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The Role of Fpr1 in Sterile Inflammation after Ischemia/Reperfusion in the Brain

Dissertation

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Content

1 Introduction

1.1 Ischemic stroke

1.1.1 Definition and epidemiology

The world health organisation has classified stroke as “rapidly developing clinical signs of focal disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than vascular origin”. It is described as sudden occurrence of disturbances of the cerebral blood circulation which leads to the lack of oxygen and glucose and consequently to death of brain tissue. Stroke represents the third leading cause of death in the western world and the most common cause of disability in adults. The incidence is around 130-340 per 100.000 (Khaw and Kessler, 2006) and due to the population shift towards the elderly, it is assumed that the frequency of stroke is even going to rise in the next few years. The “Erlanger Schlaganfallregister” published data to the lethality of stroke and they found out that 19.4% of the patients suffering from stroke died within one month, 28.5% in three months, and 37.3% after twelve months (Kolominsky-Rabas and Heuschmann, 2002). In general, strokes can be classified into ischemic infarcts which are the most common ones in 75-80% and into hemorrhagic incidences (20-25%). Primary intracerebral bleeding is the cause of stroke in 10% and subarachnoid hemorrhage in 5%, however 5-10% remain unclear (Khaw and Kessler, 2006).

1.1.2 Etiology

The occurrence of stroke can have various reasons. To develop a better understanding, the TOAST-classification was generated to define different categories. It can be distinguished between cardio-embolic cause, macroangiopathy of a cerebral artery, microangiopathy, other defined etiology and unclear etiology. An ischemic infarct can originate from a thrombus formed in the heart or the aorta which then occludes an artery of the brain. Another underlying pathology is a stenosis of the Aa. carotic interna. Microemboli can break away from a sclerosed plaque which leads to arterio-arterial embolism. Additionally, microangiopathy can lead to the occlusion of small perforating brain arteries which then causes a lacunar infarct whose origins usually lie in hypertonia or diabetes mellitus (Stapf and Mohr, 2002). There are also less common reasons that usually occur in young patients, for example dissection of brain supplying arteries, cerebral vasculitis, or coagulopathy.

1.1.3 **Pathophysiology**

The understanding of the pathophysiology in ischemic stroke is important in order to understand further consequences and therapeutic options. The occlusion of brain supplying arteries leads to an interruption of the blood supply and in consequence a lack of oxygen and glucose in the infarct core. The deprivation of these essential sources initiates an ischemic cascade leading to failure of intracellular processes crucial for cell viability. Hypoxia is the main reason for cell death of neurons after a very short timeframe of only 60-90 seconds. Since the production of high energy phosphates is no longer sustainable, the ionic membrane gradient breaks down and extracellular glutamate accumulates. As a consequence, intracellular Ca^{2+}-levels are increasing, causing cellular events crucial for tissue damage like necrosis and apoptosis (Dirnagl et al., 1999). Neurons are the most vulnerable cells in the CNS under conditions of hypoxia-ischemia. Immediate post-stroke excitotoxic mechanisms lead to lethal damage followed by inflammation and apoptosis (fig.1). Consequently, the inadequate blood supply in the core region results in irretrievable brain damage, whereas in the area around the core - the so called penumbra - where the blood flow reduction is restricted, neurons remain viable for a prolonged period of time (Fisher and Garcia, 1996). As a consequence, neurons inside the penumbra area are dysfunctional but still viable and the loss of function can be rescued with promt therapy. The type and extend of the resulting symptoms are dependent of the localization of the ischemic tissue. With the loss of the function of neurons, patients have typical neurological dysfunctions like hemiparesis, aphasia, or visual defects.

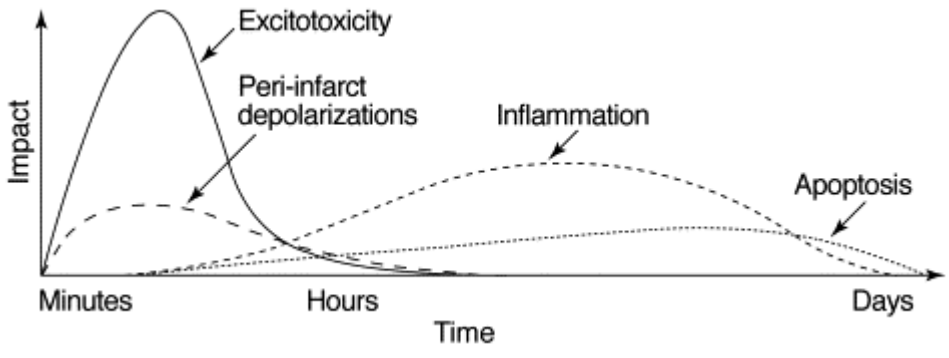

Figure 1 *Immediate excitotoxicity after the ischemic incident contributes to neuronal cell death in the ischemic core. In the further course inflammatory mechanisms lead to additional lethal cell damage (Dirnagl, Iadecola et al., 1999).*

1.1.4 **Treatment**

Finding the right therapy for patients after ischemic stroke still remains a big challenge. Currently, the thrombolysis with recombinant tissue plasminogen activator (rt-PA) and the mechanic recanalization are the only effective therapies available. But due to the limited time window of a maximum of 6hours after the onset, only few patients can benefit from rt-PA (Donnan et al., 2008; Hacke et al., 2004). Because the inflammatory response also contributes to further tissue damage after stroke, new therapeutical approaches include the suppression of inflammation.

1.2 **Cells of the central nervous system (CNS)**

There are different types of cells in the CNS, which can be conferred to as the neurovascular unit. The neurovascular unit is composed of astrocytes, neurons, endothelial cells, pericytes, and myocytes (del, 2006). These cells interact in order to detect and supply neuronal needs and therefore have important functions in stroke. After ischemia, brain cells fail to maintain the microenvironment and the blood-brain barrier is breaking down. This results in infiltration of blood-borne immune cells as inflammatory mediators into the ischemic hemisphere which exaggerate brain edema and increases cell death (Shichita et al., 2012).

Endothelial cells are situated on the inner vascular wall and are under normal conditions connected through tight junctions. Immediately after arterial occlusion proinflammatory signals are generated and oxidative stress reduces the bioavailabilty of nitric oxide (NO). Since NO is a potent vasodilator, this leads to vasoconstriction in the blood vessels and blood flow in the ischemic territory is additionally reduced (Yilmaz and Granger, 2010). Oxidative stress in endothelial cells also alters the permeability of the blood-brain-barrier which increases leucocyte extravasation and migration of other immune cells into the brain parenchyma (Engelhardt and Sorokin, 2009).

Astrocytes are supportive glial cell components in neuronal tissue which outnumber neurons by over fivefold (Sofroniew and Vinters, 2010). Their physiological functions include the maintainance of the ion and water homeostasis, the release of neurotrophic factors and metabolic support of neurons. They are also part of the blood-brain barrier. Since they provide essential metabolic support to neurons, failure of astrocyte function in cerebral ischemia results in neuron degeneration (Takano et al., 2009). It still remains unclear how

ischemia affects astrocytes, but it is assumed that neurons are not capable of surviving in the absence of astrocytes (Nedergaard and Dirnagl, 2005).

Neurons are specialized cells that transmit information to other cells throughout the whole body. They are the most susceptible cells in the brain when exposed to hypoxia. They are not only dependent on oxygen, but also on the supply of metabolic support by astrocytes. Hypoxia-ischemia leads to an increase in extracellular glutamate levels which then results in excitotoxicity and cell death in neurons (Kostandy, 2012). Another reason of neuronal cell death is energy failure in areas of reduced blood supply. Without oxygen and glucose neurons cannot perpetuate the production of ATP, which is needed to maintain ionic balance of the cell. Thus, Ca^{2+} influx and Na^{+} accumulation lead to degeneration of organelles and necrotic cell death (Dirnagl et al., 1999). It is also assumed that mitochondrial damage contributes to ischemic cell death of neurons (Lipton, 1999). Dying cells again release danger signals, which in turn activate the immune system.

Microglia are resident immune cells of the CNS and are essential for the immune surveillance of the brain and defense towards pathogens (Kreutzberg, 1996). They continuously monitor the microenvironment in the CNS. Their immunological function include phagocytosis, secretion of proinflammatory cytokines, and antigen presentation. After the ischemic attack, microglia are rapidly activated within a time period of only minutes and accumulate at the lesion site and the penumbra. Their proliferation peaks at 48-72h after the injury (Lalancette-Hebert et al., 2007). It still remains controversial whether the activation in stroke has overall beneficial or detrimental effects on the fate of the ischemic tissue. Several studies demonstrated that activated microglia release cytotoxic molecules such as nitric oxide, reactive oxygen species, and proinflammatory cytokines (i.e. TNFα or IL-1β), which promote neurotoxicity and in the end lead to neuronal cell death (Dirnagl et al., 1999; Rogove et al., 2002). On the other side, there is also experimental data from OGD (oxygen glucose deprivation)-experiments showing that activated microglia have neuroprotective functions (Neumann et al., 2006).

1.3 **Reperfusion and inflammation**

The blood flow reduction in cerebral ischemia is typically focal, affecting only parts or in rare cases complete vascular territories. In the central core region of the infarct permanent damage of neurons and cell death occurs within minutes and leads to loss of function in parts

of the affected brain region. In the direct proximity of the ischemic core is the penumbra located, where the cerebral blood flow (CBF) can still be maintained, but to a decreased extend. This results in functional impairment of the affected neurons. In this part, cells are able to survive for a certain period of time. However, the time till cell death occurs is limited and no effective neuroprotective therapy has been achieved so far. The only clinically available therapeutic approaches are targeting the recanalization of the occluded vessel, either by thrombolysis with rt-PA or by mechanic thrombolysis. The application of rt-PA lyses the thrombus and blood flow can be restored so that cells in the penumbra are supplied again with oxygen and glucose. Restored blood flow in the vessels also contributes to the influx of immune cells in injured brain tissue and initiate further inflammatory reactions. The breaking down of the blood-brain barrier leads to brain edema which contributes to acute mortality in stroke patients (Candelario-Jalil et al., 2009). Another important concern is the fear of hemorrhagic bleeding after the use of rt-PA in acute stroke patients (Kase et al., 2001). Current publications reveal that in a mouse model rt-PA decreases the infarct sizes regardless of whether vessel recanalization is successful (Ansar et al., 2014).

1.3.1 Sterile inflammation

The post-ischemic inflammation reaction consists of a sequence of different events which are triggered by ischemia and reperfusion. The blood flow reduction triggers an ischemic cascade which leads to the failure of processes and consequently cell death in brain tissue. First, ischemia causes hypoxia and glucose deprivation in the brain. The impairment of energy supply leads then to the breakdown of the ionic gradient which leads to intracellular accumulation of Ca^{2+} and Na^{+} and extracellular glutamate levels are extensively increased. As a result of the glutamate-mediated overactivation, Na^{+} and Cl^{-} enter neurons, water follows and brain edema occur (Dirnagl et al., 1999). Besides neurons, glial cells and endothelial cells are also affected after an ischemic event. The production of reactive oxygen species (ROX) activates platelets and endothelial cells, leading to microvascular occlusion (Shichita et al., 2012). Oxidative stress reduces the bioavailability of nitric oxide (NO), a potent vasodilator leading to vasoconstriction, platelet aggregation, and leucocyte adhesion (Palmer et al., 1987). As a result, blood flow to the ischemic tissue is decreased even more. Oxidative stress and inflammatory processes alter the blood-brain-barrier as well as the release of proteases from leucocytes and the downregulation of junctional proteins (Engelhard et al. 2009). In the perivascular space mast cell degranulation leads to the release of histamine and tumor necrosis factor α (TNFα) and macrophages release pro-inflammatory

cytokines (Strbian et al., 2006). The release of pro-inflammatory mediators causes the breakdown of the blood-brain-barrier. Consequently, leucocytes like neutrophils, lymphocytes, and monocytes can infiltrate easier and secondary sterile inflammation is activated. Meanwhile, dying brain cells like neurons, microglia, and astrocytes also produce inflammatory mediators (TNFα, IL-1β) and so called 'danger signals' like the nucleotides ATP or UTP. ATP for example activates microglia via P2X7 (P2X purinoreceptor 7) (Cavaliere et al., 2005). Thus, simultaneous release of pro-inflammatory mediators from infiltrating immune cells and dying brain cells exacerbate post-ischemic sterile inflammation in the brain.

