expression of IL-1β mRNA was detected by RT-qPCR. IL-1β protein expression was detected by western blot. D), E), F) Expression of IL-1β in response to IFN-γ + TNF-β and MDP, ATP or FliC was detected using western blot. In each case one representative of three independent experiments is displayed. B-actin was used as loading control. +Ctrl = positive control (LPS + MDP-activated CECs). *p < 0.05 compared to control, #p < 0.05 compared to any other sample.

(Figure 9D and E). Similarly, nigericin was also not able to induce secretion of active IL-1β (data not shown). In addition, NLRC4 inflammasome could also not be activated in these cells using IFN-γ + TNF-α priming and flagellin (FliC) activation (Figure 9F).

6.7. Non-canonical inflammasome activation in cerebral pericytes

Since no canonical activation of NLRP1, NLRP2, NLRP3 or NLRC4 inflammasomes was observed, we continued on with testing whether brain pericytes can activate caspase-dependent active IL-1β secretion through the non-canonical inflammasome pathway. We primed pericytes with IFN-γ and TNF-α and transfected them with LPS using Lipofectamine® 2000. We observed a marked increase in the expression of pro-IL-1β (Figure 10A). In addition, we detected appearance of active IL-1β in the culture medium (Figure 10A). This indicates that cerebral pericytes can secrete active IL-1β in response to intracellular LPS (*i.e.* through non-canonical inflammasome activation). The peak in the secretion of active IL-1β was seen at 2 hours after the addition of IFN-γ + TNF-α and LPS + Lipofectamine® 2000 (Figure 10B), while highest levels of pro-IL-1β could be detected after 3–5 hours (Figure 10B).

Using ELISA, we detected ~14 pg/mL IL-1β secreted by IFN-γ + TNF-α and LPS + Lipofectamine® 2000-treated cells (Figure 10C). This value corresponds to ~14 pg IL-1β secreted by 4 x 10^5 cells.

