Role of microglia in neurotropic viral infections

Ph. D. thesis

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1. Introduction

Microglia are the main immunocompetent cells of the brain that play a role in diverse physiological processes, including brain development, synaptic plasticity and memory. In turn, alterations in microglial function are linked with common brain diseases such as stroke, epilepsy, Alzheimer's or Parkinson's disease. Recognition and clearance of terminally injured neurons from the brain by microglia in line with discriminating them from salvageable cells appear to be crucial to prevent dysregulation of complex neuronal networks as seen in diverse forms of acute brain injury or chronic neurodegeneration. In line with this, microglia - as key immune cells in the brain – must protect the nervous tissue from invading pathogens including bacteria, fungi and viruses, many of which represent constant threat for the CNS in the absence of surveillance provided by specialized peripheral immune cell populations. In particular, several viral strains (e.g. herpes simplex virus type 1, HIV, Zika virus, etc.) are capable of causing both acute and chronic infection in humans and emerging data indicate their contribution to diverse forms of neurodegeneration. Several studies have proved that microglia participate in anti-viral immunity, however the mechanisms through which microglia recognize signs of infection at the cellular level and how infected cells are discriminated remained unclear.

Microglia are capable of removing synapses via complement-mediated manner and eliminate injured neurons via recognising multiple mediators including chemokines, metalloproteinases, growth factors, various metabolites or damage associated molecular patterns. Among these mediators, purinergic metabolites, such as ATP and ADP are potent chemotactic activators for microglia. Strong evidence supports that microglial purinergic receptors contribute markedly to microglial activation, migration and process motility upon acute or chronic injury, but their role during neurotropic viral infection have not been investigated. It is still unclear how microglia recognise and eliminate virus infected cells in the brain and whether/how purinergic signalling is involved in this response.

In line with this, the primary goal of my thesis was to explore microglial responses and function in a mouse neurotropic virus infection model both in *in vitro* and *in vivo*. Besides describing microglial actions in a neurotropic virus infected environment I focused on the role of microglial P2Y12 receptors.

For this purpose, I took advantage of the genetically modified strains of pseudorabies virus (PRV), a member of the subfamily Alphaherpesvirinae (similarly to human herpes simplex virus type 1), widely used for neuroanatomical tract-tracing and as a well-established model of neurotropic virus infection. When injected into peripheral organs, the „Bartha-Dup" strains of PRV display slow and highly specific retrograde transsynaptic spread in central autonomic pathways, allowing us to study directed microglial recruitment in parallel with the propagation of virus infection in the brain. Since in this model microglia may only sense signals identifying affected neurons in their vicinity, but do not become infected with

PRV even under *ex vivo* conditions unlike in the case of other neurotropic viruses, I could also investigate the functional role of microglia and microglial P2Y12 receptors together with the associated neuroinflammatory responses.

Since neurons infected with PRV-Bartha derivatives have normal electrophysiological characteristics but display increased activity, we hypothesized that microglia may detect detrimental changes in the case of individual cells before irreversible neuronal injury occurs. This model system also appeared suitable to reveal rapid and targeted recruitment of microglia to compromised neurons using *in vivo* two-photon and *in vitro* time-lapse imaging and to investigate whether nucleotides released from infected neurons mediate this effect via microglial P2Y12 receptors. It has also been unclear to date whether microglia are instrumental to control the transneuronal spread of neurotropic virus infection in the brain, which could be addressed by using selective elimination of microglia. In line with this, our model also provided us with the opportunity to explore, how do microglia and the presence of microglial P2Y12 receptor affect the recruitment of blood-borne leukocytes into the brain parenchyma. Therefore, we set up a complex set of studies to explore the role of microglia in neurotropic viral infection.

2. Aims

1) To study central inflammatory processes and the functional role of microglia in a mouse neurotropic virus infection model.

2) To identify the role of microglial P2Y12 receptor in neurotropic virus infection

3) To study inflammation and microglial responses in human post-mortem brain tissues after herpes simplex encephalitis.