1.3.2 Role of inflammatory cells

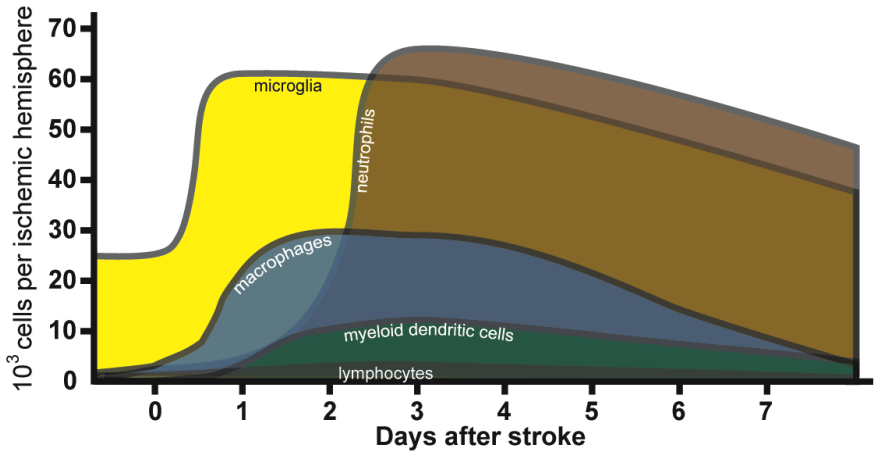

Figure 2 *Amounts of immune cells after an ischemic incident in the ischemic hemisphere (Gelderblom et al., 2009).*

Various publications in the research of MCAO (middle cerebral artery occlusion) have demonstrated that infiltrating inflammatory cells play a crucial role in post-ischemic inflammation (Dirnagl et al., 1999; Yilmaz and Granger, 2010). The most important cell populations include microglia, macrophages and neutrophils. The majority of microglia and macrophages as well as dendritic cells migrate to the ischemic hemisphere in the first 12hours after reperfusion, whereas neutrophils reach their maximum on day 3 after reperfusion (Gelderblom et al., 2009) (Figure 2). Their activation leads to the production of pro-inflammatory cytokines like interleukin-1, interleukin-6, and tumor necrosis factor α (TNFα) and they accumulate in microvessels as well as in the ischemic tissue (Huang et al., 2006). Even human studies after ischemic stroke showed that migration of neutrophils

correlates with poor neurological outcome (Akopov et al., 1996). The role of infiltrated microglia and macrophages is still under debate. First of all, they produce toxic molecules, like NO and oxygen radicals, and pro-inflammatory cytokines which results in the damage of neurons (Kaushal and Schlichter, 2008). On the other hand, macrophages phagocyte debris and microglia produce neurotrophins and plasminogen which are involved in tissue repair (Kato et al., 2000). In addition, T-cells could be detected. Not only $CD4^+$ and $CD8^+$ cells, but also $CD4^-/CD8^-$ cells were increased in the ischemic hemisphere (Gelderblom et al., 2009). Dendritic cells (DCs) surprisingly also showed a large increase from day 1 to day 3 after reperfusion. Thus, the exact role of infiltrating immune cells remains unclear and further characterization of function and mechanism is necessary. However, immune cells like $CD4^+$ and $CD8^+$ T-cells are one cause of secondary brain damage, because immunodeficient mice showed a decrease in infarct sizes and improved outcome after MCAO (Yilmaz and Granger, 2010).

1.4 The Role of cytokines and chemokines in ischemia/reperfusion

During ischemia and reperfusion, brain cells are put under stress and as a consequence, different genes are upregulated and signal molecules like cytokines are released. Cytokines are produced by different cell types and own an important role in cell signalling in the context of infection and inflammation. Basically, they can be divided into 5 groups (Murphy et al., 2016):

- Colony-stimulating factors (G-CSF, GM-CSF, M-CSF) which function as haematopoetic growth factors
- Interferons (IFN-α, IFN-β, IFN-γ) which mainly appear in antiviral host defense
- Interleukines (IL-1 - IL-37) whose main function is the communication between leucocytes
- Tumor-necrosis-factors (TNF-α) which are important for the activation of other immune cells in the context of inflammation, apoptosis, or necrosis
- Others like TGF-β, MIF which induce regulatory and anti-inflammatory processes

Cytokines which play the most important role in stroke and inflammation are IL-6, IL-1β, and TNFα (Lambertsen et al., 2012) and are therefore potential targets in future stroke therapy. IL-6 is released by microglia and neurons and increased in the peri-ischemic region. So far, there are only few studies on the role of IL-6 in experimental stroke. Its function is

ambivalent: on one side it functions as an inflammatory mediator in the acute phase and on the other side it is involved in neuroprotection and angiogenesis (Gertz et al., 2012; Suzuki et al., 2009). TNFα is the most extensively studied cytokine in experimental stroke which is synthesized in microglia and macrophages in the acute and late phase after ischemia (Gregersen et al., 2000). It is supposed to play a pathologic role in mechanisms of necrosis, apoptosis and excitotoxicity (Lambertsen et al., 2009). It could be shown that the application of neutralizing antibodies to TNFα leads to a decrease in infarct sizes and better neurological outcome after stroke (Hosomi et al., 2005). On the other hand, further studies showed a neuroprotective role of TNFα (Lambertsen et al., 2009). In conclusion, neurotoxic and neuroprotective effects of TNFα are dependent of several factors like timing, its receptors, and the activation of microglia (Sriram and O'Callaghan, 2007). Another important cytokine involved in inflammation is IL-1β which is secreted by microglia and macrophages. IL-1β induces the expression of other proinflammatory cytokines as well as its own production. The activation of astrocytes and microglia lead to an increased release of neurotoxic mediators which in the end is the reason for an increased vulnerability of neurons to ischemia. Finally, IL-1β also leads to edema formation (del Zoppo, 2009; Rothwell, 2003). Other experiments showed that the intracerebraventricular injection of IL-1β into mice is accompanied by an increase in infarct sizes (Touzani et al., 2002) and mice who are deficient in IL-1β appear to have decreased infarct sizes. This means that IL-1β has a clearly detrimental effect on the neuronal outcome (Allan et al., 2005).

Chemokines which are even smaller molecules than cytokines are released by activated cell populations and have the ability to induce chemotaxis in nearby cells which then migrate to the site of impaired tissue. The migration of cells is possible due to changes in the cytoskeleton initiated by the binding to a G-protein-coupled receptor on the outside of the cells. Chemokines can again be divided into different groups (Murphy et al., 2016):

- CXCL-chemokines (CXCL1 to CXCL16) which are involved in the migration of neutrophils
- CCL-chemokines (CCL1 to CCL28) which are mainly involved in the migration of monocytes and lymphocytes
- Others (XCL1, XCL2, CX3CL1) which activate other cell populations

Chemokines play several important roles. They do not only maintain migration, but also influence T-cell differentiation (Luther and Cyster, 2001), angiogenesis, and the maturation of T-, B- and Dendritic Cells (Rossi and Zlotnik, 2000). There is evidence that chemokines are involved in ischemia-reperfusion-models, for example in hepatic ischemia-reperfusion where hepatocytes produce chemotactic signals which then lead to increased tissue damage (Jaeschke, 2006). Although there is only few chemokines constitutively present in the CNS, in pathologies like ischemic stroke they are suggested to be upregulated. CXCL1 (chemokine C-X-C motif ligand), which is also known as growth related oncogene (GRO) alpha is one of the best examined chemokines in inflammatory processes. Its target cells are mainly neutrophils and its function was detected in bacterial peritonitis (Giron-Gonzalez et al., 2001), HIV-infection (Lane et al., 2001) and other inflammatory processes. Losy et al. (Losy et al., 2005) detected elevated CXCL1 levels in CSF in patients with cerebral ischemia. Especially in the early phase of stroke, a chemokine upregulation was shown and a positive correlation between CSF CXCL1 levels and the stroke area in CT scans after 24 hours suggest that CXCL1 contributes to the increased tissue injury after cerebral ischemia. Other publications in experimental stroke also showed the upregulation of CXCL1 as a specific inflammatory marker after 4 hours in plasma and peripheral tissue as well as after 24 hours in the brain (Chapman et al., 2009). The role of CXCL2, a different chemokine still remains unclear: on one hand CXCL2 is increased 24-72 hours after cerebral ischemia, but the administration of an antibody against CXCR2, the corresponding receptor, does not improve the outcome after 72 hours of ischemia (Brait et al., 2011). CCL2 as a monocyte chemoattractant protein of the CC-chemokine subfamily is the most potent out of this group in activating signal transduction pathways leading to monocyte migration (Sozzani et al., 1994). As it can also be produced by neurons, microglia and astrocytes, its role in neuroinflammatory processes is even more important. Although there is evidence that CCL2 is involved in ischemic stroke, its role is controversial. On one hand it is supposed to support endogenous neurovascular protection (Stowe et al., 2012) and on the other hand CCL2 overexpression leads to increased infarct volumes and monocyte and macrophage invasion (Chen et al., 2003).

1.5 Characterization of the formyl-peptide-receptor 1 (Fpr1)

Formyl-peptide-receptor 1 (Fpr1) is a seven transmembrane domain G_i-protein coupled receptor, which was first identified in phagocytic leucocytes. It is expressed on immune cells like neutrophils, monocytes, macrophages, and DCs (Yang et al., 2001), but also on

microglia, spleen, lung and liver cells (Le Y et al., 2002; Migeotte et al., 2006). Extracellular binding agonists are the N-formyl-peptide formyl-methionine-leucyl-phenylalanine (fMLP) and its analogues (Le Y et al., 2002), other formyl-peptides of bacterial or mitochondrial origin and Annexin (Perretti et al., 2001). The antagonist with the highest potential is Cyclosporin H (CsH) (Wenzel-Seifert and Seifert, 1993). The function of Fpr1 is to mediate cell chemotaxis and activation in response to bacterial formylated chemotactic peptides and therefore can be considered as host defense against microbial infection. It could be shown that mice deficient in Fpr1 are more susceptible to infection by *Listeria monocytogenes* which produce agonist peptides (Gao et al., 1999). Besides, $Fpr1^{-/-}$ mice are viable and fertile, normal in growth and development and show no differences in anatomy or behavior when compared to $Fpr1^{+/+}$ littermates. They also do not have any defects in hemostasis or healing of tail wounds (Gao et al., 1999).

