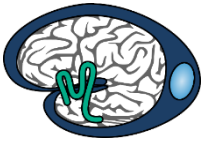

PATTERN RECOGNITION RECEPTORS AND INFLAMMASOME ACTIVATION IN THE BLOOD-BRAIN BARRIER: NEW PLAYERS IN NEUROINFLAMMATION

Summary of Ph.D. Thesis

Ádám Nyúl-Tóth

Supervisor: István Krizbai M.D., Ph.D., D.Sc.

Physiology and Pathology of the Blood-Brain Barrier
Research Group
Molecular Neurobiology Research Unit
Institute of Biophysics, Biological Research Centre
Hungarian Academy of Sciences

Doctoral School of Theoretical Medicine
Faculty of Medicine
University of Szeged

Szeged
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INTRODUCTION

Cerebral endothelial cells (CECs) — forming the blood–brain barrier (BBB) — constitute the first defence line of the central nervous system (CNS). Pericytes are contractile cells embedded in the duplication of the basement membrane of cerebral microvessels. They are both at the interface of the immune system and the CNS and thus may play an important role in the functional integration of the two systems. Despite the relative immune privilege of the brain, one of the main common attribute in different CNS diseases is neuroinflammation. 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.

During 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). Toll-like receptors (TLRs) and NOD-like receptors (NLRs) — which are the most characterized PRRs — became the focus of interest in the past decade in the field of neuroinflammation.

Activation of TLRs by pathogen- or damage-associated patterns triggers a series of signaling pathways, leading to the induction of genes which are involved in antimicrobial host defence. NLRs can also be activated by a wide range of microbial and danger signals. Furthermore, ligand recognition and activation of several members of the NLR family (*e.g.*, NLRP1, NLRP3 or NLRC4) can initiate the formation of multiprotein complexes called inflammasomes. These platforms can activate inflammatory

caspases, resulting in the production of active cytokines (interleukin (IL)-1β or IL-18) and pyroptotic cell death. Besides NLRs, inflammasomes consist of an adaptor molecule (apoptosis associated speck-like protein containing a CARD – 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. Activation of inflammasomes and IL synthesis is under the control of a complex network of signaling molecules.

Increasing evidence suggests that besides immune cells, cells of the NVU can also express and activate PRRs and inflammasomes. 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.

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 (outer membrane vesicles) released by the bacteria.**

MATERIALS AND METHODS

Cell culture and treatments

For the experiments a human cerebral endothelial cell line (hCMEC/D3) and human brain vascular pericytes (HBVPs) were used. Confluent monolayers of cells 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 μg/mL MDP, 100 μmol/L PDTC, 10 μmol/L U0126, 20 μmol/L Z-VAD-FMK, 5 mM ATP or 10 ng/mL FLiC alone or in combination for different time intervals. For intracellular LPS administration Lipofectamine®2000 was used. Pericytes were also encountered with green fluorescent protein (GFP) expressing *E. coli*.

Isolation and characterization of OMVs

OMVs were isolated from culture media of overnight growing bacterial colonies. Samples were centrifuged to settle bacterial cells. The supernatant was filtered then ultracentrifuged. Supernatant was discarded and the pellet was resolved in PBS. The

OMV content of the samples was examined by atomic force microscopy.

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

The RNA content of cells was isolated, treated with DNase and transcribed into cDNA. 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 were used for the amplification of human TLRs, NLRs, inflammasome components, cytokines and RNA-binding protein mRNAs. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control of the amplification.

Sample preparation and immunoprecipitation for western blot

Cell culture media were collected and the cells were lysed in ice cold RIPA buffer. Protein content of cell culture media samples was precipitated with either the methanol-chloroform or the TCA (trichloroacetic acid)-DOC (deoxycholic acid) method. Cell lysates were centrifuged and protein concentration of RIPA soluble fractions was determined by the bicinchoninic acid method.

For immunoprecipitation, concentrations were equilibrated with RIPA buffer, then samples were treated with anti-NOD2 or anti-NLRP3 antibodies. Protein A conjugated sepharose beads were washed in ice-cold Tris-buffered saline and used to precipitate protein-antibody complexes overnight at 4 °C. Beads were washed and resolved in Laemmli sample buffer. All samples were heated with Laemmli buffer at 95 °C for 5 minutes prior to electrophoresis.

Western blot

Protein samples were electrophoresed with standard denaturing SDS-PAGE procedures and blotted on polyvinylidene difluoride or nitrocellulose membranes. The nonspecific binding capacity of the membranes was blocked with bovine serum albumin or nonfat milk in TBS-T (Tris-buffered saline with 1 mL/L Tween-20). After blocking, membranes were incubated with the following primary antibodies in TBS-T: anti-human IL-1β, anti-caspase-1, anti-NOD2, anti-NLRP3, anti-pERK1/2 or anti-β-actin. Blots were washed, and incubated with suitable secondary antibodies in TBS-T. After further washing steps immunoreaction was visualized using Bio-Rad Clarity Chemiluminescent Substrate in a ChemiDoc MP imaging system.

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

For quantification of IL-1β secreted by brain endothelial cells and pericytes, we used a human IL-1β solid phase sandwich ELISA following the manufacturer's recommendations. Cell culture 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™ Array. After coculturing 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.