3. Materials and methods

3.1. In vivo experiments

3.1.1. Animal housing and treatment

Experiments were carried out on 12-18 weeks old C57BL/6J, P2Y12$^{-/-}$, P2RX7$^{-/-}$, Cx3Cr1$^{GFP/+}$ and Cx3Cr1$^{GFP/+}$ P2Y12$^{-/-}$ mice. All experimental procedures were in accordance with the guidelines set by the European Communities Council Directive (86/609 EEC) and the Hungarian Act of Animal Care and Experimentation (1998; XXVIII, section 243/1998), approved by the Animal Care and Use Committee of the IEM HAS.

3.1.2. Selective elimination of microglia from the brain

Mice were fed PLX5622 (Plexxikon Inc. Berkeley, USA; 1200mg PLX5622 in 1kg chow) for 3 weeks to eliminate microglia from the brain.

3.1.3. Neurotropic herpesvirus infection

Mice were randomly assigned to experimental groups and were injected either intraperitoneally or directly into the epididymal white adipose tissue with a genetically modified PRV-Bartha derivative, PRV-Bartha-Dup-Green (BDG) to induce retrograde transsynaptic infection in the brain. In a set of studies, mice were infected with BDG on 16th day of PLX5622 diet to assess the effect of microglia depletion on central propagation of virus infection. For *in vivo* two-photon imaging, Cx3Cr1$^{GFP/+}$ mice were infected with PRV-Bartha-DupDsRed (BDR) enabling the co-detection of infected neurons with microglia. After virus injection, mice were let to survive for 5 to 7 days depending on study design and were regularly monitored for neurobehavioral symptoms.

3.1.4. Two-photon imaging

To assess microglia recruitment to infected neurons in the mouse brain in real-time, Cx3CR1$^{GFP/+}$ mice were i.p. injected with 10µl of the BDR virus. The survival time was set to 7 days post-infection, when numerous infected cells were present in the cerebral cortex. After cranial window preparation, measurements were performed on a Femto2D-DualScanhead microscope (Femtonics Ltd., Hungary) coupled with a Chameleon Ultra II laser. Data acquisition was performed by MES softver (Femtonics Ltd.), two-photon image sequences were exported from MES and analysed using ImageJ.

3.1.5. Tissue processing and immunofluorescence

Brain tissues were fixed by transcardial perfusion (saline, followed by 4% PFA), then 25 μm thick free-floating brain sections were cut using a sledge microtome. The brain sections were blocked with 2% normal donkey serum and incubated with a mixture of primary antibodies overnight at 4 ^{o}C. The following day after several washing steps all slices were labelled with the matching fluorescent secondary antibodies. After mounting and covering the slices with the appropriate covering agent, images were captured with a Nikon Ni-E C2+ confocal microscope.

3.1.6. Immuno-electron microscopy

After the combined immunogold-immunoperoxidase stainings, sections were treated with osmium tetroxide, dehydrated in ascending ethanol series and acetonitrile, and embedded in epoxy resin. During dehydration sections were treated with uranylacetate. After polymerization, 70 nm thick sections were cut on an ultramicrotome, picked up on formvar-coated single-slot copper grids, and sections were examined using a Hitachi H-7100 electron microscope.

3.1.7. Correlated confocal laser-scanning microscopy, electron microscopy and electron tomography

Following multiple immunofluorescent staining, sections were analysed using a Nikon Eclipse Ti-E inverted microscope and an A1R laser confocal microscope. After imaging, sections were recovered and the immunoperoxidase reaction was developed. Electron tomography was performed using a Tecnai T12 BioTwin electron microscope equipped with a computer-controlled precision stage and an Eagle™ 2k CCD 4 megapixel TEM CCD camera. Reconstruction was performed using the IMOD software package.

3.1.8. Super-resolution (STORM) microscopy

Sections were mounted onto #1.5 thick borosilicate coverslips and covered with imaging medium immediately before imaging. STORM imaging was performed for P2Y12 (stimulated by a 647 nm laser) by using a Nikon N-STORM C2+ super-resolution system that combines 'Stochastic Optical Reconstruction Microscopy' technology and Nikon's Eclipse Ti research inverted microscope to reach a lateral resolution of 20 nm and axial resolution of 50 nm.