1.5.1 Signaling pathway of Fpr1

Fpr1 is coupled to a G-protein and ligand binding on the extracellular side results in the dissociation of the heterotrimeric G-protein on the inside into one α– and βγ-subunit (see Figure 3). The alpha-subunit activates the phosphatidyl-inositol-3-kinase (PI3K) and phospholipase C (PLC). Then PI3K converts the membrane phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). PLC then interacts with PIP3 and splits it into the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} from intracellular stores (endoplasmatic reticulum) which leads to an increase of Ca^{2+}. Studies showed that synthesis of PIP3 and Ca^{2+}-release contribute to asymmetric f-actin synthesis and cell polarization during neutrophil chemotaxis

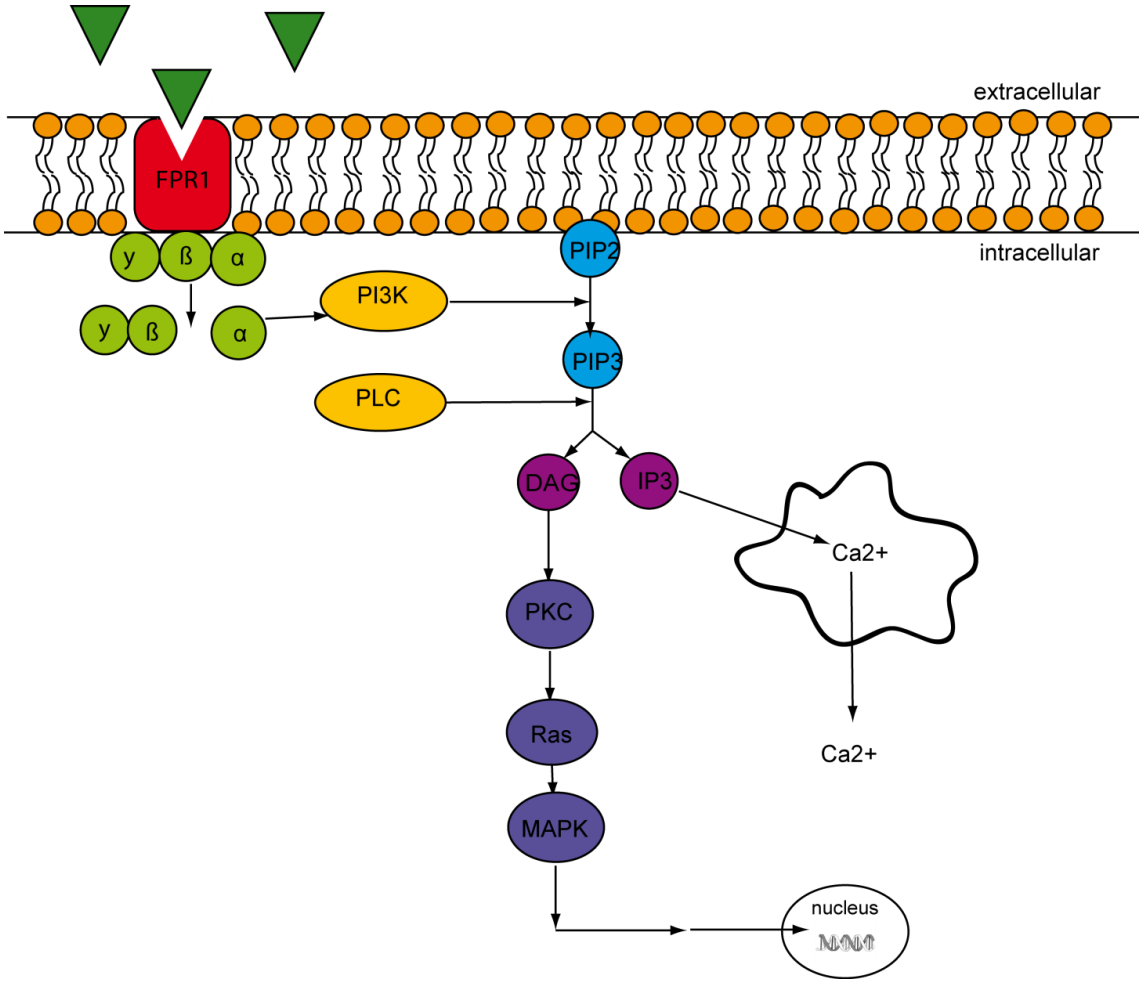

Figure 3: *Schematic illustration of the Fpr1 signaling pathways. After binding of an agonist to Fpr1-receptor, trimeric G-proteins are uncoupled from Fpr1 inside the cell and different signal transduction pathways are active. In the end they lead to actin-polymerization, adherence, chemotaxis, and gene transcription. PI3K: phosphatidylinositol-3-kinase; PIP2: phosphatidylinositol biphosphate; PIP3: phosphatidylinositol triphosphate; PLC: phospholipase C; IP3: inositol triphosphate; DAG: diacylglycerol; PKC: proteinkinase C; MAPK: mitogen-activated protein kinase.*

(Sadhu et al., 2003). DAG activates the Calcium-dependent protein kinase C (PKC). PKC leads to the activation of Ras/MAPK (mitogen-activated protein kinases) way. Also NF-κB translocation can take place to increase cytokine gene transcription to amplify the innate immune response from signals generated by infections and injuries (Chen et al., 2005). In the end, various cell responses like gene transcription, actin polymerization, adhesion, phagocytosis, and chemotaxis are possible effects (Mollica et al., 2012).

1.5.2 Sterile inflammatory processes through mitochondrial danger associated molecular patterns (DAMPs)

Neutrophils and other leucocytes are the major populations in the innate immune system and are in charge of defense against bacterial invasion (Mantovani et al., 2011). Invading microorganisms express pathogen associated molecular patterns (PAMPs), which are then recognized by different pattern recognition receptors. PAMPs can be of different origin, but most common are DNA from bacteria or formylated peptides, which bind to toll-like-receptor-9 (TLR9) or formyl-peptide-receptor-1 (Fpr1), respectively. Those receptors are expressed on cells of the innate immune system, especially on neutrophils. Binding of those PAMPs to the receptors following bacterial infection activates the immune system and systemic inflammation results. Mitochondria in cells of our organisms are evolutionary endosymbionts that are derived from bacteria and therefore show molecular and structural similarities. According to this, mitochondrial danger associated molecular patterns (DAMPs), which are released in cellular injury consist of the same motifs as bacterial PAMPs. Mitochondrial DAMPs contain formyl peptides and mitochondrial DNA. Zhang et al. demonstrated that trauma in eukaryotic cells leads to disruption of mitochondria and the release of mitochondrial DAMPs into the circulation. Formyl peptides and mitochondrial DNA activate human neutrophils through Fpr1 and TLR9, respectively. Intracellular signal transduction activates neutrophils and results in a sterile inflammation reaction. Rats that were given mitochondrial DAMPs intravenously suffered from systemic inflammation and organ injury caused by the activation of Fpr1 and TLR9. It was also shown that CsH is able to block Fpr1 competitively. In contrast to injection of mitochondrial DAMPs only, the additional injection of CsH inhibited neutrophil influx (Zhang et al., 2010). Other substances like propofol can also inhibit respiratory burst, degranulation and chemotaxis of fMLP-activated neutrophils by competitive binding to Fpr1 attenuating downstream signaling (Yang et al., 2013). Those findings suggest that blocking of Fpr1 on neutrophils or other immune cells can decrease the severity of the inflammation process.

1.6 Aim of the study

An ischemic incident in the brain is accompanied with deprivation of oxygen and glucose in the brain parenchyma and thus leading to cell death of cells in the brain within a short timeframe of minutes. After reperfusion and restored cerebral blood flow, immune cells in the ischemic hemisphere initiate further cell death. These events are triggered by the release of pro-inflammatory cytokines and other danger signals released by migrated immune cells or necrotic cells. When cells undergo necrosis, mitochondria also get disintegrated and release mitochondrial DAMPs which bind to Fpr1. Zhang et al. (Zhang et al., 2010) found out that the activation of Fpr1 through mitochondrial DAMPs is followed by the activation of innate immune pathways and human neutrophil migration which in the end can even cause a sepsis-like state in the organism. Sterile inflammation triggered after ischemia/reperfusion in stroke is postulated to be one main cause of secondary brain damage. So far, previous studies showed that Fpr1 is involved in sterile inflammatory processes after trauma. To date, the role of Fpr1 in post-ischemic inflammation after stroke has not been investigated. This study was designed to show whether Fpr1 contributes to secondary tissue damage in the context of cerebral ischemia in a mouse model. If that would be the case, further investigations in this field of research could offer new therapeutic targets. The MCAO model was applied to generally compare mice deficient in Fpr1 to their littermates. Mice with ischemic incidents were then used to analyze if they show any differences in the neurological outcome as well as the size of damaged tissue. Another focus was laid on the investigation of inflammatory processes after ischemia/reperfusion and if they vary in mice lacking Fpr1 compared to littermate controls. We also aimed to examine the expression and function of Fpr1 in neurons in *in vitro* experiments.

1.7 **Zielsetzung**

Während der ersten Minuten nach einem ischämischen Ereignis im Gehirn kommt es vor allem zu einem Mangel an Sauerstoff und Glukose, welcher zum Untergang von Hirngewebe führt. Nach wieder hergestelltem Blutfluss und Reperfusion, sind Immunzellen für das Einsetzen weiteren Zelltods verantwortlich, indem sie Botenstoffe und Warnsignale freisetzen. Es entwickelt sich eine post-ischämische Entzündungsreaktion, die zu weiterer Gewebezerstörung führt. Sobald Zellen nekrotisch werden, lösen sich auch mitochondriale Strukturen auf und Warnsignale, sogenannte DAMPs, werden ausgeschüttet. Die Aktivierung des angeborenen Immunsystems wird durch die Bindung der ausgeschütteten DAMPs an den Rezeptor Fpr1 vermittelt. Weiterhin kommt es zur Migration von neutrophilen Granulozyten, die sogar einen sepsisartigen Zustand des Organismus auslösen können. Insgesamt scheint die sterile Entzündungsreaktion nach einem Schlaganfall eine entscheidende Rolle am Ausmaß der Hirnschädigung zu spielen. Bisher konnte gezeigt werden, dass Fpr1 an der Auslösung steriler Entzündungsreaktionen nach einem Trauma beteiligt ist. Allerdings wurde die Beteiligung von Fpr1 im Schlaganfall bisher noch nicht untersucht. Das Ziel dieser Studie war es, herauszufinden, ob Fpr1 im Tiermodell des Schlaganfalls zu sekundärem Gewebeuntergang beiträgt und somit als Angriffspunkt für weitere therapeutische Ansätze in Frage kommt. Zu diesem Zweck benutzten wir das MCAO-Model, um Fpr1 knockout-Mäuse mit Wildtyp-Mäusen zu vergleichen. Mäuse nach einem ischämischen Ereignis wurden dann hinsichtlich ihres neurologischen Zustands und der Größe des Schlaganfallsgebiets untereinander verglichen. Zusätzlich untersuchten wir die Infiltration verschiedener Immunzellen nach einem Schlaganfall in Abhängigkeit von Fpr1. Ein weiterer Ansatz in *in vitro* Experimenten war die Frage, inwiefern Fpr1 auf Neuronen exprimiert wird und welche Funktion es erfüllt.

