THE ALTERATIONS OF THE CYTOKINE NETWORK IN PERINATAL HYPOXIC-ISCHEMIC BRAIN INJURY

PhD thesis

Dr. Bajnok Anna

Doctoral School of Clinical Medicine
Semmelweis University

Consultant: Gergely Toldi, MD, PhD
Official reviewers:
Éva Görbe, MD, PhD
László Szapáry, MD, PhD
Head of the Final Examination Committee:
Tamás Machay, MD, PhD
Members of the Final Examination Committee:
György Nagy MD, DSc
Zsolt Somogyvári MD, PhD

Budapest
2019

1 INTRODUCTION

Perinatal asphyxia or hypoxic-ischemic (HI) brain injury and the consequent global hypoxic-ischemic encephalopathy (HIE) continues to be a major cause of perinatal morbidity and mortality and lifelong disability, despite the advances of the past decades in prenatal diagnostic methods and neonatal intensive care. It is important to note, that the initial degree of injury and the clinical status of the neonate often do not allow accurate prediction of the eventual neurodevelopmental outcome. While some children show favorable outcome and no significant disability, others develop severe neurodevelopmental impairments such as mental retardation, sensory impairment, cerebral palsy and seizures. Identifying factors which could differentiate between mild and severe outcome would be of great clinical value.

It is becoming more and more clear, that the final outcome is influenced by several factors beyond the severity of the initial insult. One of the key factors determining the progress of brain injury is the neuroinflammatory response evoked by hypoxic-ischemic injury. Neuroinflammation is now recognized to be a common feature of many neurological disorders, including hypoxic-ischemic brain injury. However, it appears to have dual aspects. While a certain level of inflammatory response is part of the physiological recovery process of the central nervous system, excessive neuroinflammation has been shown to play an important role in facilitating further brain injury. Emerging evidence indicates, that neuroinflammation could continue for many months after the initial brain injury and this latent phase could play an important role in the long-term consequences of perinatal hypoxic-ischemic brain injury.

Following HI brain injury, microglia and astrocytes become activated and release pro-inflammatory cytokines and chemokines. Disruption of the blood-brain barrier allows infiltration of peripheral monocytes into the brain that further enhances the inflammatory response, leading to neuronal injury and apoptosis. However, the inflammatory reaction following

asphyxia is not limited to the CNS, but can also be detected in the periphery. T lymphocytes play a key role in the inflammatory response both in the brain and peripheral blood during HI brain injury, by releasing free radicals, triggering apoptotic pathways within neurons, and most importantly, producing pro- and anti-inflammatory cytokines.

An extensive dataset describes neuroinflammation to have detrimental consequences, but results have indicated over the past decade that some aspects of the inflammatory response are beneficial for CNS outcomes. Benefits of neuroinflammation include neuroprotection, the mobilization of neural precursors for repair, remyelination, and axonal regeneration.

Previous investigations in asphyxia demonstrated that pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ play an outstanding role in the pathophysiology. IL-6, IL-8, and IL-17 (Th17 cells) also have an important contribution. On the other hand, anti-inflammatory TGF-β and IL-10 have a protective role and are important for regenerative processes. Prolonged moderate hypothermia improves neurological outcome and has become standard care for term infants with hypoxic-ischemic encephalopathy over the recent years. One mechanism by which hypothermia exerts a neuroprotective effect may be by moderating the neuroinflammatory response.

The permeability of the blood-brain barrier (BBB) is higher in neonates compared to adults and is further disrupted by the hypoxic injury itself. CD49d is part of the VLA-4 antigen which mediates the migration of activated T lymphocytes through the BBB to the site of inflammation via binding to VCAM-1, expressed by endothelial cells. Although VCAM-1 is not exclusively expressed in the CNS, the level of CD49d expression can be correlated with the capacity of T lymphocytes to enter the site of inflammation, more specifically the brain tissue in the case of neuroinflammation.