3.2. In vitro experiments

3.2.1. Primary cell cultures

Primary cultures of embryonic cortical cells were prepared from mice on embryonic day 15 and astroglia/microglia mixed cell cultures were prepared from the whole brains of mouse pups, as described earlier. Microglial cells were isolated from 21-28 days old mixed cultures either by shaking or by mild trypsinization. In cultures used for time-lapse recordings, microglial cells were seeded on top of astrocytic or neuronal cell cultures in 10000 cell/cm^2 density. Neuronal or astroglia cultures were infected with either PRV-Bartha-Dup-Green (BDG) virus or PRV-Bartha-DupLac (BDL) at a final titer of 2.5x10^5 PFU/ml, as described earlier. The multiplicity of infection (MOI) was ~0,17 PFU/cell.

3.2.2. Time-lapse microscopy and cell motility data analysis

Time-lapse recordings were performed on a computer-controlled Leica DM IRB inverted fluorescent microscope. Phase contrast and epifluorescent images were collected consecutively every 10 minutes for up to 48 hours post-infection. Images were edited using NIH ImageJ software. Cell tracking: images were analyzed individually with the help of custom-made cell-tracking programs (G-track and Wintrack) enabling manual marking of individual cells and recording their position parameters into data files.

3.2.3. Cytokine measurement from media of primary cell cultures

Concentrations of IL-1α, IL-1β, TNF-α, IL-6, MCP-1, RANTES (CCL5), G-CSF and KC (CXCL1) were measured from conditioned media of primary neuronal, astroglial and microglial cell cultures by using cytometric bead array (CBA) Flex Sets. Measurements were performed on a BD FACSVerse machine and data were analysed using an FCAP Array software (BD Biosciences) as described earlier. The cytokine levels of conditional media were corrected for total protein concentrations of the samples measured by Bradford Protein Assay Kit.

3.2.4. Total RNA isolation and quantitative RT-PCR

For total RNA isolation, cell culture samples were homogenized in 500 µl TRI Reagent and isolation was performed using Tissue Total RNA Mini Kit according to the manufacturer's instructions. The primers were used in real-time PCR reaction with Fast EvaGreen qPCR Master Mix on a StepOnePlus instrument. The gene expression was analyzed using the StepOne 2.3 program. Amplicons were tested by Melt Curve Analysis on StepOnePlus instrument. Experiments were normalized to *gapdh* expression.

3.2.5. Quantification of nucleotides and adenosine

The adenine nucleotides (ATP, ADP, AMP) and adenosine (Ado) were determined in extracts from cells and culture media by using HPLC method. The HPLC system used was a Shimadzu LC-20 AD Analytical & Measuring Instruments System, with an Agilent 1100 Series Variable Wavelength Detector set at 253 nm.

3.2.6. Enzyme histochemistry for detection of ecto-ATPase activity

A cerium precipitation method was used for electron microscopic investigation of ecto-ATPase activity. The tissue blocks were then postfixed, dehydrated, treated and embedded into Taab 812 resin for ultrathin sectioning and microscopic examination.

3.2.7. Flow cytometric analysis of brain, spleen and blood samples

Cells were isolated from mouse brains by enzymatic digestion with the mixture of DNase I and Collagenase/Dispase. Spleen cells were isolated by mechanical homogenization of the spleen. Venous blood was collected from the heart before transcardial perfusion using 3.8% sodium citrate as an anticoagulant. Cells were acquired on a BD FACSVerse flow cytometer and data were analysed using FACSuite software. Total blood cell counts were calculated by using 15 μm polystyrene microbeads.

3.2.8. Statistical assessment

All quantitative measurements and analysis were performed in a blinded manner in accordance with STAIR and ARRIVE guidelines. Data were analysed using the GraphPad Prism 7.0 software. For comparing two experimental groups Student's t-test with Welch's correction or Mann-Whitney U test, for comparing three or more groups one-way or two-way ANOVA followed by Tukey's, Sidak's and Dunnett's post hoc comparison was used. $p<0.05$ was considered statistically significant.