2 Material and Methods

2.1 Cell cultures

2.1.1 Primary mixed cell culture of astrocytes and microglia

One day old pups (*Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$) were decapitated, brains dissected and transferred in a 60mm petri-dish with cold 1xHBSS/10mM-HEPES on ice. The hemispheres were separated with small forceps and cerebellum, bulbi olfactorii and the mesencephalon as well as the meninges were detached under the microscope. Each hemisphere was cut in four pieces and further preparation was done under the sterile working bench. The brains were transferred into a 15ml falcon with a 10ml glass pipette and centrifuged for 1min at 300g and room temperature. After discarding the supernatant, the tissue was resuspended in 10ml HBSS/10mM-HEPES (Gibco, #14025-050; Gibco, # 15630) and centrifuged again for 1min and 300g at RT. The supernatant was discarded again and the tissue resuspended in 5ml digestion solution, HBSS/10mM HEPES + 0.5mg/ml papain + 10µg/ml DNAse (Sigma, #P4762; Roche, #11284932001). Then it was shaken in a 37°C water bath for 25min. Afterwards, the suspension was centrifuged for 5min with 300g and RT, the supernatant discarded and the tissue resuspended in 10ml pre-warmed medium, BME + 10% FCS + 0,5% P/S (Gibco, #41010; Pan Biotech, #P40-47500; Invitrogen, #15140-122). After another centrifugation step (5min, 300g, RT), the supernatant was discarded and the pellet resuspended in 2ml medium with a fire polished glass pipette for further separation. Then, the suspension was filled up to 10ml with medium and transferred through a 70µm cell filter. The cell concentration was determined after 1:10 dilution with trypan blue (Sigma, #T8154) in a Neubauer-chamber (Marienfeld) and filled up to 300.000 cells/ml. The cells were then seeded and medium changed every 5days (substitute 3ml of old medium with 5ml of fresh medium).

2.1.2 Separation of microglia out of primary mixed culture

For further experiments microglia and astrocytes were separated after 21 to 25days. Therefore, the mixed cultures were shaken at 600-700rpm at 37°C for 2-3hours. Microglia dissociated from astrocytes and could be removed with media, which was then centrifuged for 10min at 300g and RT. The supernatant was taken off and mixed 1:2 with fresh medium to put it back on the astrocytes. The pellet was resuspended in 1ml fresh, prewarmed MEM + P/S (Gibco, #51200-046; Invitrogen, #15140-122) and the cell density of microglia

determined after counting cells in a Neubauer chamber with a 1:2 dilution of trypan blue. Then the pellet was washed with PBS (Gibco, #14190-094) for RNA-isolation or cells were seeded in 48-well plates with a density of 200.000 cells/ml.

2.1.3 **Primary neuronal cell culture**

One day before creating the cell culture, 24-well plates were covered with coverslips and coated with Poly-D-Lysine (Sigma, # P7886-100MG) 1:200 in PBS and incubated over night at 37°C. Then they were washed three times with PBS and dried under UV-light for 20min before putting 500µl neurobasal-medium + B-27 + 200mM L-glutamine + HEPES + Gentamycin (Gibco, #21103-049; Gibco, #17504-044, Gibco, #25030-024; Gibco, #15630; Gibco, #15750-060) in each well and incubated at 37°C. E16,5 pregnant mice (*Fpr1*$^{+/+}$ or *Fpr1*$^{-/-}$) were used. The abdomen of the dead mouse was cut open and the embryos put into a 10cm petri dish with cold PBS. The embryos were prepared and then put into a 60mm petri dish with dissection medium (HBSS/10mM HEPES). The whole preparation was done on ice. After decapitation of the embryos, the heads were put into a fresh petri dish with ice-cold dissection medium. The brains were dissected from the rest of the head and put into a fresh petri dish with dissection medium. Then the two hemispheres were properly separated, cerebellum and brainstem removed and the hippocampus was excised. The cortices were then collected in a petri dish and put into a 50ml falcon for digestion, hippocampi were transferred into a 15ml falcon with 10ml dissection medium. Further processing was done under the working bench: the dissection medium was taken off and 10ml, respectively 3ml digestion solution added (HBSS without Mg^{2+} and Ca^{2+} (Gibco, #14170-088), 1mg/ml trypsin (Sigma, #T8003-500MG)). For the digestion, the falcons were incubated for 6min in a 37°C water bath and shaken every two minutes. Next, the digestion solution was removed carefully and the reaction was stopped with 10ml/3ml of 1mg/ml trypsin-inhibitor (Sigma, # T9003-250MG) in Neurobasal-medium. The mixture was removed and 2ml dissection medium were added to the hippocampal neurons and dissociated with a fire-polished pipette before the cells were counted in a Neubauer-chamber with a 1:10 dilution of trypan-blue (Sigma, #T8154). To the cortical neurons 10ml of dissection medium was added and pipetted it up and down 10 times with a 10ml-glaspipette. The suspension incubated for 5min, then 7ml were carefully removed and put into a new 50ml falcon, filled up to 10ml and counted. The cells were plated in 24-well-plates with a density of 100.000 per well in 500µl medium. After 7days, experiments were started with these cell cultures.

2.2 **FACS-experiment**

For the preparation of the brain, mice were sacrificed 24h following MCAO with isoflurane (AbbVie, # B506) and perfused with 1x PBS, the brain was removed and the cerebellum separated. Hemispheres were split and the tissue was transferred into two petri dishes with 1xPBS before the selected hemispheres were transferred into a fresh petri dish and cut into small pieces. Brains were shifted to a 50ml falcon with 5ml digestion solution and shaken for 30 minutes in the 37°C water bath. From this step everything was done on ice or at 4°C. The digested tissue was thoroughly mixed with a 10ml pipette and passed through a 40µm cell strainer with a plug from a 2ml syringe. The cell strainer was washed with 40ml cold PBS and centrifuged at 310g for 10min at 4°C. The pellet was resuspended in 5ml Erylysis buffer and incubated on ice for 7min. The falcon was filled up with PBS again and centrifuged (310g, 10min, 4°C). 90% Percoll was used to prepare a 78% (A) and a 30% Percoll (B) solution. The pellet was resuspended in 2,5-2,8ml Percoll B and transferred into a fresh 15ml falcon where Percoll B was underlied with 2,5-2,8ml of Percoll A and the weight of two falcons was adjusted with Percoll B. After centrifugation (1350g, 30min, 4°C) without brakes the interface of the gradient was carefully removed with a 1ml pipette and transferred into a fresh 15ml falcon and filled up with FACS-buffer. The solution was centrifuged (700g, 10min, 4°C) and the pellet was washed twice with 10ml FACS-buffer (310g, 10min, 4°C). Cells were resuspended in 70-100µl FACS-buffer plus 2µl Fc-block (1:10). For cell staining 20µl of the cells were mixed with 20µl antibody-mix and incubated 30min at 4°C. Afterwards, 200µl FACS-buffer was added and centrifuged at 350g, 5min and room temperature. The supernatant was discarded and the pellet resuspended again in 200µl FACS-buffer and was then transferred into a FACS-tube with additional 200µl FACS-buffer. For *TruCount* (BD Biosciences) staining, a *TruCount* tube was filled with 10µl cells and 10µl CD45-APC-Cy7 (1:10) antibody and 80µl FACS-buffer. After incubation for 30min at room temperature, 300µl of FACS-buffer were added to the cells. FACS-analysis was done at the Fortessa from BD Bioscience. Digestion Solution: DMEM, 1,0mg/ml collagenase, 0,1mg/ml DNase, Erylysis-buffer: 0,15M Ammoniumchlorid, 10mM Kaliumhydrogencarbonat, 0,1mM EDTA, pH 7.2-7.4.

2.3 Stimulation experiments

2.3.1 Stimulation with brain lysate

Protein isolated from brains of three wildtype mice with MCAO was frozen at -80°C in aliquots and functioned as `brain lysate` in the following experiments. To stimulate the different cell types, incubation was performed with the following dilutions: 1:500, 1:1000, 1:5000, 1:10.000. Cell cultures were seeded in 24-well-plates (neurons), 48-well-plates (microglia) or 6-well-plates. After 6 and 24 hours incubation time at 37°C either cell death was measured with LDH-assay or stimulation was measured by analyzing different cytokine-expressionrates with qPCR.

2.3.2 Stimulation with mitochondrial DAMPs

For an optimized stimulation triggered by Fpr1, we co-stimulated neuronal cell cultures with isolated mitochondrial DAMPs. Cell cultures were seeded in the same format as in 2.1.3 and mitochondrial DAMPs were added in the following dilutions: 1:250, 1:500, 1:1000, 1:5000. After 24 hours of incubation at 37°C cell death or gen-expression was tested with LDH-assay or qPCR respectively. As positive control, fMLP (Sigma, #47729) (0.1 µM, 1µM and 10µM) was used.

2.3.3 Co-stimulation with mitochondrial DAMPs and TLR9-agonist

For co-stimulation with mitochondrial DAMPs and TLR9-agonist (CpG-ODN, Invivogen, # tlr9-1668) we stimulated the cells seeded similar as described above and added mitochondrial DAMPs and CpG-ODN simultaneously. CpG-ODN was added in the following concentrations: 0.1µM, 1µM and 5µM. Cell death was measured with LDH-assay.

2.4 Molecular biological techniques

2.4.1 RNA-isolation out of primary cell cultures

For the isolation of RNA out of astrocytes, microglia and neurons, the RNeasy mini kit from Qiagen (Qiagen, #74104) was used as described in the manufacturer’s protocol.

Microglia were used after shaking and separating them from the astrocytes and washing with pre-warmed PBS. Astrocytes were incubated another 24 hours at 37°C after shaking. And neurons were used after one week for the Isolation of RNA.

2.4.2 Synthesis of cDNA from isolated RNA

The RNA-concentrations of each probe were measured using the RNA Pico Chip from Agilent. Dilution was performed in a way that all RNA-concentrations were the same for one experiment. For the transcription the Maxima First Strand cDNA Synthesis Kit for RT-qPCR from Thermo Scientific was used. To each sample 4µl reaction mix and 2µl enzyme (reverse transcriptase) were added and then filled up to a total amount of 20µl with RNase-free water. Afterwards the PCR-tubes were put into the cycler and cDNA was regenerated with the following setting: 10min incubation at 25°C, 30min reverse transcription at 50°C, 5min inactivation of the enzyme at 85°C and cooling to 4°C.

2.4.3 Protein isolation from primary cell cultures

The cell cultures were washed with pre-warmed PBS and then lysis-solution added (1 ml RIPA reagent, 4mM PEFA, 1x complete buffer, 1x phosphatase inhibitor single-use cocktail) (ThermoScientific, #89900; Roche, #11429868001; Roche, #11497698001; ThermoScientific, #78428). After the incubation time of 5min on ice, the cells were transferred to a 1,5ml-Eppendorf tube with the help of a cell scraper. Then they were treated with ultrasound 10 times before it was centrifuged for 10min at 13.400rpm and 4°C. The supernatant was transferred in a new 1.5-Eppendorf-tube and frozen at -80°C. For the determination of the amount of proteins the BCA protein assay kit from Pierce (ThermoScientific, #23225) was used.