The interplay between the kynurenine system and cytokines is a regulator of both innate and adaptive immune responses, and it plays an important role in the interactions between the central

nervous and the immune systems. IDO, the rate-limiting enzyme in the degradation of tryptophan plays a central role in regulating these interactions. Induced by pro-inflammatory stimuli (such as IFN-γ), IDO is primarily produced by APCs and has several immunosuppressive effects, thus maintaining the balance between pro- and anti-inflammatory impulses. The rate of TRP degradation, expressed by the ratio of KYN to TRP (K/T) allows a good estimate of its enzymatic activity. The induction of IDO and the kynurenine system results in the inhibition of T cell functions, the activation of regulatory T cells and the inhibition of natural killer cells. The alterations of the kynurenine system appear to play a role in the pathophysiology of a broad spectrum of neurological disorders, including ischemic brain injury, however its role has previously not been investigated in perinatal asphyxia.

The perinatal period also carries the highest risk for stroke in the entire childhood. Neonatal arterial ischemic stroke (NAIS) by definition is an arterial ischemic stroke, with clinical symptoms occurring in the neonatal period supported by radiological findings. NAIS generally occurs in term neonates and presents a major risk for life-long motor, cognitive, and/or behavioral disabilities ranging from fine motor impairments to unilateral cerebral palsy, which develops in around 20-30% of affected neonates. Thus NAIS is a leading cause of cerebral palsy.

Interestingly, in almost all cases of NAIS the intra-cranial arteries developing from the carotid arterial tree are affected, i.e. the proximal parts of the anterior cerebral artery (ACA), the middle cerebral artery (MCA) and the posterior cerebral artery (PCA)—while the basilar artery and the extra-cranial arteries are left unaffected. The diagnosis of NAIS is often delayed, due to the prenatal onset or absence of specific signs, therefore the primary focus regarding therapeutic interventions is focused on prevention and post-insult anti-inflammatory mechanisms. Another challenge regarding the diagnosis of NAIS is the fact that the risk factors and clinical signs of global hypoxic-ischemic encephalopathy due to perinatal asphyxia show a significant overlap with NAIS and the two often co-occur. Differentiating between the two

syndromes is a complex question and neuroinflammation following ischemia appears to be a common feature of the two.

The pathophysiology of NAIS is poorly understood and disease specific preventive measures, prognostic factors and therapeutic strategies are not available. According to the classic pathophysiological hypothesis, most ischemic lesions are a result of thromboembolic events, where the presumable source of the thrombi are the placenta or the umbilical vessels. While in some instances this could be the case, this hypothesis does not give a plausible explanation to why NAIS is almost exclusively affecting the intracranial arterial territories developing from the carotid arterial tree, while the incidence of basilary arterial or extracerebral infarcts is negligible. In addition, there is angiographic evidence indicating a possibility of local arterial wall defects and in situ thrombus generation. Based on preclinical findings a new pathophysiological hypothesis is being raised, which considers the complex and bidirectional relationship of coagulation and inflammatory pathways. It describes the development of NAIS as the result of a multiple hit mechanism, originating from both perinatal inflammation and hypoxia–ischemia (HI). In-vivo data from rats suggests, that maternofetal inflammation induces focal arteritis specific to the intracranial arteries developing from the carotid arterial tree, which are susceptible to NAIS. The level of several pro-inflammatory cytokines, i.e. TNF-α, IL-1β and MCP-1 was higher in the susceptible intra-cerebral arteries compared to extra-cerebral arteries. Neural tissue damage could be aggravated by previous sensitization due to inflammation, which could lead to increased oxidative stress and an increased production of pro-inflammatory cytokines. The primary aim of current research in the field of perinatal stroke is to gain a better understanding of the pathomechanism of the disease with specific regard to the activation of the inflammatory pathway, that could present a possibility for more specific diagnosis, intervention and even prevention

2 AIMS

In this study our aims were the following:

1) To assess the differences in the prevalence and cytokine production of T lymphocyte subsets between moderate and severe HIE, in order to identify the players of the inflammatory response that may influence the severity of the neuroinflammation

