Final Report NKFIH K116158

Title: Role of brain endothelial cells and pericytes in the inflammatory responses of the neurovascular unit

The main goal of the research was to understand the role of brain endothelial cells and pericytes in the inflammatory responses of the neurovascular unit. To achieve this the following specific aims were set:

1. To elucidate the regulation of inflammasome components and their function in pericytes and cerebral endothelial cells.

2. To determine how pericytes exposed to pathological condition regulate the function of the NVU.

3. To investigate the regulation of inflammasome activation in cerebral endothelial cells and pericytes in vivo.

Pattern recognition receptors are the main sensing receptors of the innate immune system. A selected number of pattern recognitin recptors are able to form inflammasomes whose main function is the production of interleukin-1beta and -18, key mediators of inflammation. These receptors are mainly expressed in immune cells, however, there is evidence, that they can be expressed in other cell types as well including cells of the CNS.

After we have characterized the expression profile of pattern recognition receptors in cerebral endothelial cells and we have demonstrated that inflammasomes can be activated in cerebral endothelial cells (Nagyőszi et al., J Neurochem. 2015 Nov;135(3):551-64.) we started to systematically investigate according to the research plan the pattern recognition receptor expression of brain pericytes. We detected expression of NOD1, NOD2, NLRC5, NLRP1-3, NLRP5, NLRP9, NLRP10 and NLRX mRNA in non-treated cells. Among the ten known human TLRs, TLR2, TLR4, TLR5, TLR6 and TLR10 was found to be expressed. Expression levels of the majority of these receptors 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.

As a next step we aimed at understanding the transcriptional regulation of NLRs, inflammasome-associated proteins and TLRs in cerebral pericytes in response to oxidative

stress, bacterial wall components and inflammatory cytokines. Inflammatory mediators induced the expression of NLRA, NLRC4 and TLR9 and increased the levels of NOD2, TLR2, inflammasome-forming caspases and inflammasome-cleaved interleukins. Oxidative stress, on the other hand, upregulated expression of TLR10 and NLRP9. Activation of selected pattern recognition receptors can lead to inflammasome assembly and caspase-dependent secretion of IL-1β. TNF-α and IFN-γ increased the levels of pro-IL-1β and pro-caspase-1 proteins; however, no canonical activation of NLRP1, NLRP2, NLRP3 or NLRC4 inflammasomes could be observed in human brain vascular pericytes. On the other hand, we could demonstrate secretion of active IL-1β in response to non-canonical inflammasome activation, a mechanism which hasn't been detected in the CNS so far.

We continued our investigations with the characterization of non-canonic inflammasome activation in pericytes. We have shown that the non-canonic activation of inflammasomes in pericytes occurs 2-4 hours after activation with LPS loaded in lipofectamine particles. Furthermore, we have demonstrated that pericytes are able to take up E-coli bacteria and they activate inflammasomes in a similar time frame.

A very exciting finding was that not only whole bacteria, but outer membrane vesicles derived from E-coli can also activate inflammasomes in pericytes. This indicates that non-CNS inflammatory processes may have a remote effecton pericyte function. These results were published in: Nyúl-Tóth Á et al. Brain Behav Immun. 2017 Aug;64:220-231. doi: 10.1016/j.bbi.2017.04.010. (IF2017: 6,306/D1).

Our further investigations revealed that inflammasomes can be activated through the non-canonical pathway not only in pericytes but in cerebral endothelial cells as well. The amount of released IL-1b is even higher compared to the canonical activation. In order to determine the functional consequences of non-canonical inflammasome activation we monitored the transendothelial electrical resistance (TEER) of primary porcine brain endothelial cell (PBEC) monolayers and we found a significant increase in the permeability of the blood-brain barrier. This change was due to the reduction of the main tight junction protein claudin-5, as demonstrated by immunfluorescence staining. Further we have shown that kinurenic acid can reduce inlammasome activation in cerebral endothelial cells.

By extending our studies to other cellular components of the neurovascular unit we showed that inflammasome activation in primary human astrocytes occurs by the canonical pathway only.