2.4.4 **Isolation of mitochondria from the CNS**

We used a protocol which was previously described by Zhao and Herdegen (Zhao and Herdegen, 2009). The protocol is shown in a flow chart in Figure 4. For the isolation of mitochondria, the hippocampus and cortex of single mice were dissected on ice and placed into a pre-cooled glass-dounce homogenizer with 1ml isolation medium, 2ml respectively (150mM Mannitol, 20mM Tris, 1mM EGTA, 1mM EDTA, 0.3% BSA) (Sigma, # M4125;

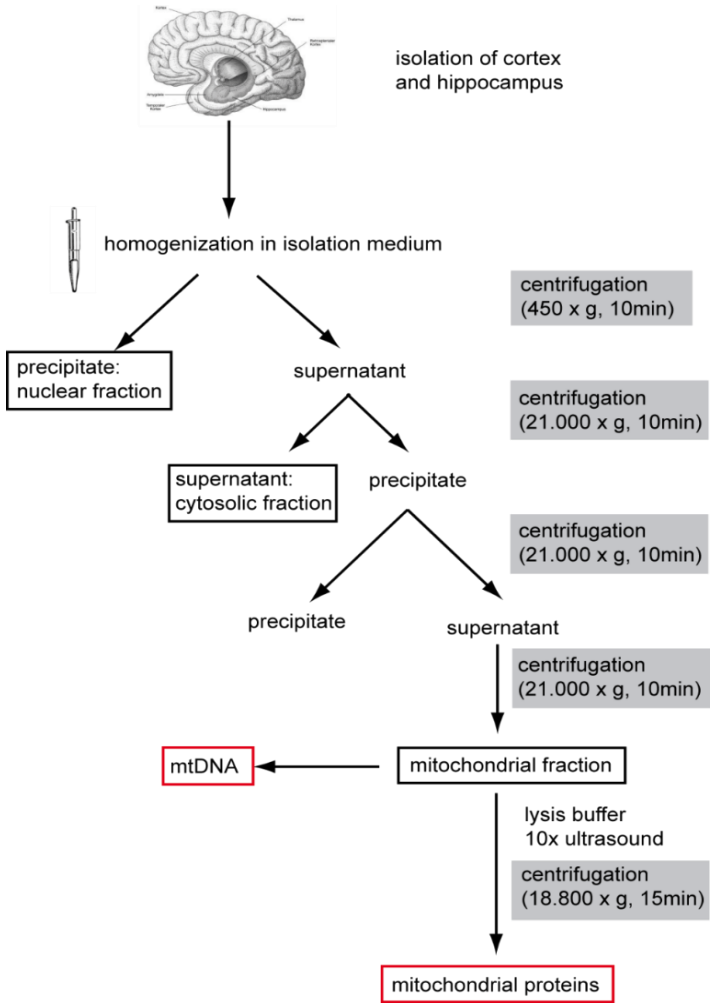

***Figure 4**: Sequence of steps for the isolation of mitochondria from cortex and hippocampus*

Sigma, # T1503; Sigma, # E3889; Merck, #1.08417.0100; Sigma, # A7906) and rotated up and down 10 times. The homogenated brains were then incubated for 30min on ice and then centrifuged for 10min at 450g and 4°C. The supernatants were transferred into new tubes and centrifuged at 21.000g and 4°C for 10min. The cytosolic fraction was now in the supernatant and the mitochondria in the pellet. The pellet was washed with 1ml isolation medium once and with 1ml PBS once (21.000g, 10min, 4°C), resuspended in lysis-buffer

(cell lytic (Sigma, # C2978) + 1x phosphatase and protease inhibitor) and incubated for 30min on ice. Afterwards the suspension was treated with ultrasound 10 times and centrifuged at 18.800g and 4°C for 15min. The supernatants were frozen at -80°C and the protein concentration determined with the BCA protein assay kit. To determine the purity of the different fractions (nuclear, cytosolic and mitochondrial) we did Western Blot analysis.

2.4.5 **Reverse transcription quantitative PCR (RT-qPCR)**

cDNA was diluted 1:25 with RNase-free water and then 4.5µl of the solution were pipetted in each well of a 96-well-PCR-plate. The primer was diluted 1:11 with the mastermix and 5.5µl of this mixture added to the wells. As housekeeping gene sdha was used. PCR was done with the Light Cycler96 from Roche. The following primers were used: sdha, Fpr1, TLR9, IL-6, IL-1ß, CCL2, CXCL1, CXCL2, MMP2, MMP3, MMP9. The following primers were used:

Table 1: *Primers used for RT-qPCR and their purchase order numbers.*

Sdha	applied biosystems	Mm01352366_m1
Fpr1	applied biosystems	Mm00442803_s1
TLR9	applied biosystems	Mm00446193_m1
IL-6	applied biosystems	Mm00446190_m1
IL-1β	applied biosystems	Mm00434228_m1
CCL2	applied biosystems	Mm00441242_m1
CXCL1	applied biosystems	Mm00433859_m1
CXCL2	applied biosystems	Mm00436450_m1
MMP2	applied biosystems	Mm00439498_m1
MMP3	applied biosystems	Mm00440295_m1
MMP9	applied biosystems	Mm00442991_m1

2.5 **Western Blot**

For Western Blot analysis, the frozen protein lysates were slowly defrosted on ice. Lysates were then mixed 4:1 with LDS and digested for 10min at 72°C. The 4-12% gel was put in the chamber which was then filled up with running buffer. Then equal amounts of protein were loaded into the wells of the Bis-Tris-gel and run for 1hour at 60mA. Meanwhile the membrane was activated for 5min with methanol and washed twice with water. To transfer the protein from the gel to the membrane, blotting was performed for 30min at 2.5mA. Then the membrane was blocked for 1hour in 3% milkpowder in TBST. Staining with the primary antibody was done overnight in 3% blocking solution at 4°C. The membrane was washed

four times for 10min and incubated for 1hour with the secondary antibody at room temperature before it was washed again four times with TBST. For signal development, the Super Signal West Pico solution from Thermo Scientific was used. After the reagent was removed, the blots were developed in the darkroom.

2.5.1 Determination of cell death via LDH-assay

To determine cell death in cell cultures, the LDH-production in supernatants was measured. For this process we used the Cytotoxicity Detection Kit from Roche.

2.6 Immunocytochemistry

Immunological stainings were produced to determine the purity of the cell cultures and also the expression of different proteins. Therefore we put coverslips into the wells of the cell cultures. After a certain time (seven days for neurons and 18-22days for astrocytes and microglia), cells were removed carefully and washed with pre-warmed PBS. Next, the cells were fixed with 300µl 4% PFA for 12min and then permeabilized with 300µl 0.2% Triton for 4min. For blocking we used 3% BSA/PBS + 2%NGS for at least 30min. The antibodies were prepared in the required dilutions with blocking solution and one drop was put onto parafilm in a wet-chamber. Next, coverslips were placed onto the drop upside down and incubated at 4°C overnight. On the next day, the coverslips were placed back into the 24-well-plate and washed three times with PBS for 5min each before they were incubated with the secondary antibody for 3-5hours. Next, they were washed twice with PBS and stained with DAPI (1:1000) for 5min. After one more washing step with PBS the coverslips were fixed on a microscope slide with fluoromount (Biozol Diagnostica).

2.7 *In-vivo* stroke model (MCAO)

Fpr1$^{-/-}$ mice (Gao et al., 1999) were only mated with *Fpr1*$^{-/-}$ mice and *Fpr1*$^{+/+}$ with *Fpr1*$^{+/+}$ respectively, so that only homozygous mice could be used for experiments. All mice (*Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$, male, 20g – 25g, 12 weeks old) were randomized and all scientists blinded before performing surgery. The temporary occlusion of the middle cerebral artery (tMCAO) was done as described in Gelderblom et al., 2009. The artery occlusion for one hour was done with a nylon filament (6-0). The mice's weight was between 20 and 25g and they were 12 weeks old. Anesthesia was done with isofluran 1-2% vol/vol in oxygen and analgesia achieved with buprenorphine (0.03mg/kg of body weight intraperitoneally every

12hours for 24hours). Mice were scored for neurological deficits directly after stroke (1hour) and on each following day until killing on a scale from 0 to 5 (0, no deficit; 1, preferential turning, 2, circling; 3, longitudinal rolling; 4, no movement; 5, death). In addition, an extended score was determined for posture, grip and climbing (1. Forelimb symmetry and posture 0-3, 2. Gripping test of the forepaws 0-3, 3. Climbing 0-3). 0 means normal and 3 means that the mouse cannot perform the task. To compare mice with each other, all three scores were added. In addition, the corner test was performed on day 1 and 3 after ischemia-reperfusion. Mice were sacrificed either after 24hours or 3days after reperfusion with the use of isoflurane. Only mice with a neurological score more than or equal to 1 after reawakening were included for stroke size analysis, and only animals with a visible cortical infarct were included for FACS analysis of infiltrating cells. For analysis of infarct sizes, brains were harvested, cut into 1mm slices (Braintree Scientific, 1mm) and vital staining using 2% (w/v) 2,3,5-triphenyl-2-hydroxy-tetrazolium chloride (TTC, Sigma) in phosphate buffer was performed. Infarct volumes were determined by blinded examiners using NIH ImageJ and statistics (T-test, Graph Pad Prism).

2.8 **Statistics**

All data is shown as mean ± SD or SEM. To determine whether two groups are statistically different, we used the unpaired student's t-test including the Mann-Whitney U test. For the analysis of two different variables at different time points and the interaction between, 2-way ANOVA with Bonferroni posthoc test was used. 1-way ANOVA with Bonferroni posthoc test was used for the analysis of multiple groups and one time point. If the p-value was smaller than 0.05, results were considered as statistically significant. For better readability, some SD values are shown only one sided, but should be considered as symmetric.

3 Results

3.1 Expression of Fpr1 on cells of the CNS on RNA-level

So far, it is not exactly known on which cells Fpr1 is expressed. Several studies could show that Fpr1 is expressed on different cells in the CNS like dendritic cells, neutrophils (Prossnitz, 1997), macrophages (Gavins, 2010), monocytes (Prossnitz, 1997), microglia, and astrocytes (Slowik et al., 2012). In order to demonstrate the expression on cells of the CNS, we isolated RNA from different cell types of the CNS and immune cells to compare

A

Cell type	Ct (*Fpr1*)	ΔCt (β-actin – *Fpr1*)
Neutrophils	23,66	2,71
Macrophages	32,13	7,95
Microglia	32,45	10,36
Neurons	33,55	12,09
Astrocytes	30,69	12,37

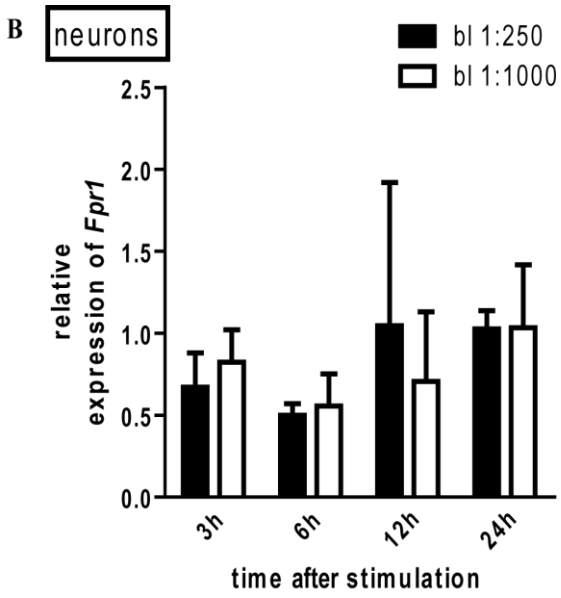

***Figure 5**: **A**, Ct and ΔCt values of different cell types after qPCR. **B**, relative expression of Fpr1 in stimulated wildtype neurons with brain lysate in different concentrations (1:250 and 1:1000) compared to negative control. Bars show expression after 3, 6, 12, and 24hours after stimulation, shown are mean values +SD. Analysis with 2-way ANOVA and Bonferroni post-hoc test.*

the different expression rates. Real time PCR was performed with primers for Fpr1 and the housekeeping gene β-actin. The measured Ct value (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Since we did not have a positive control that showed an expression rate of 100%, we were not able to determine absolute values and instead we used the ΔCt values as relative values to compare expression rates with each other (Fig. 5A). The lower the ΔCt value, the higher is the expression of Fpr1. Figure 5A demonstrates that neutrophils show the highest rate of Fpr1-expression of the analysed cell types followed by macrophages and microglia as immune cells of the brain. Neurons and astrocytes show a lower amount of amplified RNA. To

examine whether neurons might start expressing more Fpr1 after the ischemic incident, we placed them under stimulated conditions. After stimulation with isolated brain lysate in different concentrations, expression rates were normalized to a negative control. As shown in figure 5B, the distribution of expression rates does not show any correlation and differences between the rates are not significant.