2) To assess the alterations of plasma cytokine levels in comparison with intracellular cytokine levels in moderate and severe HIE

3) To describe the plasma levels of the substances of the kynurenine system (TRP, KYN and KYNA) and assess IDO activity based on the KYN/TRP ratio in moderate and severe HIE

4) Based on the pooled data collected in the first month of life from four NAIS patients, we aimed to assess the gross differences in the cytokine production of T lymphocytes and plasma cytokine levels between moderate, severe HIE and NAIS. Similar data has not been published in humans before, therefore, although the number of NAIS cases is small, the presented data could serve as a base for future, larger-scale case-control studies.

3 METHODS

3.1 Patients

We enrolled 33 term neonates admitted to the regional neonatal intensive care unit at the First Department of Pediatrics at Semmelweis University, Budapest, Hungary with the initial diagnosis of perinatal asphyxia requiring therapeutic hypothermia. The diagnosis of moderate-to-severe hypoxic-ischemic encephalopathy and the eligibility for cooling were assessed according to the TOBY criteria. All enrolled neonates were outborn and hypothermia was initiated upon admission, between 1-5 h of life. Rectal temperature was maintained between 33-34 °C.

2 ml venous blood samples were collected between 3-6 h of life (at admission), as well as at 24 h, 72 h and 1 wk of life during intensive care treatment, adjusted to blood sampling related to clinical care. A further venous blood sample was obtained at 1 mo of age during a routine outpatient follow-up appointment.

Neonates with congenital abnormalities or CNS malformations, maternal chorioamnionitis or perinatal infections were excluded from the study. Blood cultures and ear swabs were obtained at admission from all infants and bacterial infection was excluded. Clinical or culture-proven sepsis was not detected in any of the participating infants. All infants received regular preventive intravenous antibiotics, i.e. ampicillin and gentamicin during the hypothermic treatment. Neonates received standard intensive care treatment.

Neonates were monitored by aEEG and MRI examinations were performed within the first week of life if possible, and within 12 days of life in all cases. MRI data were interpreted based on criteria defined by Rutherford et al. Following the MRI scan, four neonates were diagnosed with neonatal arterial ischemic stroke. Data from these neonates was pooled and compared to global HIE. One neonate was excluded from the study due to a suspected metabolic disease.

The remaining 28 neonates were divided into two groups (moderate and severe HIE) depending on the severity of hypoxic-ischemic encephalopathy, determined by initial and recovery time of amplitude-integrated EEG (aEEG) monitoring and MRI results. In the cases where MRI could not be performed due to the critical condition of the patient grouping was done based on the aEEG results. The severe group (n = 11) consisted of newborns with moderate-to-severe HIE signs on MRI scans AND burst-suppression OR continuous extremely low voltage OR flat tracing background activity on aEEG OR normalization of aEEG after 48th hour of life or never, OR early death (< 28 days). Neonates that met none of the above listed criteria constituted the moderate group (n = 17) (normal MRI scans or mild HIE signs on MRI scans AND continuous or discontinuous normal voltage background activity on aEEG OR normalization of aEEG activity before 48th hour of life).

In the severe group, 3 infants deceased before one month of age due to severity of the insult. Available data from these neonates were included at the relevant time points within the severe group. Therefore, 72 h, 1 week and 1 month data were missing in case of 2 infants and 1 month data were missing from 1 infant.

Our study was reviewed and approved by the Hungarian Medical Research Council (TUKEB 6578-0/2011-EKU) and written informed consent was obtained from parents of all participants. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

3.2 Flow cytometry

Plasma was separated from peripheral blood samples by centrifugation. Plasma samples were aliquotted and immediately frozen and stored at -80 °C for later determination of cytokine concentrations and HPLC measurements.