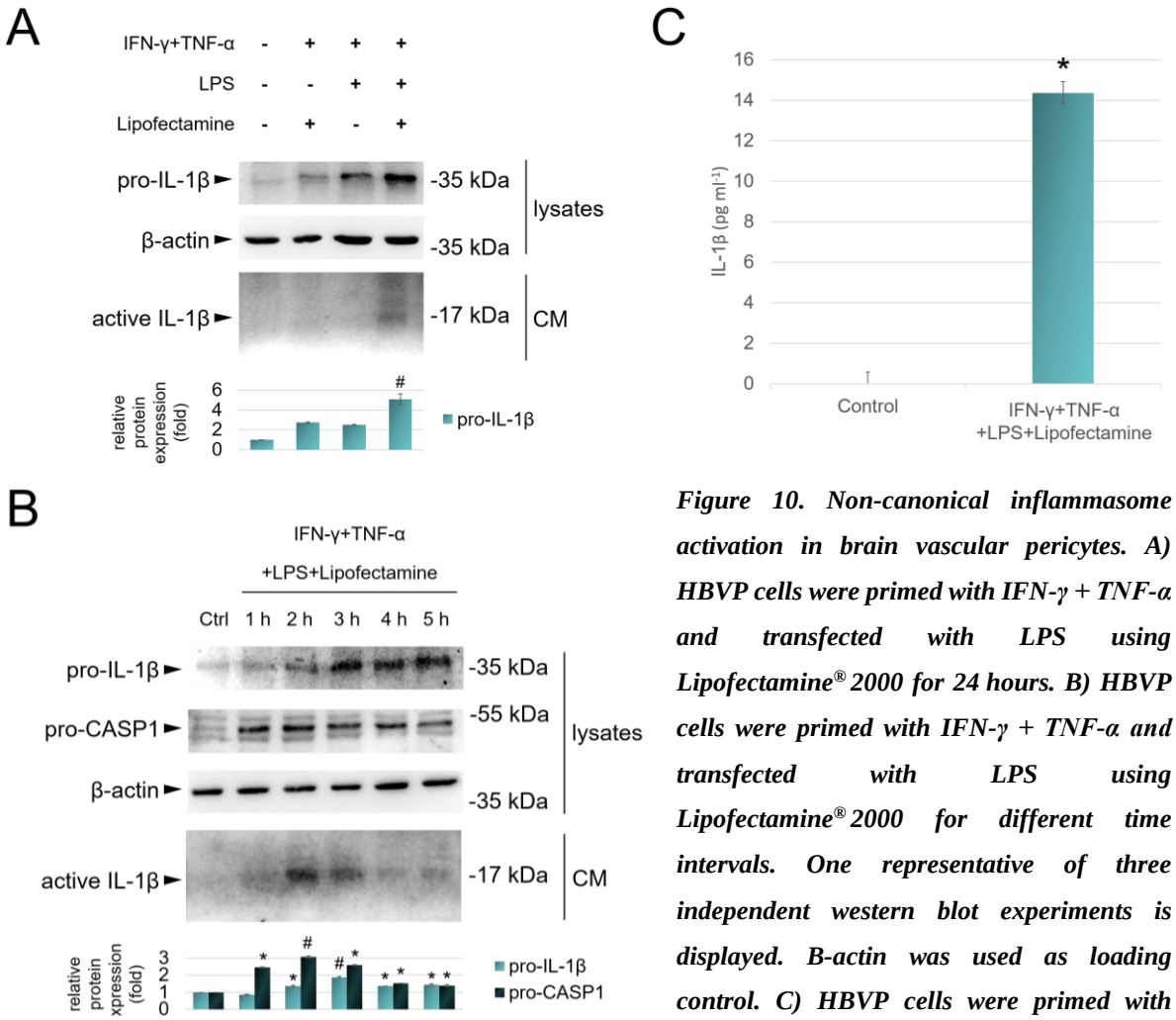


Figure 10. Non-canonical inflammasome activation in brain vascular pericytes. A) HBVP cells were primed with IFN-γ + TNF-α and transfected with LPS using Lipofectamine® 2000 for 24 hours. B) HBVP cells were primed with IFN-γ + TNF-α and transfected with LPS using Lipofectamine® 2000 for different time intervals. One representative of three independent western blot experiments is displayed. B-actin was used as loading control. C) HBVP cells were primed with IFN-γ + TNF-α and transfected with LPS using Lipofectamine® 2000 for 4 hours. Secreted IL-1β was detected from the culture medium using ELISA. *p < 0.05 compared to control, #p < 0.05 compared to any other sample.

6.8. Inflammatory activation of cerebral pericytes in response to *E. coli* infection

Thereafter, we infected HBVPs with GFP-expressing *E. coli* bacteria in the absence or presence of IFN-γ and TNF-α priming stimuli. In order to detect phagocytized bacteria, we stained GFP-*E. coli*-infected HBVP cells with anti-α-actin or anti-PDGFRβ antibodies and Hoechst (Figure 11A). Orthographic projections of the three-dimensional merged images indicated that GFP staining (corresponding to bacteria) co-localized with α-actin or PDGFRβ staining (corresponding to pericytes), suggesting presence of intracellular bacteria which could induce secretion of active IL-1β through the non-canonical inflammasome pathway.

We further tested the inflammasome activation in response to *E. coli* infection. The presence of bacteria potentiated the priming effect of IFN-γ + TNF-α on both pro-IL-1β and pro-caspase-1, and this effect was more pronounced at 4 hours than at 2 hours after infection (Figure 11B). In addition, bacterial infection not only upregulated the pro-form of IL-1β, but

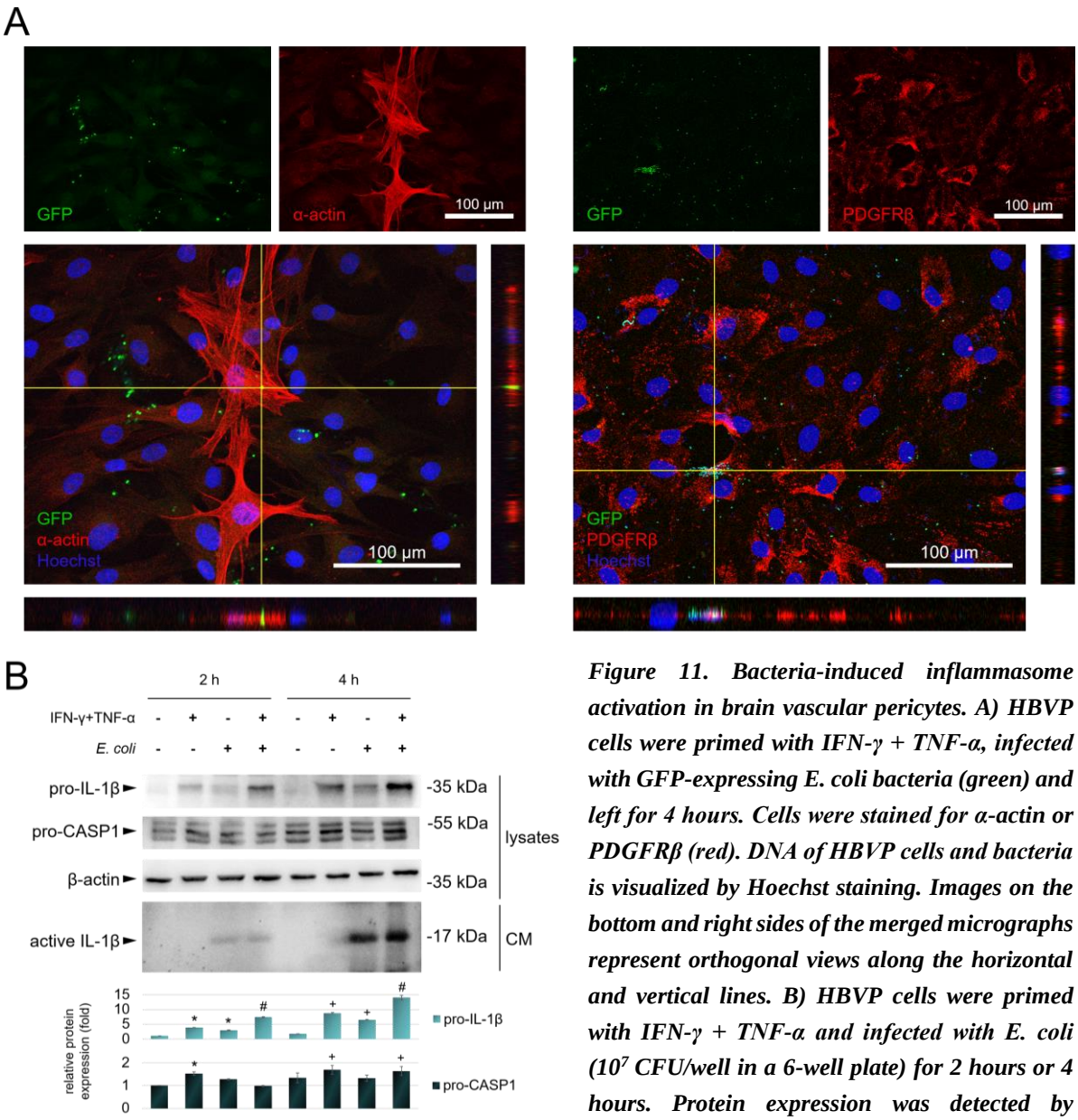
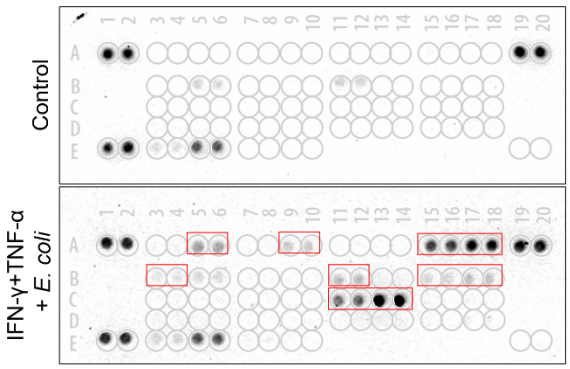