3.2 Fpr1 as trigger for inflammation reactions in ischemic hemispheres

The following experiment was performed to reveal whether Fpr1 plays a role in triggering the inflammation reaction after stroke in our model. Therefore, we compared the production of chemokines and cytokines, which are involved in inflammation reactions, in *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ littermates. RNA from the ischemic hemisphere was taken 24hours after reperfusion to measure the gene expression of cytokines like IL-6, IL-1β, and TNFα and chemokines like CCL2, CXCL1, and CXCL2 in qPCR. The interaction between gene expression in *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ mice was analyzed with an unpaired t-test as shown in figure 6. In all of the six experiments there is a marginal trend towards a downregulation of chemokine and cytokine gene expression in *Fpr1*$^{-/-}$ mice, but none of the differences was statistically significant ($p \geq 0.05$).

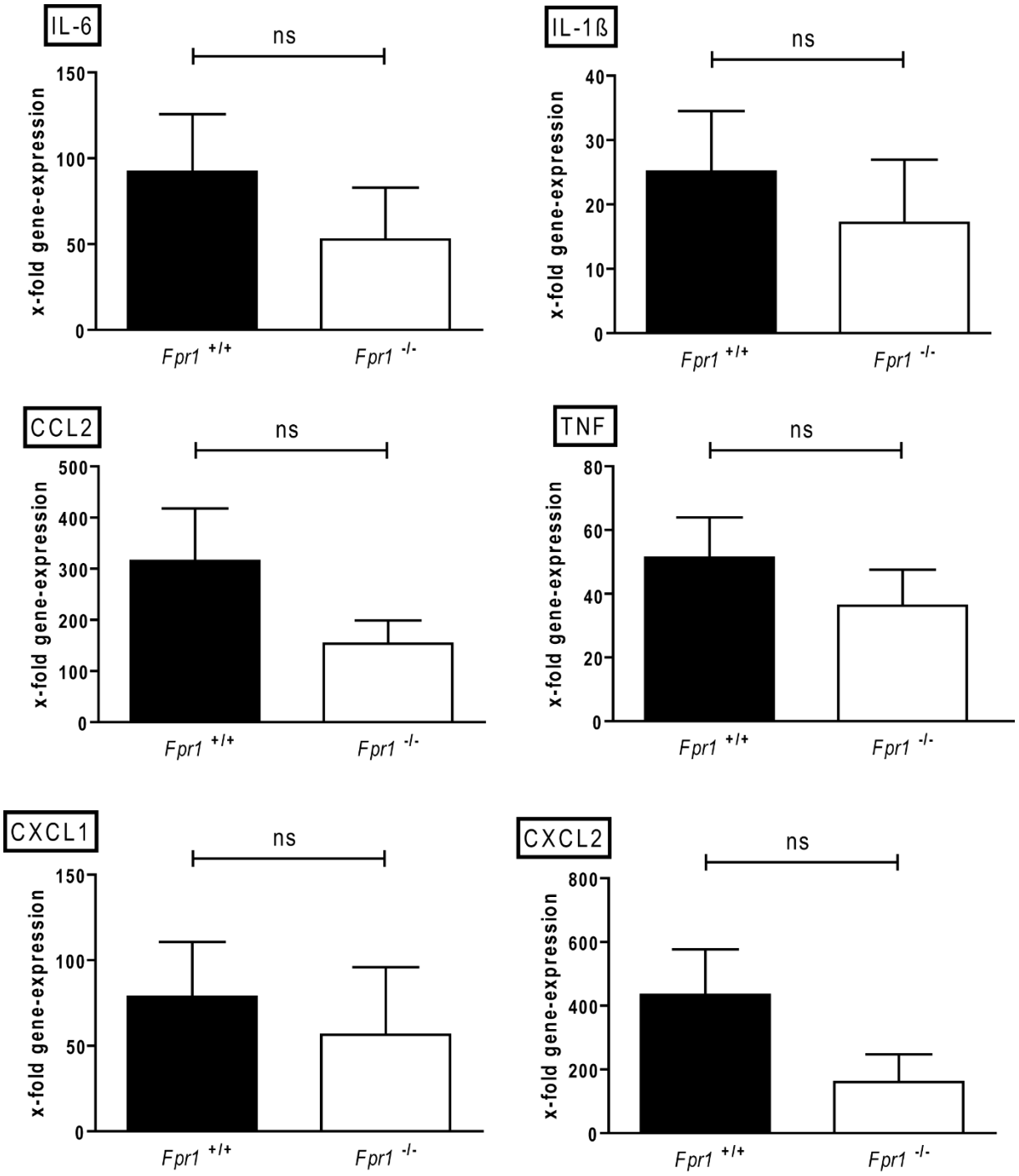

***Figure 6**: Gene expression of IL-6, IL-1β, CCL2, TNFα, CXCL1, and CXCL2 in the ischemic hemisphere 24 hours after reperfusion; shown is the mean value + SD (error bars can be considered symmetric); n=6 for each group (Fpr1$^{+/+}$ and Fpr1$^{-/-}$); analysis with unpaired t-test; $p \geq 0.05$.*

3.3 Stimuli as trigger of cell death on neurons of *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ mice

Zhang et al. (2010) showed that mitochondrial DAMPs released after tissue trauma caused inflammation reactions associated to Fpr1. Other previous studies showed that sterile inflammation after ischemia-reperfusion in the brain lead to long-term tissue damage and associated dysfunction (Dirnagl et al., 1999; Gelderblom et al., 2009; Yilmaz and Granger, 2010). Considering these facts, we aimed to investigate whether Fpr1 also plays a role in cell death of neuronal cells after ischemia and reperfusion.

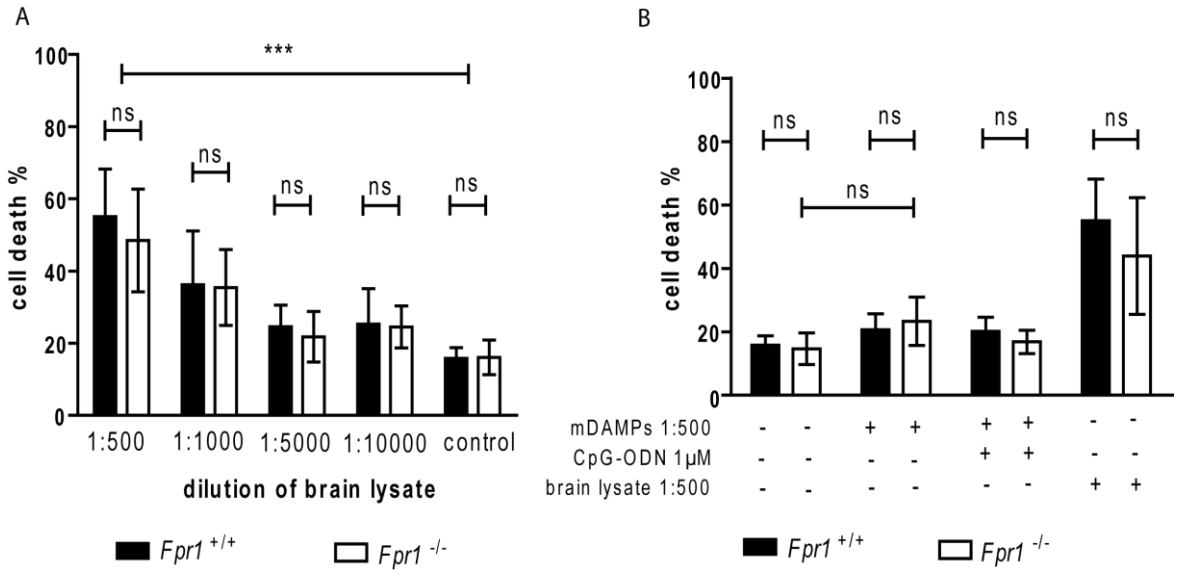

***Figure 7**: **A,** Neurons of Fpr1$^{+/+}$ and Fpr1$^{-/-}$ primary cell cultures were stimulated with brain lysate isolated from a stroke hemisphere in different dilutions; shown are mean values ±SD; n=7; analysis with 2-way ANOVA and Bonferroni post-hoc test; **B**, Neurons of Fpr1$^{+/+}$ and Fpr1$^{-/-}$ primary cell cultures were stimulated with mitochondrial DAMPs, CpG-ODN and mitochondrial DAMPs, brain lysate as positive control and none of those as negative control; shown are mean values ±SD; n=7; analysis with 2-way ANOVA and Bonferroni post-hoc test.*

We isolated protein from a stroke hemisphere (brain lysate) and stimulated neurons in the primary cell cultures with different concentrations of it. Then, cell death was measured after 24hours with LDH-assay. As shown in Figure 7A, with higher concentration of brain lysate, more cell death in neurons occurs, especially if compared to the negative control. However, there is no significant difference between *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ littermates. In order to gain a better understanding of the components of the brain lysate leading to cell death, we performed further stimulation experiments. The cell cultures were stimulated with isolated mitochondrial DAMPs from mouse brain, which are supposed to bind to Fpr1. In addition, CpG-ODN, which binds to TLR9 and activates the following signalling cascade was added.

There is no significant difference in cell death between the stimulation with mDAMPs, mDAMPs together with CpG-ODN and the negative control (Figure 7B). Stimulation of neuronal cells of *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ mice also did not show any significant differences in cell death.

3.4 FACS-analysis of infiltrating immune cells 24h post-perfusion

FACS-experiments were performed with stroke hemispheres of *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ mice as described in section 2.2 in order to compare the absolute and relative amount of different infiltrating immune cells in the littermates. We designed two different types of sets with antibody cocktails to differentiate leucocyte subpopulations. Leucocytes were separated from cell debris by the expression of CD45. Then, B-cells (B220^{+}) were separated from T-cells (CD3^{+}) and CD3^{+} cells were then separated into NK, NK T-cells, γδ-Tcells, CD4^{+}, CD8^{+} and double negative T-cells (Fig. 8). In the second subset we also selected CD45^{+} cells out of all cells and then separated microglia (CD45low, CD11b^{+}) from the other immune

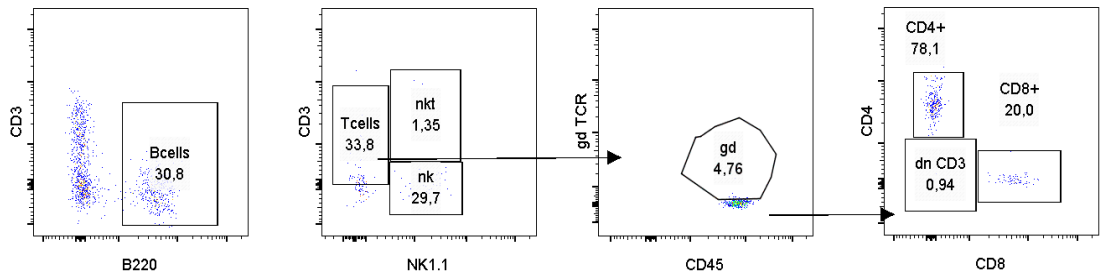

***Figure 8**: Gating strategy of CD45^{+} leucocytes into the subpopulations: B220^{+} B-cells, CD3^{+} Tcells, NK- and NKT-cells, CD45^{+} γδT-cells, CD4^{+} and CD8^{+} T-cells, and double negative T-cells.*

cells, which were then divided into neutrophils (Ly6g^{+}, CD11b^{+}) and lymphocytes (Ly6g^{-}, CD11b^{-}). Further division resulted in dendritic cells and monocytes. Monocytes could also be separated in inflammatory (Ly6c^{+}) and non-inflammatory (Ly6c^{-}) cells (Fig. 9). Figure 10 represents the distribution of infiltrating immune cells in the ischemic hemisphere 24hours after reperfusion. In figure 10A all lymphocytes, B-cells, as well as T-cells and its subpopulations are shown as fractions of all lymphocytes infiltrating into the stroke brain.