3.2. Human samples

To investigate microglia recruitment in response to neurotropic virus infection in the human brain, paraffin-embedded (FFPE) post-mortem brain tissues from five patients with known HSV-encephalitis aged 42-66 years were analyzed (ethical approval ETT-TUKEB 62031/2015/EKU, 34/2016 and 31443/2011/EKU (518/PI/11)). Tissue samples from two additional patients with no known neurological disease were used as controls. After deparaffinisation, immunohistochemistry was performed by using an array of antibodies to study microglia, neurons and leukocytes), and representative pictures were captured using a Nikon Ni-E C2+ microscope.

4. Results

4.1. Microglia are instrumental for anti-viral immunity in the central nervous system

To study whether microglia respond to local cues and are recruited to virus-infected neurons, we made use of the precisely controlled, retrograde transsynaptic spread of the PRV derivative, Bartha-DupGreen (BDG) to central autonomic nuclei from peripheral targets. In the hypothalamic paraventricular nucleus (PVN), microglial numbers increased three-fold in response to infection and infected neurons were surrounded by numerous Iba1-positive cells, 6 days after intraperitoneal (i.p.) virus injection. To investigate whether microglia are involved in the control of neurotropic virus infection, we performed selective depletion of microglia, by feeding mice the CSF1R antagonist PLX5622. After three weeks of depletion, 96% of microglia were eliminated from the brain as evidenced by the lack of the microglial markers Iba1 and P2Y12. Selective elimination of microglia resulted in a three-fold increase in the number of PRV-immunopositive and disintegrating neurons, compared to that seen in the microglia competent infected group. The absence of microglia was also associated with the development of diverse neurological symptoms in infected mice starting on the 5th day of infection when infected neurons were numerous in the brain stem, the hypothalamus and the autonomic-associated nuclei in the limbic system. These symptoms included heavy breathing, muscle spasms and seizure-like episodes, which were absent in control mice at similar time after the onset of the infection.

To investigate the nanoscale interaction between microglia and infected neurons, we visualized viral proteins using super-resolution microscopy together with the microglial phagosome/lysosome marker CD68. In control mice, recruited microglia surrounded the soma of infected neurons tightly and the already phagocytosed neuronal parts containing viral particles were present inside phagosomes. The absence of microglia resulted in increased extracellular viral protein levels and accumulation of PRV-immunopositive cell debris. Confirming these observations, electron microscopy revealed a direct contact between microglial processes and the cell membrane of the infected neurons as well as the uptake of infected neurons by microglia. On the contrary, we could not detect virus-dependent immediate-early GFP signal or any PRV-immunopositive viral capsids in microglia, indicating that productive infection does not develop in these cells, in line with earlier reports.

4.2. Microglia recruitment is initiated rapidly to virus-infected neurons in the brain

After we confirmed the instrumental role of microglia in controlling neurotropic virus infection, we aimed to investigate whether the recruitment of microglia occurs early enough to allow the isolation of infected neurons before the breakdown of neuronal cell membranes. We made use of the immediate-early marker, GFP, which allows time-mapping the different phases of infection at a single neuron level. In the early phase of the infection, compromised neurons express only GFP. GFP-positive neurons expressing low levels of viral structural proteins already appeared more surrounded by microglia compared to the neurons expressing GFP only. This suggests that recruitment of microglia is induced within a few hours of infection, by the time viral structural proteins are produced. In the latest phase of neuronal infection, only high levels of viral proteins could be detected in the neuronal cytoplasm, which paralleled increases in the number of microglia around them. Next we aimed to investigate microglial recruitment in vivo in real-time. Since microglia are very sensitive to any disturbances in the brain, we first optimized our cranial window surgical protocol, to avoid microglial activation keeping the dura mater intact. To be able to detect both microglial recruitment and viral infection we injected PRV-Bartha-DupDsRed (BDR) virus into $Cx3Cr1^{+/GFP}$ riporter mouse. In order to image this phenomenon, mice were allowed to survive 7 days after virus injection, resulting in virus infection in upper layers of the cerebral cortex. During imaging we monitored the expression levels of DSRed fluorophore to follow the different infection stages parallel with microglial recruitment in real time in vivo. Following imaging 3D reconstruction from 2P Z-stack revealed that microglial processes formed a barrier-like structure, with several contact points around the cell body of the infected neuron. Microglia recruited to infected cells showed increased process velocity compared to microglia distant from sites of virus infection, indicating that microglia may respond to local signals induced by infected neurons.