Figure 11. Bacteria-induced inflammasome activation in brain vascular pericytes. A) HBVP cells were primed with IFN-γ + TNF-α, infected with GFP-expressing E. coli bacteria (green) and left for 4 hours. Cells were stained for α-actin or PDGFRβ (red). DNA of HBVP cells and bacteria is visualized by Hoechst staining. Images on the bottom and right sides of the merged micrographs represent orthogonal views along the horizontal and vertical lines. B) HBVP cells were primed with IFN-γ + TNF-α and infected with E. coli (10^7 CFU/well in a 6-well plate) for 2 hours or 4 hours. Protein expression was detected by western blot. One representative of three independent experiments is displayed. B-actin was used as loading control. *p < 0.05 compared to control (2 hours), +p < 0.05 compared to control (4 hours), #p < 0.05 compared to any other sample.

A



B

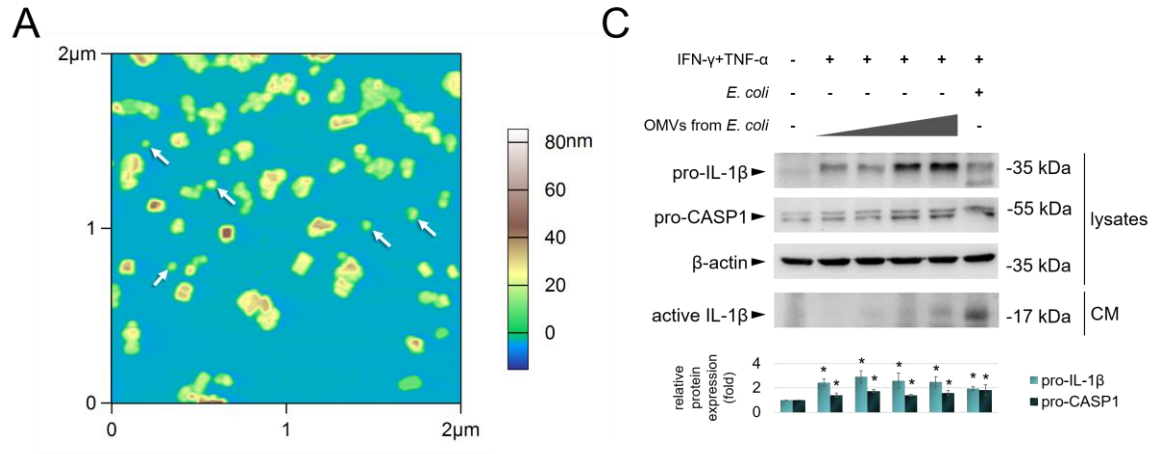
Coordinate	Target/Control	Entrez Gene ID	Alternate Nomenclature	Inflammatory activation with IFN-γ+TNF-α + *E. coli*
A1, A2	Reference Spots	N/A	_____	
A3, A4	CCL1/I-309	6346	P500, SCYA1, SCYA2, TCA-3	
A5, A6	CCL2/MCP-1	6347	MCAF	+++
A7, A8	MIP-1α/MIP-1β	6348/6351	CCL3/CCL4	
A9, A10	CCL5/RANTES	6352	_____	++
A11, A12	CD40 Ligand/TNFSF5	959	CD154, CD40LG, gp39, TRAP	
A13, A14	Complement Component C5/C5a	727	C5/C5a	
A15, A16	CXCL1/GROα	2919	CINC-1, KC	++++
A17, A18	CXCL10/IP-10	3627	CRG-2	+++++
A19, A20	Reference Spots	N/A	_____	
B3, B4	CXCL11/I-TAC	6373	β-R1, H174	+
B5, B6	CXCL12/SDF-1	6387	PBSF	
B7, B8	G-CSF	1440	CSFβ, CSF-3	
B9, B10	GM-CSF	1437	CSFα, CSF-2	
B11, B12	ICAM-1/CD54	3383	_____	+
B13, B14	IFN-γ	3458	Type II IFN	
B15, B16	IL-1α/IL-1F1	3552	_____	++
B17, B18	IL-1β/IL-1F2	3553	_____	+++
C3, C4	IL-1ra/IL-1F3	3557	_____	
C5, C6	IL-2	3558	TCGF	
C7, C8	IL-4	3565	BCDF, BSF1	
C9, C10	IL-5	3567	_____	
C11, C12	IL-6	3569	BSF-2	++++
C13, C14	IL-8	3576	CXCL8, GCP1, NAP1	+++++
C15, C16	IL-10	3586	CSIF	
C17, C18	IL-12 p70	3592/3593	CLMF p35	
D3, D4	IL-13	3596	_____	
D5, D6	IL-16	3603	LCF	
D7, D8	IL-17A	3605	CTLA-8	
D9, D10	IL-17E	64806	IL-25	
D11, D12	IL-18/IL-1F4	3606	IGIF	
D13, D14	IL-21	59067	_____	
D15, D16	IL-27	246778	IL-27 A	
D17, D18	IL-32α	9235	_____	
E1, E2	Reference Spots	N/A	_____	
E3, E4	MIF	4282	GIF, DER6	
E5, E6	Serpin E1/PAI-1	5054	Nexin, PLANH1	
E7, E8	TNF-α	7124	TNFSF1A	
E9, E10	TREM-1	54210	CD354	
E19, E20	Negative Control	N/A	_____	