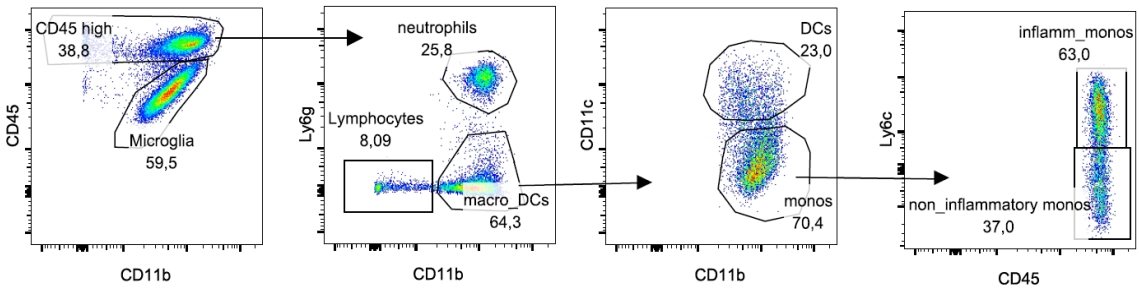

***Figure 9**: Gating strategy of CD45$^+$leucocytes and their division in other immune cells: neutrophils (Ly6g$^+$), lymphocytes (Ly6g$^-$, CD11b$^-$), dendritic cells (CD11c$^+$, CD11b$^+$), and monocytes (CD11b$^+$) with the division into inflammatory (Ly6c$^+$, CD45$^+$) and non-inflammatory monocytes (Ly6c$^-$, CD45$^+$).*

There are no significant differences between the *Fpr1*$^{+/+}$ mice and their littermates in the amount of infiltrating lymphocytes. Figure 10B compares the relative amount of all immune cells located in the hemisphere 24 hours after stroke. Obviously, the biggest amount of immune cells are microglia with more than 60% since they are the only resident immune cells in the brain, followed by monocytes with 12% in *Fpr1*$^{+/+}$ and 8.5% in *Fpr1*$^{-/-}$ mice, respectively. Neutrophils, lymphocytes and dendritic cells appear with less than 10% in both mice. Again, the differences in *Fpr1*$^{+/+}$ and *Fpr1*$^{-/-}$ mice are not significant. To detect the relation between microglia and the other immune cells, we referred the amount of immune cells to the amount of microglia, which is shown in figure 10C. It demonstrates again the big difference in cell counts between microglia on one hand and monocytes, neutrophils, lymphocytes and dendritic cells on the other hand. The comparison of absolute cell numbers (figure 10D) showed that most lymphocyte subpopulations are infiltrating to a higher amount in *Fpr1*$^{-/-}$ mice, especially in B-cells and CD4$^+$ T-cells. Figure 10E also demonstrates absolute cell numbers of the other immune cells and microglia represent the highest population with about 176 000 cells per hemisphere compared to only 32 000 neutrophils and 34 000 monocytes in *Fpr1*$^{-/-}$ mice. Interestingly, cell amounts are again higher in *Fpr1*$^{-/-}$ mice compared to their littermates in all immune cell populations, although the differences are not significant. In summary, these results suggest that relative cell amounts are approximately the same in both littermates and differences are not significant.

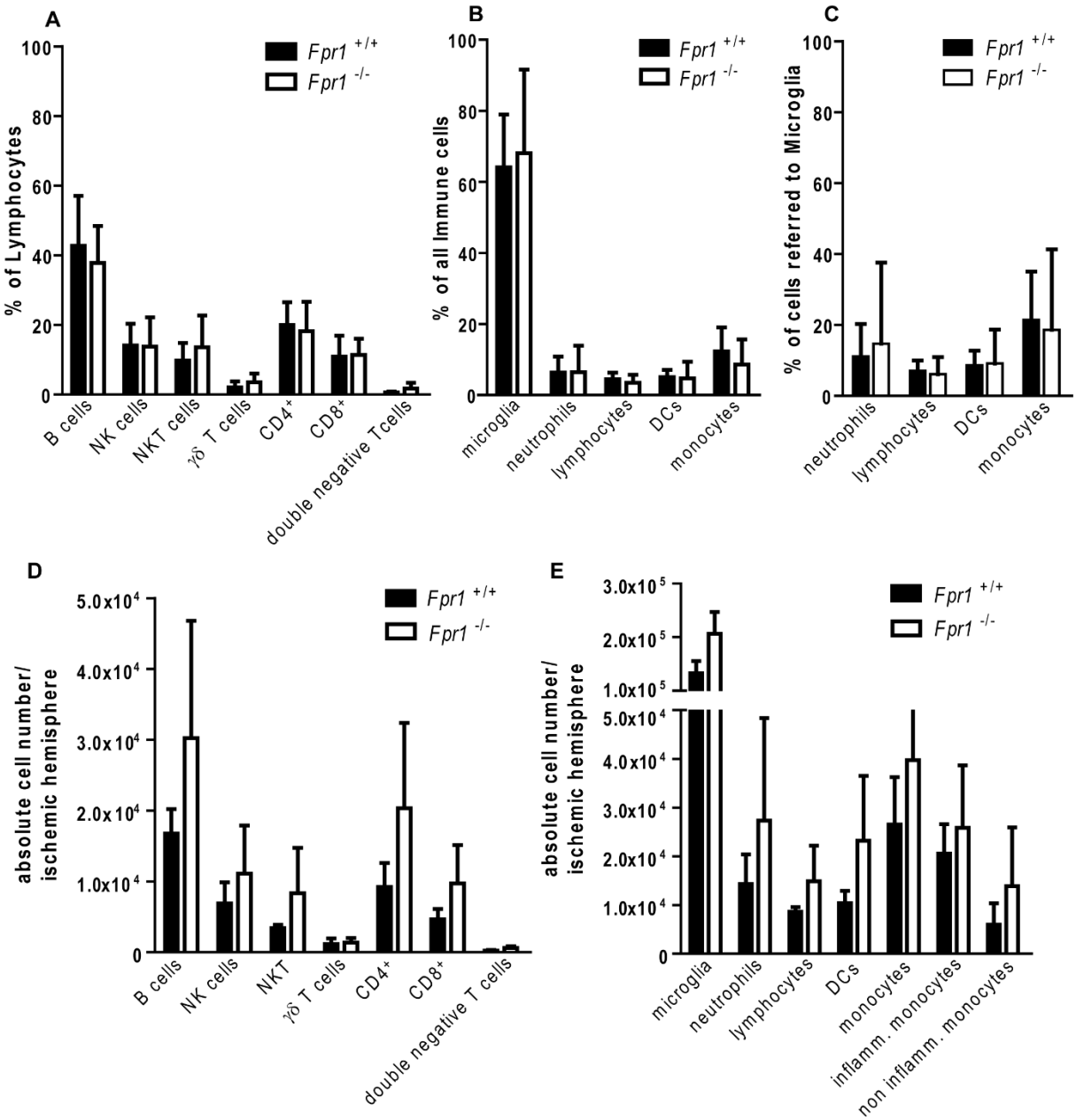

***Figure 10**: FACS-analysis of infiltrating immune cells into the stroke hemisphere 24h after reperfusion; **A**, FACS-analysis of B- and T-cells and their percentage of all Lymphocytes; shown are mean values +SD; n=4; analysis with 2-way ANOVA and Bonferroni post-hoc test; **B,** infiltration of all CD45-positive leucocytes and their distribution into the different cell types; shown are mean values +SD; n=4; analysis with 2-way ANOVA and Bonferroni post-hoc test; **C,** CD45-positive immune cells referred to the amount of microglia in percent; shown are mean values +SD; n=4; analysis with 2-way ANOVA and Bonferroni post-hoc test **D**, absolute cell numbers of different lymphocyte-subpopulations per ischemic hemisphere; **E**, absolute cell numbers of all immune cells per ischemic hemisphere.*

3.5 Immunological stainings

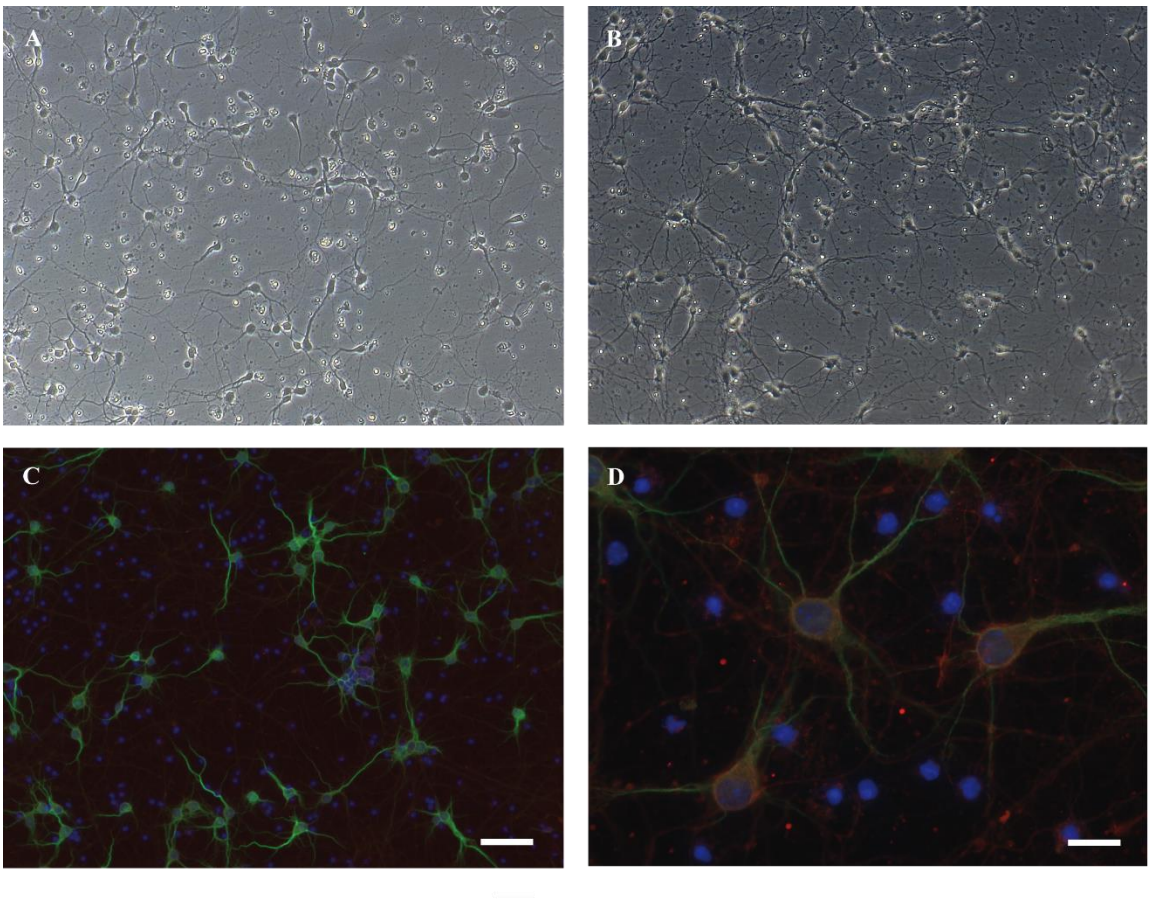

***Figure 11**: Primary neuronal cell cultures and immunocytochemical staining. **A** cortical mice neurons after 5days, **B** cortical mice neurons after 5days plus 24hours of stimulation, **C** and **D**: immunocytochemical staining of MAP2 (green) and Fpr1 (red) in 7days old cortical mice neurons, cell nucleus stained with DAPI (blue) (scale-bar = 50µm, scale-bar = 20µm).*

3.5.1 Immunocytochemistry of *Fpr1*$^{+/+}$ neuronal cells

Figure 11A shows the cortical mice neurons that were used after 7 days of incubation at 37°C for the stimulation experiments. After stimulation, we observed more cell death and neurons in a bad condition (Figure 11B). In order to analyse the expression of Fpr1, we additionally performed immunocytochemical stainings after 7 days (figure 11C and D). MAP2 was used as a neuronal marker and DAPI for the cell nucleus. It seems that Fpr1 is expressed in neuronal cell nuclei (Figure 11D). However, there is also unspecific staining in between the cells.