Remaining cells were resuspended in RPMI (Roswell Park Memorial Institute)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with PMA (Phorbol 12-myristate 13-

acetate) (50 ng/ml), ionomycin (1 microg/ml) and BFA (Brefeldin A) (10 microg/ml) for 6 h at 37 °C to allow intracellular accumulation of cytokines. For surface marker staining, samples were then incubated with the following fluorochrome-conjugated anti-human monoclonal antibodies: CD4 PE-Cy7 (Phycoerythrin-Cyanine 7) and CD8 APC-Cy7 (Allophycocyanin-Cyanine 7) (panel 1), or CD4 APC-Cy7 and CD49d PerCP (Peridinin-Chlorophyll-Protein) (panel 2), respectively, according to the manufacturers' instructions (all from BioLegend, San Diego, CA, USA).

Red blood cells were lysed and PBMCs were permeabilized using FACSLysing and FACSPermeabilizing solutions (BD Biosciences, San Jose, CA, USA). Cells were washed and resuspended in PBS (phosphate buffer saline) and divided into two equal aliquots and stained according to the manufacturers' instructions for intracellular cytokines using the following conjugated anti-human monoclonal antibodies or the appropriate isotype controls: IL-6 PE (Phycoerythrin), IL-17A PerCP, IL-10 APC (Allophycocyanin), IFN-γ FITC (Fluorescein Isothiocyanate) (for panel 1), or TNF-α PE-Cy7, FoxP3 PE, TGF-β APC, IL-1β FITC (for panel 2), respectively (all from BioLegend). Following labeling, cells were washed and resuspended in PBS for flow cytometry analysis.

Samples were analyzed immediately on a FACSAria flow cytometer (BD Biosciences) equipped with 488 and 633 nanometer excitation lasers. Data were processed using the FACSDiVa software (BD Biosciences). 100,000 cells were recorded. Evaluators of flow cytometry data were blinded to the clinical status of the neonates.

3.3 Immunoassays

Plasma samples were stored at -80 °C until analysis. The plasma levels of the following cytokines, chemokines and growth factors were determined using Bio-Plex Pro Assays (Bio-Rad Laboratories, Hercules, CA, USA): IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, TNF-α, TGF-β, G-

CSF, GM-CSF, MCP-1, MIP-1b and VCAM. Bio-Plex Pro Assays are immunoassays formatted on magnetic beads that utilize principles similar to those of a sandwich ELISA. Capture antibodies against the biomarker of interest are covalently coupled to the beads. A biotinylated detection antibody creates the sandwich complex and the final detection complex is formed by the addition of a streptavidin-phycoerythrin (SA-PE) conjugate, where PE serves as the fluorescent reporter. Reactions are read using a Luminex-based reader.

3.4 High-performance liquid chromatography (HPLC)

Plasma samples were stored at -80 °C until analysis. Directly prior to analysis, samples were thawed, vortexed and 300 µl of plasma was "shot" onto 700 µl of precipitation solvent (containing 3.57 w/w% perchloric acid and 2.857 mM 3-nitro-L-tyrosine as internal standard (Scharlau, Barcelona, Spain)). Samples were then centrifuged (13000 G for 10 minutes at 4 °C) and the supernatants were collected. For the quantification of KYN, KYNA, and TRP concentrations of samples, an Agilent 1100 HPLC system was used (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a fluorescent detector, which was used to determine the concentration of KYNA and TRP, and a UV detector which was applied for the determination of KYN and the internal standard. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm × 4.6 mm I.D. (Phenomenex Inc., Torrance, CA, USA) after passage through a Hypersil ODS precolumn, 20 × 2.1 mm I.D., 5 µm particle size (Agilent Technologies) with a mobile phase composition of 0.2 M zinc acetate/acetonitrile 95/5 v/v% with a pH adjusted to 6.2 with glacial acetic acid, applying isocratic elution. The flow rate and the injection volume were 1.5 ml/minute and 20 µl, respectively. The fluorescent detector was set at excitation and emission wavelengths of 344 nm and 398 nm, and after 3.5 minutes of each run, the wavelengths were changed to 254 nm and 398 nm. The UV detector was set at a wavelength of 365 nm. L-TRP, L-KYN sulfate salt, KYNA and zinc acetate

dihydrate were purchased from Sigma-Aldrich and acetic acid was purchased from VWR International (Radnar, PA, USA).