Figure 12. Expression of cytokines in HBVPs exposed to IFN-γ + TNF-α and E. coli bacteria. A) HBVP cells were primed with IFN-γ + TNF-α, infected with E. coli bacteria and left for 4 hours. Cell lysates were applied onto a human cytokine array and expression of proteins displayed on the array coordinates was detected. B) Semi-quantitative analysis of protein expression level in comparison to the control. Proteins with increased expression in comparison to control cells are marked in red.

also induced secretion of active IL-1β. This was apparent already after 2 hours, but even more IL-1β was detected after 4 hours (Figure 11B). Besides IL-1β, in response to IFN-γ + TNF-α and *E. coli* infection, cerebral pericytes upregulated the expression of other ILs and chemokines as well, like IL-1α, IL-6, CCL2 (monocyte chemoattractant protein-1 – MCP-1), CCL5 (regulated on activation, normal T cell expressed and secreted – RANTES), CXCL1, CXCL8 (IL-8), CXCL10 (IFN-γ-induced protein-10 – IP-10) and CXCL11 (Figure 12). Moreover, expression of adhesion molecules (*e.g.* ICAM-1) was also increased (Figure 12).

6.9. IL-1β secretion from cerebral pericytes in response to bacterial OMVs

LPS can not only access the intracellular compartment of the cells by phagocytosis of bacteria, but also by uptake of LPS-containing OMVs shed by bacteria. Therefore, we isolated OMVs from *E. coli* (Figure 13A and B) and treated HBVPs with varying amounts of vesicles in the presence of IFN-γ and TNF-α. Significant upregulation of pro-IL-1β and pro-caspase-1 was observed in response to OMVs (Figure 13C). Moreover, OMVs induced secretion of active IL-1β in a concentration-dependent manner, although to a lower extent than the bacteria themselves.



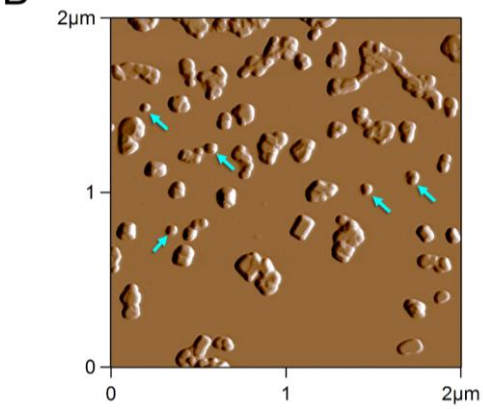


Figure 13. Bacterial OMV-induced inflammasome activation in brain vascular pericytes. A), B) Atomic force microscopy height image (A) and deflection image (B) showing OMVs isolated from E. coli bacteria. Arrows indicate single vesicles scattered among aggregated OMVs and precipitated salt. C) HBVP cells were primed with IFN-γ + TNF-α and treated with OMVs isolated from E. coli bacteria. Increasing concentrations of OMVs were the following: 0.3 mg/mL, 3 mg/mL, 30 mg/mL and 150 mg/mL (in 800 mL/well in a 6-well plate). One representative of three independent western blot experiments is displayed. B-actin was used as loading control. *p < 0.05 compared to control.

7. DISCUSSION

The BBB has been suggested to separate and to connect at the same time the immune and central nervous systems (Banks, 2015). Besides restricting immune cell trafficking into the CNS, cells of the BBB express immune receptors, respond to PAMPs and DAMPs, secrete immunoactive substances and participate in neuroinflammatory processes. The barrier itself is formed by CECs; however, — by regulating endothelial barrier functions — pericytes and astrocytes are also integral parts of the BBB (Wilhelm and Krizbai, 2014). Among these three cell types, astrocytes (Chauhan *et al.*, 2009; Johann *et al.*, 2015; Lee *et al.*, 2013; Minkiewicz *et al.*, 2013), but not CECs and pericytes have been characterized for PRR expression (Table 4).