To further explore whether microglial contacts with the cell membranes of infected neurons can be formed in the early phases of virus infection, we visualized microglia-neuron contacts with confocal microscopy in $Cx3Cr1^{+/GFP}$ mice, followed by the investigation of selected neurons with correlated electron microscopy and electron tomography. 3D reconstruction from confocal Z-stack revealed the formation of microglial contacts around the cell body and the main dendrites of infected neurons, before the appearance of mature virions in the neuronal cytoplasm. At this stage of infection, neuronal cell membranes were intact with normal chromatin structure seen in the nucleus. Microglial processes surrounding infected neurons showed CD68-immunopositivity, indicating the phagocytic activity of microglia. Besides, the electron tomography revealed the formation of specific membrane interactions between infected neurons and microglia suggesting the recognition and contact of the intact cell membranes by recruited microglial processes.

4.3. Virus infection triggers the recruitment and phagocytic activity of microglia *in vitro*

We studied microglia recruitment to sites of infection in vitro as well. For this purpose we first established co-cultures of neurons and GFP-positive microglia from $Cx3Cr1^{+/GFP}$ mice and performed time-lapse imaging over a 48h period. We observed that microglia contacted the cell body and the main processes of uninfected neurons without causing injury or showing phagocytic activity. In contrast, microglia added to virus-infected neurons were recruited to and phagocytosed infected cells. Next, we aimed to study the behavior of microglia which required co-cultures of microglia and astrocytes, which are more sparsely distributed cells compared to neurons. Analysing individual microglia trajectories revealed that in sparsely distributed control astrocyte culture microglia migrated longer distances. However in PRV-infected cultures recruited microglia formed prolonged cell-to-cell contacts. This phenomenon was associated with a reduction of cell velocities in infected cultures indicating that signals from infected cells direct microglial migration, scanning behavior, and subsequent phagocytic activity. Similar microglial responses were seen in neuronal/microglial co-cultures.

Importantly, the development of productive infection was never observed in microglia in vivo or in vitro even after the direct exposure of the cells to high viral titers or following extensive phagocytic activity, as evidenced by the absence of the immediate-early GFP signal and PRV proteins from microglia outside phagosomes.

4.4. Nucleotides released from infected cells trigger microglia recruitment and phagocytosis via microglial P2Y12 receptor

Next, we checked whether purine nucleotides such as ATP that are chemotactic for microglia at a short time scale could be released from compromised cells. We found that cultured neurons released ATP after virus infection, which was associated with reduced ATP, ADP, AMP and adenosine levels in cell lysates, within hours upon the expression of the immediate-early marker, GFP, which precedes the expression of viral structural proteins required for productive infection. We associated the changes in purinergic metabolites with increased ecto-ATPase levels in infected cells, but those were not due to apoptosis or necrosis, since at the early stages of infection neurons expressing high levels of GFP showed no uptake of propidium iodide (PI). Parallel with increased ecto-ATPase levels we observed insreased NTDPase1 expression in microglia at sites of virus infection in the brain, indicating that microglia respond to changes in the levels of purine nucleotides.

To investigate the mechanisms mediating microglial responses to purine nucleotides released from infected cells, we assessed microglial responses in co-cultures of $P2X7^{-/-}$ or $P2Y12^{-/-}$ microglia and wild type astrocytes. Similarly to that seen in wild type microglia, motility of $P2X7^{-/-}$ cells decreased when exposed to infected cells and trajectories showed characteristic localized pattern due to frequent

scanning activity, indicating that P2X7 deficiency does not prevent the recognition of virus-infected cells by microglia. In contrast, virus-exposed P2Y12-deficient microglia showed increased motility with trajectories characteristic of random walk behaviour and lacking the localized pattern, suggesting that these cells are unable to display targeted recruitment in response to infection. Furthermore, wild type and P2X7$^{-/-}$ microglia showed a markedly increased phagocytic activity in infected cultures, which was fully abolished in P2Y12-deficient microglia. Thus, P2Y12 is a key contributor to the recognition of compromised cells by microglia and to microglial phagocytosis of virus-infected cells in vitro.