To answer the questions of the second specific aim we investigated inflammasome mediated communication between cells of the neurovascular unit. We asked how pericytes exposed to pathological conditions which activate inflammasomes influence the endothelial barrier. We exposed pericytes for 4h to inflammasome activation and we have shown that conditioned media of pericytes treated as described above (but not conditioned medium from untreated pericytes) are able to reduce electrical impedance of a cerebral endothelial monolayer – an indication of deteriorated barrier properties. Our further immunofluorescence and Western-blot analyses demonstated that this may be caused by a decreased expression of the tight junction proteins claudin-5 and ZO-1. Expression of other endothelial markers including VE-cadherin or PECAM were not influence by the pericyte conditioned medium. In addition we have revealed that activated pericytes induce IL-1b secretion in endothelial cells towards the apical compartment suggesting a pericyte mediated inflammasome activation in endothelial cells. An antibody array study identified several cytokines (including CXCL10 and CXCL11) which were upregulated in endothelial cells in response to activated pericyte conditioned medium. The results were validated using qPCR.

Investigating the pericyte – endothelial communication in opposite direction we found that inflammasome activation in endothelial cells induces NLRP3 expression and upregulates a-sma expression in pericytes. In addition we investigated cytokine release in pericytes in response to endothelial activated conditioned media. Cytokine profiling identified several pro-inflammatory cytokines, to be elevated in response to the activated conditioned medium including MIF, Serpin E1, CXCL12, IL-8, CCL2, ICAM and CXCL1 (manuscript in preparation).

Our studies focused on pattern recognition receptors in pericytes revealed the expression of NLRP2 which is also an inflammasome forming pattern recognition receptor and might be activated by non-canonical inflammasome activation, characteristic of brain pericytes. our results indicate that non-canonical inflammasome activation in HBVP increases NLRP2 expression at mRNA and protein level. Besides celular localization NLRP2 signal was detected in the culture medium as well raising the possibility of secretion of NLRP2. Experiments including proteomic analysis of the supernatant are running to test this hypothesis. Our ex vivo experiments revealed an upregulation of NLRP2 in perivascular cells in mouse brain in response of micro-occlusion induced in vivo by fluorescently-labelled microspheres.

Our in vivo experiments were focused on the identification of inflammasomes which can be induced in vivo in cells of the neurovascular unit by pathological conditions. First we started investigating inflammatory conditions. We have shown that intracarotid injection of E-coli induced a marked increase in the expression of NLRP2 in hippocampal astrocytes and our preliminary studies indicate that infections can significantly amplify inflammasome activation in stroke. Our further experiments revealed that, somewhat unexpected, the level of NLRP2 decreased in microvessels isolated from old rats (24 months old) compared to those from the young (3 months old).

We investigated the effect of target deprivation on inflammasome activation as well. Oculomotor (III.) and hypoglossal (XII.) nerve axotomy caused central (III. and XII. nucleus) inflammation by increasing the levels of NLRP3 and ASC proteins. Co-staining studies revealed that NLRP3 was mainly upregulate in neurons and astrocytes (and partially in microglia) but not in endothelial cells or pericytes. This phenomenon was more pronounced in the XII. nucleus and was highly reduced by post-operative treatment with diazoxide. It is noteworthy that target deprivation induced a translocation of NLRP3 from the nucleus to the cytolasm. These results indicate that loss of synaptic inputs of motoneurons can lead to inflammasome activation. Since besides a substantial number of neurodegenerative disorders loss of synaptic inputs of motoneurons characterizes old age as well NLRP3 may play a role in age-related dysfunction of motor neurons as well (manuscript in preparation).

We have summarized our knowledge on the role of inflammasome activation in the neurovascular unit and role of interendothelial junctions during ageing in two review publication (Wilhelm I et al., Am J Physiol Heart Circ Physiol. 2017 Nov 1;313(5):H1000-H1012. doi: 10.1152/ajpheart.00106.2017. Epub 2017 Aug 11. (IF2016: 3,324/Q1), Costea et al., Int J Mol Sci. 2019 Nov 3;20(21). pii: E5472. doi: 10.3390/ijms20215472 (IF2018: 4,181/Q1).