7.1. Expression and regulation of PRRs expressed in CECs and brain pericytes

Here we show that CECs and brain pericytes express several NLRs and TLRs. Among the 22 NLRs tested, NOD1, NOD2, NLRC5, NLRP1, NLRP3, NLRP5, NLRP9, NLRP10 and NLRX mRNAs were expressed in both cell types of the BBB. In addition to these, NLRC4, NLRP12 and NLRA were further present in CECs, while in pericytes NLRP2 was also detected under physiological conditions. These receptors can sense bacterial components (*e.g.*, MDP recognized by NOD2, NLRP1 and NLRP3), toxins and cellular danger molecules (*e.g.*, extracellular ATP-induced K^+ flux which is a NLRP3 and also a NLRP2 activator) (Kim *et al.*, 2016). Our group previously detected TLR2, TLR3, TLR4 and TLR6 expression in CECs (Nagyoszi *et al.*, 2010). By using RT-PCR, expression of TLR2, TLR4, TLR5, TLR6 and TLR10 of the 10 known human TLR genes was detected in brain pericytes in basal conditions. Thus TLR2, TLR4 and TLR6 were expressed in both cell types. These receptors confer responsiveness to diverse microbial molecules, including LPS, flagellin and zymosan (Vidya *et al.*, 2018).

The majority of these receptors (NOD1, NOD2, NLRC5, NLRP1, NLRP3, NLRX1, TLR4 and TLR6) were expressed in levels comparable in CECs and brain pericytes. Some PRRs were more abundantly expressed in CECs than in pericytes (NLRP5, NLRP9 and TLR2),

while expression of NLRP10 was much higher in pericytes than in CECs. Importance of these differences between CECs and brain pericytes still needs to be elucidated.

Table 4. Expression of TLRs and NLRs in cells of the NVU, cerebral and non-cerebral pericytes. Data were collected from our study (bold) and literature. * = expressed only upon stimulation or in pathological conditions.

	TLRs		NLRs	
	mRNA	protein	mRNA	protein
cells of the NVU				
CECs	TLR2, TLR3, TLR4, TLR6	TLR2, TLR6	**NOD1, NOD2, NLRC4, NLRC5, NLPR1, NLRP3, NLRP5, NLRP9, NLRP10, NLRP12, NLRA, NLRX**	**NOD2, NLRP3**
astrocytes	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, TLR10	TLR3, TLR4	NLRC4, NLRP1, NLRP2, NLRP3	NLRC4, NLRP1, NLRP2, NLRP3
microglia	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7,TLR8, TLR9	TLR1, TLR2, TLR3, TLR4	NOD1, NOD2, NOD3, NLRC4, NLRC5, NLRP1, NLRP2, NLRP3, NAIP	NLRP1, NLRP3
neurons	TLR1, TLR2, TLR3, TLR4	TLR3, TLR4, TLR7, TLR9	NLRC4, NLRP1, NLRP3	NLRC4, NLRP1, NLRP3
brain pericytes	**TLR2**, TLR4, **TLR5, TLR6, TLR9*, TLR10**	TLR4	NOD1, NOD2, **NLRC4*, NLPR1, NLRP2, NLRP3, NLRP5, NLRP9, NLRP10, NLRA*, NLRX**	NOD1
peripheral pericytes				
lung	TLR4	TLR3, TLR4		
liver	TLR2, TLR3, TLR4, TLR7, TLR9	TLR2, TLR3, TLR4	NLRP3	NLRP3
kidney	TLR2, TLR4			
placenta	TLR2, TLR4		NLRP3	

Among NLRs and TLRs, besides the four TLRs described by our group (Nagyoszi *et al.*, 2010), only NLRP3 and NLRA have been formerly identified in brain endothelial cells (Girvin *et al.*, 2002; Reimer *et al.*, 2010), while in pericytes NOD1, NOD2 and TLR4 have been previously described (Guijarro-Muñoz *et al.*, 2014; Kovac *et al.*, 2011; Navarro *et al.*, 2016b). Even in peripheral pericytes only a few of these receptors have been detected (Table

4). NLRP3 has been detected in placental and kidney pericytes and hepatic stellate cells (fat-storing pericytes of the liver) (Leaf *et al.*, 2016; Stark *et al.*, 2013; Weiskirchen and Tacke, 2014). TLR2 and TLR4 have been described in lung, kidney and placental pericytes (Edelman *et al.*, 2006; Edelman *et al.*, 2007; Leaf *et al.*, 2016; Stark *et al.*, 2013), while hepatic stellate cells express TLR2-4, TLR7 and TLR9 (Weiskirchen and Tacke, 2014). Thus, our results pinpoint towards a more important role of the cerebral endothelium and pericytes in innate immunity than previously thought.

It is well known that a wide range of pathological conditions can regulate the expression of PRRs, especially oxidative stress and inflammatory stimuli. Most of our knowledge about these regulation mechanisms is based on information originating from cells of the immune system. Here we show that these stimuli are able to regulate PRRs — including NOD2, NLRP3, NLRP5, NLRP9 and NLRP10 — in CECs and pericytes as well. Despite a relatively high NOD1 expression in CECs, NOD1 was not inducible by inflammatory stimuli in this cell type. On the other hand, NOD1 expression was enhanced by 1.5-2 folds in brain pericytes in response to TNF-α, IFN-γ or IL-1β. In a recent study, similar to our results, NOD1 was shown to be upregulated in brain pericytes in response to TNF-α or IFN-γ (Navarro *et al.*, 2016b); however, low expression and low induction of NOD2 gene was observed. In contrast, according to our results, expression of NOD2 is highly stimulated by inflammatory cytokines in both CECs and pericytes. The discrepancy between the two results might come from different culture conditions (*e.g.*, different serum and growth supplements). The large increase seen by us in NOD2 expression in response to inflammatory stimuli in both CECs and pericytes had been previously observed in other cell types as well (Gutierrez *et al.*, 2002; King *et al.*, 2009; Rosenstiel *et al.*, 2003).