3.5 Statistical analysis

Data are expressed as median and interquartile range. Comparisons between sample populations were performed with Mann-Whitney tests, as a test of normality (performed according to Kolmogorov-Smirnoff) indicated non-normal distribution of data. Comparisons between the paired values (samples collected at different time points) in the same population were made with Friedman tests. p values less than 0.05 were considered significant. Outliers were identified using Grubbs' tests and were excluded from analyses. Statistics were calculated using the GraphPad Prism 5 software (La Jolla, CA, USA).

4 RESULTS

4.1 Comparisons between moderate and severe HIE

4.1.1 IL-1β

We found an elevated prevalence of IL-1β-expressing CD4 lymphocytes at 6 h after birth in severe HIE compared to moderate HIE. These cells also showed a higher rate of extravasation to the CNS at 6 h in severe HIE, indicated by the decrease in the prevalence of CD49d-expressing CD4+ IL-1β+ cells in the peripheral blood. The prevalence of these cells did not differ at later time points.

Intracellular levels of IL-1β, indicated by the mean fluorescence intensity (MFI) of IL-1β within each cell subset showed a peak at 6 h and were comparably lower at the following time points in both HIE groups. The plasma levels of IL-1β changed similarly in the moderate HIE, and showed a similar tendency in the severe group, but did not reach the level of significance. No differences were present in the plasma level of IL-1β between moderate and severe HIE. These data support the role of IL-1β in the initiation of the neuroinflammatory response.

4.1.2 IL-6

The prevalence of IL-6 producing CD4+ T lymphocytes and the intracellular IL-6 levels were comparable in the two HIE groups, no significant difference was present. Interestingly, in both of the HIE groups CD4+ lymphocytes expressed the highest level of IL-6 at 24 h, indicated by the peak of the MFI of IL-6 at this time point. IL-6 production decreased afterwards, suggesting that IL-6 may also play an important role in the initial phase of the neuroinflammatory response.

We found a difference in the plasma level of IL-6, which was higher in severe HIE at 1 wk than in moderate HIE. We found a decrease in plasma IL-6 levels in the moderate group by 1 mo, which was not significant in the severe one.

4.1.3 IL-17

The prevalence of IL17-producing CD4+ T cells, also known as Th17 cells increased by 24 h compared to 6 hours of life and remained elevated during the first month of life. At 1 wk this value was significantly higher in the severe group than in the moderate group. Intracellular IL-17 production (indicated by the MFI of IL-17) was also higher in the severe group compared with the moderate group at 72 h in CD4+ lymphocytes.

At 6 h, the prevalence of IL-17 producing CD8+ lymphocytes was higher in moderate HIE, than in severe HIE. In the moderate group, the MFI of IL-17 in CD8+ cells peaked at 72 h and decreased by 1 mo. IL-17 production (indicated by the MFI of IL-17) Intracellular IL-17 levels were higher in the severe group compared with the moderate group at 24 h in CD8+ lymphocytes

No differences could be detected between the two study groups in the plasma levels of IL-17.

4.1.4 TNF-α

The TNF-α production of CD4+ lymphocytes (MFI of TNF-α) was increased at all time points compared to 6 h in both HIE groups. By 1 mo, the MFI of TNF-α in CD4+ cells was higher in the severe group than in the moderate, suggesting the presence of an ongoing inflammatory response, which could contribute to the long-term consequences of perinatal asphyxia. Plasma levels of TNF-α did not differ.

The prevalence of CD49d-expressing CD4+ TNF-α+ lymphocytes, which are prone to enter the CNS is lower in severe HIE at 6 h compared to moderate HIE and also compared to later time points, indicating increased extravasation. The prevalence of CD49d-expressing CD4+ TNF-α+ cells then increased and became higher in severe HIE than in moderate HIE by 72, which could be a result of increased CD49d production.