Immunofluorescence studies

Pericytes and CECs were grown on coverslips and after priming they were encountered with *E. coli* bacteria for 4 hours or LPS and MDP for 24 hours. Samples were washed extensively then were fixed. After washing steps with PBS, samples were

permeabilized then nonspecific binding capacity was blocked with normal goat serum in PBS. Anti-α-actin, anti-PDGFRβ or anti-NLRP3 and anti-claudin-5 antibodies were applied on the coverslips in normal goat serum in PBS overnight. After washing, coverslips were incubated with Cy3-labeled anti-rabbit, anti-mouse or Alexa488-labeled IgG secondary antibodies. After three further washing steps, samples were mounted with FluoroMount-G media. Fluorescent signals were examined with an Olympus Fluoview FV1000 confocal laser scanning microscope.

Statistical analysis

Statistical tests were performed with open source R Statistical Software version 3.4.0. All data presented 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. Changes were considered statistically significant at $p < 0.05$. Each experiment was repeated at least three times.

RESULTS

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

In CECs and brain pericytes several members of the NOD-like and Toll-like receptor families were expressed under control conditions as summarized in Table 1. Furthermore, all necessary inflammasome components and caspase substrate ILs were also present in both endothelial cells and pericytes.

Table 1. Expression of TLRs and NLRs in CECs and 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
CECs	TLR2, TLR3, TLR4, TLR6	TLR2, TLR6	**NOD1, NOD2, NLRC4, NLRC5, NLPR1, NLRP3, NLRP5, NLRP9, NLRP10, NLRP12, NLRA, NLRX**	**NOD2, NLRP3**
brain pericytes	**TLR2**, TLR4, **TLR5, TLR6, TLR9*, TLR10**	TLR4	NOD1, NOD2, **NLRC4*, NLPR1, NLRP2, NLRP3, NLRP5, NLRP9, NLRP10, NLRA*, NLRX**	NOD1

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. Therefore, we treated CECs and pericytes with relevant concentrations of H_2O_2, LPS, MDP, LPS + MDP, IFN-γ, TNF-α or IL-1β. IL-1β, TNF-α and LPS + MDP were the most potent to induce gene expression changes as visible in Table 2.

Interestingly, H_2O_2 treatment decreased the expression of NLRP5 in a significant manner in CECs. In contrast to CECs, oxidative stress radically upregulated NLRP9 and TLR10 in brain pericytes.

Table 2. Changes in the mRNA expression of the most important NLRs and IL-1β in response to inflammatory stimuli in CECs and cerebral pericytes.

	CECs			pericytes		
	IL-1β	TNF-α	LPS+MDP	IL-1β	TNF-α	LPS+MDP
NOD1	-	-	-	↑	↑	-
NOD2	↑	↑	↑	↑	↑	↑
NLRP1	-	-	-	-	-	-
NLRP3	↑	↑	↑	↑	↑	↑
IL-1β	↑	↑	↑	↑	↑	↑

Canonical inflammasome activation in CECs

We found that NOD2 and NLRP3 protein levels increased significantly in response to LPS priming followed by MDP activation stimuli in CECs. 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. In our western blot experiments 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. Cleaved, active form of IL-1β could also be detected in the cell lysates. 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, the combined treatment resulted in an almost 10-fold increase, reaching ~5 pg/ml (~5 pg secreted by 1.5 x 10^6 cells). Secretion of active IL-

1β was mediated by caspases (*i.e.,* inflammasomes), as Z-VAD completely inhibited IL-1β release.

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. 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. Expression of caspase-1 mRNA and pro-caspase-1 protein, on the other hand, was potentiated by IFN-γ. Priming of IL-1β was maximal after 2–4 hours on the mRNA and protein levels in response to IFN-γ and TNF-α. 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. In addition, NLRC4 inflammasome could also not be activated in these cells using IFN-γ + TNF-α priming and flagellin (FliC) activation.

Non-canonical inflammasome activation in cerebral pericytes

Since no canonical activation of inflammasomes was observed, we continued on with testing the non-canonical inflammasome pathway. We primed pericytes with IFN-γ and TNF-α and transfected them with LPS using Lipofectamine®2000. We detected appearance of active IL-1β in the culture medium. 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, while highest levels of pro-IL-1β could be detected after 3–5 hours. Using ELISA, we detected ~14 pg/mL IL-1β secreted by IFN-γ + TNF-α and LPS + Lipofectamine®2000-treated cells. This value corresponds to ~14 pg IL-1β secreted by 4 x 10^5 cells.

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. 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. 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. In addition, bacterial infection not only upregulated the pro-form of IL-1β, but also induced secretion of active IL-1β. This was apparent already after 2 hours, but even more IL-1β was detected after 4 hours. In these conditions, besides IL-1β, 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.

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* 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. Moreover, OMVs induced secretion of active IL-1β in a concentration-dependent manner, although to a lower extent than the bacteria themselves.

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 and AIM2 inflammasomes in neurons, NLRP2 inflammasome in astrocytes and the NLRP3, NLRC4 and AIM2 inflammasomes in microglia. 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 and in brain pericytes despite the demonstrated expression of IL-1β mRNA.

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 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. Inflammasome inhibitors are emerging as therapeutic agents in inflammatory diseases and therefore, our results identify CECs and brain pericytes as potential targets in the treatment of neuroinflammatory disorders.

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LIST OF PUBLICATIONS

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

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**
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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**

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:

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**

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