4.5. Recruitment of microglia and elimination of virus-infected neurons are mediated by microglial P2Y12 *in vivo*

Next, we investigated whether nucleotides released from compromised neurons are involved in the recruitment of microglia in vivo. We found that all microglia surrounding the cell body and the processes of infected neurons in either C57BL/6 or Cx3Cr1$^{+/GFP}$ mice expressed P2Y12 receptors. STORM super-resolution microscopy, allowed us to see that microglial P2Y12 receptor numbers increased over two-fold in response to infection and P2Y12 clusters in microglial processes contacting infected neurons were localized around the membrane of the infected cell. Interestingly, we also observed that microglial processes of the same cells, which did not contacted with infected neurons did not show the clustering of P2Y12 receptors, neither did the receptor numbers increase within the clusters. This indicates that microglial P2Y12 clustering is contact specific and may be an integral part of microglial scanning behaviour.

We investigated the contribution of purinergic signaling to antiviral immunity in vivo by inducing virus infection in mice lacking P2X7 or P2Y12 receptors. We found that an absence of P2Y12 resulted in >50% reduction in the numbers of microglia recruited to infected neurons in the PVN, whereas a non-significant trend to reduction was seen in P2X7$^{-/-}$ mice. In case of P2Y12$^{-/-}$ mice our results were very similar to that seen after microglia depletion. The number of infected neurons containing viral structural proteins increased over 3-fold in the absence of P2Y12 receptor, but we did not detect any changes in P2X7$^{-/-}$ mice. Around PRV-immunopositive neurons in P2Y12$^{-/-}$ mice we observed clusters of microglia, indicating that lack of P2Y12 receptor impairs microglial recruitment and compromises phagocytic responses, but does not fully block microglial migration to already disintegrated cells. Contrary to microglia depleted virus infected mice we did not observe any neurological symptoms in P2Y12 deficient mice, which suggest that the absence of microglia but not microglial P2Y12 may be responsible for the adverse neurological symptoms in our model.

In a subsequent study we directly compared virus infected and microglia depleted P2Y12$^{-/-}$ and wild type mice, to better understand the role of P2Y12 receptor in the spread of infection and possible underlying mechanisms concerning neurologycal symptoms. We compared levels of extracellular viral proteins, which were markedly increased identically both in microglia depleted and P2Y12 decifient

mice compared to wild type group. Newt we compared the levels of CD68-positive phagosomes, and we have seen that P2Y12$^{-/-}$ microglia showed markedly decreased amount of CD68 compared to wild type.

4.6. Microglia recruit leukocytes into the brain in response to virus infection independently of P2Y12-mediated signalling

Next, we aimed to investigate the recruitment of blood-borne cells to the brain during virus infection and wondered whether microglia and P2Y12-mediated actions are involved in this process. As expected, numerous CD45^{high} immunopositive leukocytes were recruited to sites of virus infection. Based on the higher CD45 expression and using microglia/macrophage specific marker Iba1 and microglia specific P2Y12, we could discriminate blood-borne infiltrated leukocytes from activated microglia. We observed that purinergic signaling via microglial P2Y12 receptors contributes to leukocyte infiltration identical what we have seen in wild type infected brains. Using both immunofluorescent labeling and fluorescence-activated cell sorting (FACS) we found that the number of CD45-positive blood-borne leukocytes did not change in P2Y12 deficient mice after infection. We concluded that even though P2Y12-mediated mechanisms play a major role in controlling the spread of infection, the absence of this receptor does not impair leukocyte infiltration in PRV infected brains.

In contrast with our previous results in P2Y12 deficient mice, selective elimination of microglia surprisingly resulted in a marked decrease in CD45-positive leukocytes around virus-infected areas in spite of the increased number of infected neurons in the brain. Using FACS analysis we uncovered that from all infiltrated CD45-positive cell populations, CD45^{high}, Cx3Cr1+, CD11b+, Ly6C^{high} monocyte numbers were markedly reduced in microglia depleted, virus-infected animals. Note that extensive virus infection in the brain was also associated with an increased number of circulating granulocytes in microglia-depleted mice, suggesting that peripheral myeloid populations were capable of responding to central viral infection, but the recruitment to the brain was inhibited by the absence of microglia. Next, we investigated the possible causes behind the absence of the inflammatory Ly6C^{high} monocyte population. For this purpose, we made use of the Intercellular Adhesion Molecule 1 (ICAM), which labels activated vessels in infected areas, but surprisingly we could not detect any significant difference in vascular activation or the integrity of BBB as assessed by IgG staining, between microglia-depleted infected and control infected animals.