4.1.5 Other pro-inflammatory cytokines

The MFI of IFN-γ in CD4+ cells was elevated in severe HIE compared to moderate HIE at 72 h.

Plasma G-CSF levels were higher in the severe group than in the moderate group at 24 h and 1 wk. In moderate HIE, plasma G-CSF levels showed a decrease by 1 wk and remained low during the rest of the first month of life.

Plasma MCP-1 levels within the moderate group were elevated at 24 h, 72 h and 1 wk compared to 6 h.

4.1.6 TGF-β

In the moderate group, the prevalence of CD49d-expressing CD4+ TGF-β+ cells increased after 72 h and remained elevated until the end of the first month of life. This indicates a greater potential for TGF-β producing cells to enter the CNS in from 1 wk onwards moderate HIE. CD4+ cells also expressed higher levels of TGF-β from 24 h onwards in the moderate group. Although similar tendencies were present in the severe group alterations did not reach significant levels.

4.1.7 Tregs

The prevalence of Tregs was somewhat higher in severe HIE at 24 h, which might be a result of a compensatory mechanism, however, the biological significance of this increase is difficult to determine.

4.1.8 Other anti-inflammatory cytokines

In the moderate group, plasma IL-10 levels decreased by 1 mo and were lower than at 6 and 24 h. In the severe group, plasma IL-13 levels decreased by 72 h and were lower than at 6 and 24 h in the following timepoints. Plasma IL-13 and IL-5 levels were higher in the moderate than in the severe group at 72 h.

4.1.9 The kynurenine system

The plasma levels of KYN were higher in moderate HIE at 1 mo than in severe HIE. The components of the kynurenine system

otherwise showed similar alterations in time within the two HIE groups. TRP levels showed a gradual increase in both groups by the end of the first month of life. Parallelly, plasma KYN and KYNA levels showed a gradual decline until 1 mo. In line with the above alterations, the enzymatic activity of IDO, indicated by the ratio of KYN and TRP (K/T ratio) also showed a decline by the end of the first month after the perinatal HI injury. This indicates, that enhanced IDO activity is more relevant in the early phase of neuroinflammation.

4.1.10 ROC analysis

We performed ROC analyses to assess which parameters have the potential to discriminate between a moderate and a severe insult at an early stage. The only significant results of the ROC analyses were related to intracellular IL-1β. The prevalence of CD4+ IL-1β+ cells at 6 h ($p = 0.018$, ROC AUC = 0.784) and that of CD4+ IL-1β+ CD49d+ cells at 6 h ($p = 0.027$, ROC AUC = 0.767) was able to differentiate severity with a reasonable sensitivity and specificity.

4.2 Comparisons between HIE and NAIS

4.2.1 Pro-inflammatory cytokines

Similar to our observations in HIE, CD4+ T lymphocytes produced the highest level of IL-1β at 6 hours in NAIS as well. This was indicted by the elevated MFI value of IL-1β, which decreased significantly by 24 hours. We found no difference between NAIS and HIE groups in the intracellular production of IL-1β.

The MFI of IL-6 in CD8+ cells at 72 h was lower in NAIS than in severe HIE. However, by 1 mo the MFI of IL-6 in CD8+ cells decreased in both HIE groups but became higher in NAIS.

At 6 h the prevalence of CD8+ lymphocytes producing IL-17 was higher in NAIS than in severe HIE and CD8 cells also expressed higher levels of IL-17 at 6 h in NAIS than moderate HIE.

In NAIS, CD4+ lymphocytes expressed the highest level of IFN-γ at 24 h. Intracellular IFN-γ level decreased significantly by 72 h, by when it became lower in NAIS than in either HIE groups. The prevalence of IFN-γ+ CD4 cells did not differ between the study populations.

Our results indicate a marked inflammatory response in NAIS at 72 hours, characterized by the elevated plasma levels of several cytokines, i.e. IL-5, IL-17 and MCP-1 compared to HIE. Plasma MCP-1 level at 72 h was higher than at 6 h than at all other time points within the NAIS group. By 1 mo however, inflammatory response appears to decrease in NAIS, as plasma levels of IL-4, IL-12 and IL-17 are lower compared to HIE groups. The level of IL-4 was lower at 1 mo than at 72 h within the NAIS group. Plasma IL-12 and IL-17 levels showed a similar decreasing tendency at 1 mo, however this difference was not significant, probably due to the very low number of cases.