Transcriptional regulation of other PRRs, including inflammasome components, is not completely characterized in non-immune cells. Our results demonstrate that NLRP3, NLRP5, NLRP9 and NLRA can be highly upregulated by inflammatory mediators in CECs, as previously shown in other cell types (Niebuhr *et al.*, 2014; Tangi *et al.*, 2012). In brain pericytes we observed that a distinct set of genes is upregulated by inflammatory mediators (including NOD2, TLR2, but also NLRA, NLRC4, NLRC5, TLR9, inflammasome-forming caspases and inflammasome-cleaved interleukins), while expression of TLR10 and NLRP9 is induced by

oxidative stress. To our best knowledge, we are the first to show significant induction of NLRP10 transcription in response to TNF-α in both CECs and pericytes.

Inflammasome components may also be responsive to microbial components like LPS or MDP. While expression of NLRP1 was unaffected by LPS in both CECs and cerebral pericytes, caspase-1, NOD2 and NLRP3 were upregulated by LPS in CECs, but not in pericytes. It is noteworthy that LPS and MDP treatments had synergistic effects, a combined treatment eliciting a much higher increase in NLRP3, NOD2, TLR2, AIM2, IL-1β and IL-33 mRNA levels in CECs, than treatment with either substance alone. Interestingly, the strong inductive effect of LPS→MDP on NLRP3 was mediated by MAPK and NF-κB signaling, but these signaling pathways were not involved in the expression regulation of NOD2 in CECs. Caspase-1 was regulated only by the NF-κB pathway in CECs.

Taken together, as shown by the correlation matrix comparing the two cell types studied, CECs and brain pericytes have a similar pattern of regulation of NLRs, TLRs and inflammasome components. Aside from a few exceptions (NLRP5 in endothelial cells, NLRP5 and TLR2 in pericytes), PRRs and ILs expressed by both cell types react similarly to inflammatory stimuli or oxidative stress on the gene expression level.

7.2. Activation of inflammasomes in CECs via the canonical pathway

Activation of certain PRRs can lead to the inflammasome-dependent secretion of IL-1β, which is the main mechanism of release of this inflammatory cytokine. Recent studies indicate that inflammasomes are activated in the majority of brain diseases (infections, ischemia, neurodegenerative disorders, traumatic brain injury, *etc.*), the immune response being more detrimental than beneficial in the majority of the cases. In inflammatory processes of the brain infiltrating immune cells and almost all cells of the CNS (microglial cells, astrocytes, neurons and CECs) may take part (Lénárt *et al.*, 2016). It is well known that among cells of the BBB, astrocytes are able to assemble inflammasomes. They can activate NLRP1, NLRP3, NLRC4, AIM2 and possibly NLRP2 inflammasomes, which may play role in several

neurodegenerative diseases (Lénárt *et al.*, 2016). However, possible inflammasome activation in CECs and brain pericytes has not been tested so far.

Nevertheless, we detected expression of NLRP1 and NLRP3 inflammasome components together with inflammasome-processed ILs (IL-1β, IL-18, IL-33 and IL-37) in both CECs and pericytes. Inflammasome activation might need both priming and activating signals through activation of TLRs and/or NLRs. Concerted activation of TLRs and NLRs not only induced an increase in IL-1β mRNA, but led to a significant increase in the released active IL-1β as well in CECs. This release was caspase-dependent, indicating inflammasome activation. The amount of IL-1β secreted by LPS→MDP-treated CECs was lower than that observed in immune cells (Palova-Jelinkova *et al.*, 2013) ; however, this may be enough to achieve relevant local concentrations to regulate the NVU.

Induction by LPS→MDP was mediated by ERK1/2 – similarly to human PBMCs (Windheim *et al.*, 2007) or monocytes (Liang *et al.*, 2013) –, suggesting that ERK1/2 is an important element in the regulation of IL-1β expression. On the other hand, inhibition of NF-κB was not only unable to inhibit IL-1β mRNA induction, but even increased its expression in CECs. This result was unexpected because Shimogaki *et al.* could demonstrate a reduction in IL-1β transcription in PBMCs (Shimogaki *et al.*, 2014).

IL-1β expression can be regulated by RNA binding proteins as well. The most investigated RNA binding regulator proteins are AUF1, TIA1, TTP, HuR and SRC3 (Anderson 2010). All the above mentioned RNA binding proteins could be detected in CECs at transcriptional level. Of these, TIA1 was downregulated in CECs by LPS→MDP, suggesting that in response to inflammatory agents a releasing mechanism may occur that liberates inflammatory cytokines from translational inhibition (Haneklaus *et al.*, 2017; Piecyk *et al.*, 2000).

7.3. Activation of inflammasomes in brain pericytes via the non-canonical pathway

In contrast to the canonical inflammasome activation in CECs, we detected different mechanisms of inflammasome activation in brain pericytes. In a recent study, upregulation of IL-1β mRNA was detected by microarray analysis in LPS-treated HBVPs; however, protein expression and secretion have not been further evaluated (Guijarro-Muñoz *et al.*, 2014). In

another study, no IL-1β release could be detected in mouse pericytes in control conditions or LPS treatment (Kovac *et al.*, 2011). However, secretion of active IL-1β is a predominantly inflammasome-dependent phenomenon; therefore, it might require an activating signal besides LPS-induced priming.