4.7. Recruitment of P2Y12-positive microglia and leukocytes at sites of infection in the human brain during herpes simplex encephalitis

To investigate microglia recruitment and neuroinflammatory changes in the human brain, we analysed herpes simplex type 1 (HSV-1) encephalitis temporal lobe samples. Similarly to what we have seen in PRV infected mice brain tissue, in human samples both HSV-1 and HSV-2 infected neurons were surrounded by P2Y12-positive microglia cells, which we confirmed further with another microglia specific marker Tmem119. We also found that infected cells were contacted by 1-3 microglia (on average 1.5 microglia / HSV1+ cell). CD68 labeling in recruited amoeboid cells indicated active phagocytosis at sites of virus-infected neurons. Microglia showed negative results for HSV antigens suggesting that viral infection does not develop in these cells. With CD45-immunohistochemistry and Giemsa staining, we identified recruited leukocytes close to HSV1-positive neurons. To discriminate leukocytes from recruited microglia we used Tmem119 and P2Y12. Interestingly, we observed a strong correlation between the number of recruited leukocytes and the amount of HSV-1 infected neurons.

We found that moderate HSV1 infection (less than 50 HSV1-positive cells / mm2) was mostly associated with the activation of local microglia.CD68-positive cells with either ramified or amoeboid morphology were also observed in these areas. At areas of advanced HSV1 infection (50-500 HSV1-positive cells / mm2), numerous CD45-positive cells were observed in the brain parenchyma, which was associated with markedly increased numbers of CD68-positive-macrophages (likely to be of both microglial and blood-borne origin). In line with this, the number of ramified microglia, and the total number of P2Y12-positive or Tmem119-positive cells was reduced.

5. Discussion

Even though modern medical science can treat many infectious diseases, neurotropic virus infections cause major problems in the human population. Neurotropic herpesviruses can establish lifelong latent infections and can also contribute to severe diseases including encephalitis and neurodegeneration.

Immune responses induced by both activated microglia and recruited peripheral immune cells can irreversibly disrupt the complex features of the CNS, often leaving patients or affected animals with various neurological conditions that can contribute to long-term neurodegeneration or even death. Previous studies have already demonstrated that microglia are among the first to respond to any noxious stimuli in the CNS, and participate in anti-viral immunity. However, the exact mechanisms how microglia respond to neurotropic infections are still unclear. The aim of my thesis was to explore central (microglia) and peripheral (infiltrating leukocyte) immune cell actions upon neurotropic virus infection of the brain and identify some of the mechanisms behind microglial elimination of infected neurons.

For this reason, first I have established a neurotropic virus infection model to study microglial responses to neuronal injury. In this model I induced neurotropic virus infection using genetically modified PRV-Bartha derivatives, which spread exclusively among synaptically linked neurons, in a retrograde way. The advantage of the model is that PRV injection does not require any in situ manipulation of the brain parenchyma, keeping microglia intact. Also, it allows time-mapping of the different phases of infection at a single neuron level. With the help of this model, we demonstrated that microglia are recruited to and isolate infected neurons within hours and microglia are able to identify injured neurons way earlier than the integrity of the cell membranes is compromised.