Different CNS diseases associated with inflammatory processes show a pronounced regional preference. This led us to investigate the possible heterogeneity of the neurovascular unit. During our investigations we have observed that there are considerable regional differences in the gene expression of BBB associated genes. Therefore, using in silico, in vitro, and ex vivo techniques we compared the expression of BBB-associated genes and proteins (i.e., markers of CECs, brain pericytes, and astrocytes) in the cortical grey matter and white matter. In silico human database analysis (obtained from recalculated data of the Allen Brain Atlas), qPCR, Western blot, and immunofluorescence studies on porcine and mouse brain tissue indicated an increased expression of glial fibrillary acidic protein in astrocytes in the white matter compared

with the grey matter. We have also found increased expression of genes of the junctional complex of CECs (occludin, claudin-5, and α-catenin) in the white matter compared with the cerebral cortex. Accordingly, occludin, claudin-5, and α-catenin proteins showed increased expression in CECs of the white matter compared with endothelial cells of the cortical grey matter. In parallel, barrier properties of white matter CECs were superior as well. These differences might be important in the pathogenesis of diseases differently affecting distinct regions of the brain (Nyúl-Tóth et al., Am J Physiol Heart Circ Physiol. 2016 Jun 1;310(11):H1702-14. We have summarized our results and compared them with literature data in a review (Wilhelm et al., Tissue Barriers. 2016 Jan 28;4(1):e1143544 (D1) and we summarized our knowledge about the role of the BBB in pharmaceutical targeting of the brain (Krizbai et al., Curr Pharm Des. 2016;22(35):5442-5462 (Q1).

We performed additional studies regarding the role of pericytes in several CNS diseases associated in which inflammation may play an important role like stroke or tumor metastasis formation. We have demonstrated with two-photon microscopy in anesthetized mice that initial vasoconstriction in response to spreading depolarization is coincident in space and time with the large extracellular accumulation of potassium, as shown with a potassium indicator fluorescent dye (Menyhárt, Farkas et al., Neurobiol Dis. 2018 Nov;119:41-52. doi: 10.1016/j.nbd.2018.07.026 (IF2018: 5,160/D1).

During our further investigations on the neurovascular unit, we were first to describe transcellular migration of metastatic cells through the blood-brain barrier (Herman et al., . J Cell Mol Med. 2019 Apr;23(4):2619-2631. doi: 10.1111/jcmm.14156 (IF2018: 4,658/Q1).

Using two photon microscopy and high resolution (STED) imaging we discovered the existence of direct interactions between metastatic tumor cells and brain pericytes both in vivo and in vitro and we have described different novel vascular responses in initial stages of metastasis formation. These include including vessel constriction, endothelial plug formation up- and downstream of the tumor cell, vacuolization of the endothelium and new vessel formation (Hasko et al., Acta Neuropathol Commun. 2019 Aug 19;7(1):133. doi: 10.1186/s40478-019-0788-1 (IF2018: 5,883/D1). We identified miRNAs involved in brain metastasis formation f triple negative breast cancer cells (Sereno, Hasko et al., Mol Oncol. 2020 Jan 13. doi: 10.1002/1878-0261.12632 (IF2018: 5,962/D1). In addition we have characterized the biomechanical properties of the interaction between melanoma cells and the neurovascular unit

by using atomic force and single-cell force spectroscopy. Varga B et al., J Mol Recognit. 2017 Jun;30(6). doi: 10.1002/jmr.2603. (IF2016: 2,175/Q3).

We have summarized our knowledge on the role of the neurovascular unit in the formation of brain metastases. Wilhelm I, Fazakas C, Molnár K, Végh AG, Haskó J, Krizbai IA. Foe or friend? Janus-faces of the neurovascular unit in the formation of brain metastases. J Cereb Blood Flow Metab. 2018 Apr;38(4):563-587. doi: 10.1177/0271678X17732025 (IF2018: 6,040/D1).

Our research highlights the importance of pericytes and cerebral endothelial cells in brain pathology, especially those associated with inflammation. We have identified key mechanisms of inflammasome activation in cells of the neurovascular unit and these mechanisms can be be potential therapeutic targets. We published with the support or partial support of this grant 17 publications totalling 70,3 impact factors. Of the 17 publications 8 are of category D1 (6 in which the PI is principal author) and 6 of Q1 category (5 in with the PI is principal author).