Henceforth, based on our gene expression results, priming was achieved using combination of IFN-γ and TNF-α treatments upregulating NOD2 (an NLRP1 inflammasome component), NLRP2, NLRP3, caspase-1 and IL-1β expression. For activation, we used MDP (targeting NLRP1 and NLRP3 inflammasomes) or extracellular ATP (a NLRP3 and NLRP2 activator). However, no detectable active IL-1β was formed in brain pericytes in response to these stimuli. Interestingly, non-cerebral pericytes can activate NLRP3 inflammasomes to secrete active IL-1β in the placenta and kidney (Leaf *et al.*, 2016; Stark *et al.*, 2013), suggesting organ-specific mechanisms.

Since NLRC4 expression was also induced upon stimulation with IFN-γ and TNF-α, we next tested whether FliC – an NLRC4 inflammasome activator – could induce secretion of active IL-1β in HBVPs. Similar, to MDP or ATP, flagellin was not able to promote formation of active IL-1β in cerebral pericytes. This might be explained by absence of NAIP expression, which is indispensable for NLRC4 inflammasome formation. We confirmed absence of NAIP expression in cerebral pericytes both in control conditions and in the presence of inflammatory mediators (IFN-γ, IL-1β and TNF-α). Besides the best-characterized inflammasome-forming NLRs (NLRP1, NLRP3, NLRC4 and AIM2), some other proteins (NLRP2, NLRP6 and pyrin) can also form inflammasomes. According to our results, NLRP1, NLRP2, NLRP3 and NLRC4 inflammasomes were not activated in brain pericytes, NLRP6 and AIM2 were not expressed in brain pericytes, while pyrin was not tested. In addition, there may be other inflammasome pathways, which are less well characterized and have not been tested in these cells. Nevertheless, by addressing four types of inflammasomes, we did not detect canonical inflammasome activation in brain pericytes, although the priming effect was clearly seen.

Besides the canonical pathway, there is a non-canonical way of inflammasome activation induced by Gram-negative bacteria, although the precise mechanisms are unknown (Diamond *et al.*, 2015; Vanaja *et al.*, 2015). During this process, LPS enters the cytosol where it is sensed in a TLR4-independent way (Kayagaki *et al.*, 2013). As a result, caspase-11 in mouse (Diamond *et al.*, 2015) or caspase-4/5 in human cells is activated (Casson *et al.*, 2015;

Viganò *et al.*, 2015). These caspases are in fact the intracellular receptors of LPS (Shi *et al.*, 2014). After cytoplasmic LPS detection by caspase-4/5, NLRP3 inflammasome was shown to be the cleavage platform for IL-1β in monocytes (Baker *et al.*, 2015). Caspase-4 is abundantly expressed in HBVPs in basal conditions and caspase-5 was also detected. When transfected into the cytosol, LPS induced secretion of active IL-1β, indicating that – in contrast to the canonical pathway – the non-canonical inflammasome activation could be induced in human brain pericytes. In fact, our recent experiments have shown that CECs are also able to activate the non-canonical inflammasome pathway (data not published).

The amount of IL-1β secreted in response to activation of the non-canonical pathway in cerebral pericytes was somewhat higher than that detected in CECs after canonical inflammasome activation, but much lower than that released by microglia or astrocytes in response to inflammatory or metabolic stress (Burm *et al.*, 2015; Jian *et al.*, 2016). Nevertheless, the local effect of IL-1β secreted by pericytes or CECs might indeed be relevant, since astrocytes can sense IL-1β concentrations as low as 10 pg/mL (Meini *et al.*, 2008). Pericytes located in the vascular basement membrane are in close contact on the one hand with the basolateral side of CECs and on the other hand, with endfeet of astrocytes which surround and cover the outer surface of capillaries. Therefore, pericytes might possess the role of transmitting signals (*i.e.*, inflammation) from CECs to astrocytes.

7.4. Inflammasome activation and cytokine production in brain pericytes in response to *E. coli* infection or OMVs emitted by the bacteria

It was previously shown that brain pericytes are phagocytic cells (Balabanov *et al.*, 1996; Pieper *et al.*, 2014), and our confocal microscopy images suggested presence of bacteria in the cytoplasm of *E. coli* infected pericytes. Moreover, both IFN-γ and TNF-α – used by us for priming of inflammasome components – have been shown to increase the phagocytic activity of brain pericytes (Pieper *et al.*, 2014). Besides phagocytosis of bacteria, endocytosis of LPS or LPS-containing vesicles can also induce non-canonical inflammasome activation. *E. coli* bacteria shed significant amounts of LPS into the surrounding medium during growth (Mattsby-Baltzer *et al.*, 1991), which can be internalized by various cell types via CD14- and scavenger receptor-mediated mechanisms (Dunzendorfer *et al.*, 2004; Vishnyakova *et al.*,

2003). Recently, OMVs have been identified as vehicles delivering LPS into the cytosol (Vanaja *et al.*, 2016). *E. coli* produces OMVs which contain several virulence factors, including LPS. Our results indicate that in response to OMVs released by *E. coli* bacteria, inflammasomes could be activated in cerebral pericytes probably through the non-canonical pathway.

Brain pericytes are also able to secrete several other proinflammatory cytokines and chemokines (Guijarro-Muñoz *et al.*, 2014; Kovac *et al.*, 2011). LPS-induced upregulation of cytokines and chemokines (Navarro *et al.*, 2016a) was highly overlapping with IFN-γ + TNF-α and *E. coli* infection-induced cytokine expression in human brain pericytes, which includes CCL2, CXCL1, CXCL8, CXCL10 and IL-6. In addition, among cells of the BBB, pericytes are the most sensitive to TNF-α, responding to this cytokine by release of MIP-1α and IL-6 (Matsumoto *et al.*, 2014). In response to TNF-α, pro-IL-1β expression is also upregulated; however, secretion of the active cytokine requires cytosolic LPS leading to inflammasome activation through the non-canonical pathway.