4.2.2 Anti-inflammatory cytokines

The prevalence of IL-10+ CD8 lymphocytes was lower in NAIS than in the severe HIE at 6 h and 72. Although the prevalence of IL-10+ CD8 lymphocytes remained consistently lower in NAIS, the difference was not significant level at the 24 h time point. On the other hand, the prevalence of IL-10+ CD4 cells was higher at 24 h in NAIS than in moderate HIE.

At 1 wk the prevalence of CD49d-expressing TGF-β+ CD4 lymphocytes, which are prone to enter the CNS was elevated in NAIS compared to both HIE groups, and all other time points within the NAIS group. By 1 mo of age however, the prevalence of TGF-β+ CD4 lymphocytes became lower in NAIS than in HIE.

5 CONCLUSIONS

1) IL-1β and IL-6 producing T lymphocytes appear to play an important role in the early phase of the adaptive immune response in perinatal HIE.
2) TNF-α production is sustained during the first month of life in T cells and is higher in severe HIE, which could contribute to a worse outcome
3) The elevated prevalence of Th17 lymphocytes in severe HIE could indicate the role of this subset in the delayed progression of HI brain injury
4) Elevating TGF-β production and increased extravasation CD4+ T could play an important regulatory role in HIE by initiating the reparative processes
5) The assessment of the prevalence and CD49d expression of IL-1β+ CD4+ cells at 6 h appears to be able to predict severity at an early stage in HIE
6) In NAIS the inflammatory response is more enhanced at 72 h than in HIE, indicated by the higher level of several plasma cytokines (i. e. IL-5, IL-17, MCP-1).
7) At 1 wk, there is a marked increase in the extravasation of TGF-β producing CD4+ T cells in NAIS both compared to the 6 h value and HIE, which could indicate an enhanced reparative process
8) By 1 mo the inflammatory response is attenuated in NAIS, indicated by lower plasma cytokine levels and the lower prevalence of TGF-β producing CD4+ T cells

6 PUBLICATIONS

Publications directly related to the PhD dissertation

Cumulative impact factor: 10.386

1) Bajnok A, Berta L, Orbán C, Tulassay T, Toldi G. (2018) Cytokine production pattern of T lymphocytes in neonatal arterial ischemic stroke during the first month of life-a case study. J Neuroinflammation. 15(1):191. **IF: 5.193**

2) Bajnok A, Berta L, Orbán C, Veres G, Zádori D, Barta H, Méder Ü, Vécsei L, Tulassay T, Szabó M, Toldi G. (2017) Distinct cytokine patterns may regulate the severity of neonatal asphyxia-an observational study. J Neuroinflammation. 14(1):244. **IF: 5.193**

Publications not related to the PhD dissertation:

Cumulative impact factor: 42.072, as first author: 6.615

1) Bajnok A, Ivanova M, Rigó J Jr, Toldi G. (2017) The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia. Mediators Inflamm. 2017:8045161. **IF: 3.549**

2) Bajnok A, Kaposi A, Kovács L, Vásárhelyi B, Balog A, Toldi G. (2013) Analysis by flow cytometry of calcium influx kinetics in peripheral lymphocytes of patients with rheumatoid arthritis. Cytometry A. 83(3):287-93. **IF: 3.066**

3) Orbán C, Vásárhelyi Z, Bajnok A, Sava F, Toldi G. Effects of caffeine and phosphodiesterase inhibitors on activation of neonatal T lymphocytes. Immunobiology. 223(11):627-633. **IF: 2.873**

4) Dulic S, Vasarhelyi Z, Bajnok A, Szalay B, Toldi G, Kovacs L, Balog A (2018). The Impact of Anti-TNF Therapy on CD4+ and CD8+ Cell Subsets in Ankylosing Spondylitis. Pathobiology. 85(3):201-210. **IF: 1.592**