The established neurotropic virus infection model combined with selective microglia depletion and P2Y12R specific KO lines allowed us to study the role of microglia and purinergic signalling in the context of virus mediated brain pathology. Our data, obtained from *in vitro* and *in vivo* studies show that microglia recruitment and clearance of compromised cells require cell-autonomus P2Y12 signalling in microglia, triggered by nucleotidases released from affected neurons. We show that selective elimination of microglia by CSF1R blockade results in a marked increase in the spread of infection and egress of viral particles into the brain parenchyma, which is largely independent from the presence of P2Y12. In agreement with other recent studies using the same microglia elimination model in other neurotropic virus infection models, such as mouse hepatits virus (MVH) or West-Nile virus, we observed, that depletion of microglia results in increased mortality. However, in contrast to these studies, we detected the appearance of various neurological symptoms in the absence of microglia in virus infected animals. The absence of microglia also resulted in non-synaptic spread of the virus, resulting advanced infection of cortical areas. This implicates that microglial barrier formation around infected cells is essential in controlling viral spread, and timely elimination of infected neurons is essential to prevent contact infection. Interestingly, while microglia-depleted mice had higher number of infected neurons compared

to that seen in P2Y12 deficient animals, the levels of extracellular virus proteins were not different. From this, we concluded that, while microglia are present, phagocytic actions were not effective without normal P2Y12 receptor signaling, implying that the presence of P2Y12 receptors are essential for successful phagocytosis.

We also identify microglia as key contributors to monocyte recruitment to the brain during virus infection, as in the absence of microglia the recruitment of monocytes were completely abolished in virus infected animals. Our method of microglia depletion was highly selective and did not affect immune cells in the periphery. Interestingly, in case of virus infected P2Y12 deficient mice, we detected identical monocyte recruitment, to that seen in control animals. These data suggest that other microglial chemotactic factors, such as MCP-1 or RANTES may be responsible for driving leukocyte migration to sites of infection in the brain independently from the presence of P2Y12 receptor.

Since we found that microglia responses are involved in leukocyte recruitment during virus infection, we examined whether this may be due to differences in blood-brain barrier permeability influenced by microglial actions. Inflammatory cytokine measurements from microglia depleted, virus infected brain homogenates have implicated marked changes in cytokine levels, which could be an indication of BBB disruption. However, we found that loss of microglia did not alter the extent of BBB injury or vascular activation based on IgG staining and immunofluorescent intercellular adhesion molecule 1 (ICAM) labelling. Our data show, that the in the absence of microglia monocyte recruitment is inhibited or at least not facilitated. As microglia deficiency does not markedly influence BBB injury in this experimental model, microglia might influence leukocyte recruitment by factors modulating the properties of the intact endothelial barrier.

In addition to that seen in mice, we found that P2Y12-positive microglia were found recruited to HSV-1 infected neurons in human post mortem brain tissues. As we have seen in mice, P2Y12-positive microglia recruited to and formed barriers around HSV-1 infected neurons. We also have found increased leukocyte recruitment, which can be correlated with the severity of the infection. Our findings, that microglia control neurotropic virus infection via P2Y12 in mice and the recruitment of P2Y12-positive microglia to HSV-1 cell in the human brain suggest that microglia P2Y12 plays a major role in anti-viral immunity in the CNS.

According to recent studies major evidence suggest that neurotropic viruses, such as HSV-1 can contribute to several forms of neurodegeneration, like the development of Alzheimer's disease. Collectively, our data could support understanding the mechanisms though which microglia control the elimination of injured neurons in the brain, and facilitate the development of new targeted therapies in common brain diseases.

6. List of publications

6.1. Publications related to this thesis

1. **Fekete R**, Cserép C, Lénárt N, Tóth K, Orsolits B, Martinecz B, Méhes E, Szabó B, Németh V, Gönci B, Sperlágh B, Boldogkői Z, Kittel Á, Baranyi M, Ferenczi S, Kovács K, Szalay G, Rózsa B, Webb C, Kovacs GG, Hortobágyi T, West BL, Környei Z, Dénes Á (2018) *Microglia control the spread of neurotropic virus infection vira P2Y12 signalling and recruit monocytes through P2Y12-independent mechanisms*. **Acta Neuropahtologica** 136(3):461-482. doi: 10.1007/s00401-018-1885-0.

2. Szalay G, Martinecz B, Lénárt N, Környei Z, Orsolits B, Judák L, Császár E, **Fekete R**, West BL, Katona G, Rózsa B, Dénes Á (2016) *Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke.* **Nature Communications** 7:11499. doi: 10.1038/ncomms11499.

6.2. Other publications

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