All these findings suggest that pericytes might have a well-established role in neuroinflammatory processes.

8. SUMMARY

Despite an acknowledged role of inflammation in a large number of CNS disorders, only a few inflammasomes have been characterized so far in the CNS. These include NLRP1 (Abulafia *et al.*, 2009; de Rivero Vaccari *et al.*, 2008) and AIM2 (Adamczak *et al.*, 2014) inflammasomes in neurons, NLRP2 inflammasome in astrocytes (Minkiewicz *et al.* 2013) and the NLRP3, NLRC4 and AIM2 inflammasomes in microglia (Denes *et al.*, 2015; Halle *et al.*, 2008; Hanamsagar *et al.*, 2011). Our results indicate that besides the above mentioned cell types, CECs and brain pericytes may also assemble inflammasomes. Here we also show that one inflammatory signal (like LPS) alone may only induce a minimal release of IL-1β protein. Inflammasome assembly and activation through the canonical way (induced by priming with LPS and induction by MDP) is needed for the secretion of active IL-1β in CECs, while pericytes use the non-canonical pathway (dependent on cytosolic LPS) to activate inflammasomes. This explains why no LPS-induced IL-1β secretion could be previously detected in murine brain vessel endothelial cells (Verma *et al.* 2006) and in brain pericytes (Guijarro-Muñoz *et al.*, 2014; Kovac *et al.*, 2011) despite the demonstrated expression of IL-1β mRNA (Fabry *et al.*, 1993; Guijarro-Muñoz *et al.*, 2014).

Taken together:

- We have characterized the expression profile of Toll-like and NOD-like receptors and of inflammasome components in control and stimulated human CECs and brain pericytes. Our findings indicate that these cells can sense diverse microbial components, toxins and endogenous danger signals released upon tissue damage.
- Moreover, we demonstrated for the first time that inflammasomes can be activated in CECs in a canonical, partially MAPK-dependent manner, resulting in the secretion of active IL-1β.
- We found that cerebral pericytes respond to inflammatory cytokines; however, in response to canonical inflammasome activating signals, expression of inflammasome components is upregulated without secretion of active IL-1β.
- According to our results, in brain pericytes release of IL-1β is well-controlled and seems to depend on intracellular LPS, which induces a prompt release of active IL-1β through the non-canonical inflammasome pathway. To our best knowledge, this is the first

evidence of non-canonical inflammasome activation in cells of the CNS. This suggests that cerebral pericytes activate potent inflammatory reaction — which might possibly be harmful — in a strictly regulated manner.

- Finally, we have shown that beside IL-1β, brain pericytes express a wide range of other inflammatory cytokines in response to bacterial infection.

Activation of inflammasomes in CECs and brain pericytes is a less characterized, but presumably important mechanism of the BBB to regulate the neuro-immune axis. Recent evidence indicates involvement of inflammasomes in diseases related to the NVU, including neurodegenerative diseases, stroke and retinopathy (reviewed in: Mohamed *et al.*, 2015; Tohidpour *et al.*, 2017). Inflammasome inhibitors are emerging as therapeutic agents in inflammatory diseases (Coll *et al.*, 2015) and therefore, our results identify CECs and brain pericytes as potential targets in the treatment of neuroinflammatory disorders.

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10. ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, *Dr. István Krizbai*, leader of the Physiology and Pathology of the Blood-Brain Barrier Research Group, who introduced me to the field of molecular neuroscience. He encouraged and supported me all along during my B.Sc., M.Sc. and Ph.D. studies. I am especially grateful to *Dr. Péter Nagyőszi* and *Dr. Imola Wilhelm* for their instructions and help in my work.

I express my thanks to *Dr. Pál Ormos, Dr. László Zimányi* the former and recent directors of the Institute of Biophysics; as well as to *Dr. László Siklós*, head of the Molecular Neurobiology Research Unit and to all my colleagues in the whole institute for the pleasant working atmosphere.

I am very grateful to my former and recent colleagues in the group, *Dr. Attila E. Farkas, Dr. Csilla Fazakas, János Haskó, Mihály Kozma, Ádám Mészáros, Dr. Judit Molnár* and *Kinga Molnár* for their instructions, help and the motivating teamwork all the time.

I would like to thank all collaborators for the expert contribution in the experiments, especially *Dr. Krisztina Nagy* and *Dr. Péter Galajda* for their work and help in the microbiology parts of the study; and to *Dr. Gergely A. Végh* and *Dr. György Váró* for atomic force microscopy images.

Special thanks to my whole family for their eternal love and support. I am also grateful to all my friends who stood by me, even if not mentioned here by name.

Finally, I thank for the financial supports provided by the Doctoral Scientific Scholarship of the Doctoral Institute of University of Szeged, the ÚNKP-16-3/1. VI. 2. New National Excellence Program of the Ministry of Human Capacities and the Gedeon Richter Centenary Foundation. This work was supported by the National Research, Development and Innovation Office (grant numbers: GINOP-2.3.2-15-2016-0020, GINOP-2.3.2-15-2016-0034 and GINOP-2.3.2-15-2016-0030).

11. APPENDIX – REPRINTS OF THE SCIENTIFIC PUBLICATIONS