5) Orbán C, D Szabó, A Bajnok, B Vásárhelyi, T Tulassay, A Arató, G Veres, G Toldi. (2017) Altered activation of peripheral CD8+ T cells in pediatric Crohn's disease. Immunol Lett, 185:48-51. **IF: 2.483**

6) Legány N, G Toldi, C Orbán, N Megyes, A Bajnok, A Balog. Calcium influx kinetics, and the features of potassium channels of peripheral lymphocytes in primary Sjögren's syndrome. (2106) Immunobiology 221:1266-1272. **IF:2.720**

7) Orbán C, D Szabó, A Bajnok, B Vásárhelyi, T Tulassay, A Arató, G Veres, G Toldi. (2016) Altered calcium influx of peripheral Th2 cells in pediatric Crohn's disease: infliximab may normalize activation patterns. Oncotarget. 7(29):44966-44974. **IF: 5.168**

8) Orban C, E Perez-Garcia, A Bajnok, G McBean, G Toldi, A Blanco-Fernandez. Real time kinetic flow cytometry measurements of cellular parameter changes evoked by nanosecond pulsed electric field. (2016) Cytometry Part A 89:472-479. **IF: 3.222**

9) Folyovich A, Biczó D, Bajnok A, Bessenyei D, Kis I, Gimesi-Országh J, Béres-Molnár AK, Toldi G. (2016) Higher Incidence of Stroke on the Last Day of the Month in Hungary-a Role for Psychosocial Factors and Financial Insecurity? J Stroke Cerebrovasc Dis. 25(5):1192-1195. **IF: 1.517**

10) Toldi G, ZE Vásárhelyi, J Rigó Jr, C Orbán, Z Tamássy, A Bajnok, T Shima, S Saito, A Molvarec. Prevalence of Regulatory T-Cell Subtypes in Preeclampsia. (2015) American Journal of Reproductive Immunology 74:110-115. **IF: 2.916**

11) Grozdics E, Berta L, Bajnok A, Veres G, Ilisz I, Klivényi P, Rigó J Jr, Vécsei L, Tulassay T, Toldi G. (2014) B7 costimulation and intracellular indoleamine-2,3-dioxygenase (IDO) expression in peripheral blood of healthy pregnant

and non-pregnant women. BMC Pregnancy Childbirth. 14:306. **IF: 2.190**

12) Orbán C, A Bajnok, B Vasarhelyi, T Tulassay, G Toldi. (2014) Different calcium influx characteristics upon Kv1.3 and IKCa1 potassium channel inhibition in T helper subsets. Cytometry Part A, 85: 636-641. **IF: 2.928**

13) Folyovich A, Biro E, Orban C, Bajnok A, Varga V, Beres-Molnar AK, Vasarhelyi B, Toldi G. Relevance of novel inflammatory markers in stroke-induced immunosuppression. (2014) BMC Neurology 14:41. **IF:2.040**

14) Folyovich A, Biró E, Orbán C, Bajnok A, Vásárhelyi B, Toldi G. Kv1.3 lymphocyte potassium channel inhibition as a potential novel therapeutic target in acute ischemic stroke. (2014) CNS & Neurological Disorders-Drug Targets 13:801-806. **IF: 2.628**

15) Orbán C, E Biro, E Grozdics, A Bajnok, G Toldi. (2013) Modulation of T lymphocyte calcium influx patterns via the inhibition of Kv1.3 and IKCa1 potassium channels in autoimmune disorders. Frontiers in Immunology 4: 3.

16) Toldi G, Bajnok A, Dobi D, Kaposi A, Kovács L, Vásárhelyi B, Balog A. (2013) The effects of Kv1.3 and IKCa1 potassium channel inhibition on calcium influx of human peripheral T lymphocytes in rheumatoid arthritis. Immunobiology. 218(3):311-6. **IF: 3.180**