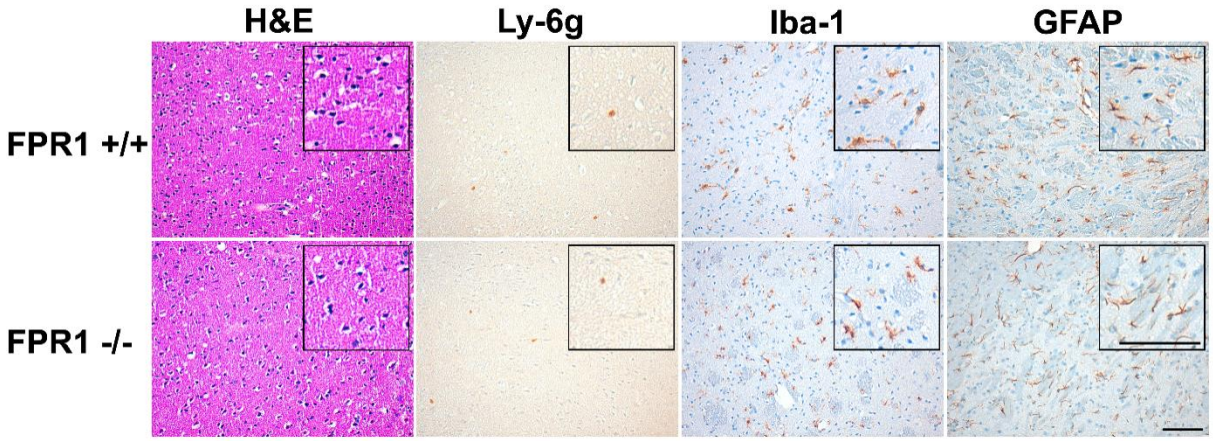

***Figure 12**: Immunohistochemical staining of Fpr1$^{+/+}$ and Fpr1$^{-/-}$ mice brains' tissue with different antibodies (Ly-6, Iba-1, GFAP). (scale-bar = 50µm)*

3.5.2 Immunohistological staining of brains from *Fpr1* littermates

Brains of *Fpr1*$^{+/+}$ mice and their littermates were used after MCAO for immunohistological stainings. Antibodies for different cell types like neutrophils (Ly-6g), microglia (Iba-1) and astrocytes (GFAP) were used to compare the appearance and distribution of the immune cells in *Fpr1*$^{+/+}$ with *Fpr1*$^{-/-}$ after stroke (Fig. 12). In summary, no relevant differences in the amount of stained cells can be seen in the different types of brain tissue.

3.6 Impact of Fpr1 on infarct sizes in MCAO

3.6.1 No reduction of infarct volumes 24h after reperfusion in *Fpr1*$^{-/-}$ mice

The main focus was laid on *in vivo* experiments and we raised the question if mice lacking Fpr1 are protected in stroke. To evaluate if the existence of the receptor leads to bigger infarct volumes compared to mice without Fpr1, we used the MCAO model. All mice used for the *in vivo* stroke model were put under the same conditions and underwent occlusion for 60 minutes. After 24 hours we used different scores (extended neurological score, Corner test, neurological score) to test the neurological outcome of the mice. For the analysis of infarct sizes we performed TTC-stainings of the brain sections, one representative example is shown in Figure 13E. Cerebral blood flow was measured before, during, and after reperfusion (Fig. 13F). Figure 13A reveals that there has been a slight increase of the infarct volume in mice missing the Fpr1 receptor. Although infarct volumes in *Fpr1*$^{+/+}$ mice with 60.3 mm^3 in the mean were quite higher than in *Fpr1*$^{-/-}$ mice with only 49 mm^3, the

differences are not statistically significant and also vary a lot from very small to rather big. Consistent with these results, the neurological outcome of the *Fpr1*$^{-/-}$ mice (Figure 13B-D) is not significantly better than of the littermates expressing Fpr1. The Corner test which was initially described by Zhang et al. (Zhang et al., 2002), is a sensorimotor functional test, which is increasingly used in stroke models. Naïve mice without any neurological deficits do not have a preferred side, but mice with cortical damage after MCAO preferentially turn contralateral which in our set-up means to the right. In our experiment, *Fpr1*$^{+/+}$ mice prefer the right side in 92% vs. 90% in *Fpr1*$^{-/-}$ mice. The higher the score in the extended neurological score, the worse is the neurological outcome. Here *Fpr1*$^{-/-}$ mice show a marginal better outcome compared to the littermates (extended score: 1.8 vs. 2.5 and neurological score: 1.5 vs. 1.75) shown in Figure 13C and D. Overall, these results indicate that there is a trend that mice lacking the Fpr1 receptor might slightly be protected in ischemic stroke, but all results are not statistically significant.

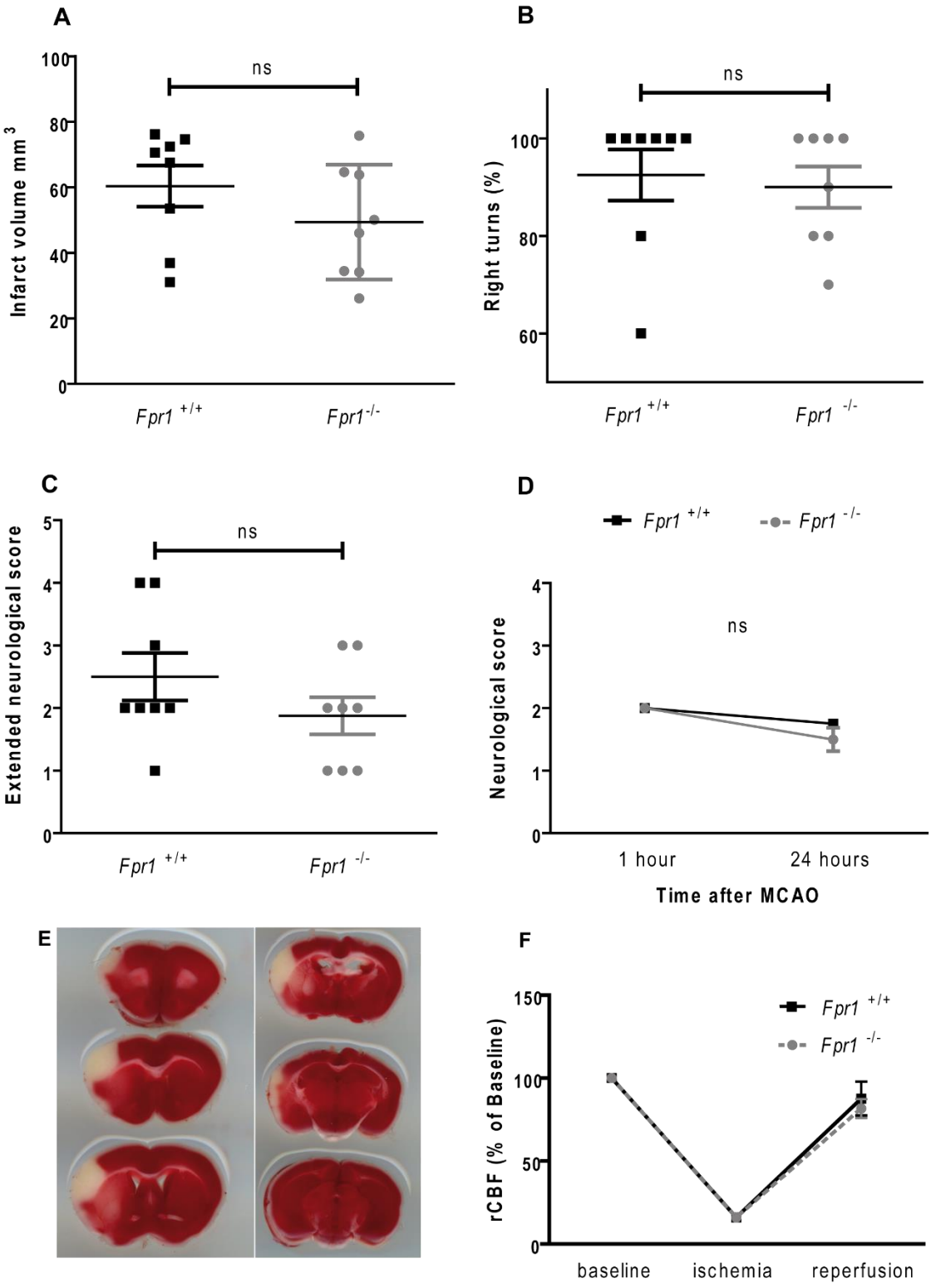

***Figure 13**: Correlation of Fpr1 with infarct sizes in the MCAO model 24 hours after reperfusion. **A** infarct volumes of Fpr1$^{+/+}$ mice in comparison to Fpr1$^{-/-}$ mice (n=8), analysis with t-test. **B** right turns in Corner test for sensorimotor function (n=8). **C** Extended neurological score for more complex motoric function testing (n=8). **D** Neurological score 1 hour and 24 hours after reperfusion (n=8). **E** Representative TTC-stained coronal 1 mm thick brain section of the bregma area 24 hours after MCAO. **F** transtemporal laser Doppler as control for the cerebral blood flow before, during and after ischemia.*

3.6.2 **No reduction of infarct volumes 3d after reperfusion in *Fpr1*$^{-/-}$ mice**

Another set of *in vivo* experiments was performed three days after reperfusion. The setup of the experiments was the same as in 3.6.1, except that infarct volumes were measured three days after ischemia and reperfusion, Corner test was performed on day one and day three, and berlin scores and neurological scores were determined on all days. Figure 14A shows infarct volumes of *Fpr1*$^{+/+}$and *Fpr1*$^{-/-}$ mice after three days. The size of the infarct area in mice with the Fpr1 receptor averages at 53.6mm^3, whereas mice lacking the Fpr1 receptor show a mean volume of 48.6mm^3. Obviously, there is again a difference in size, but in this experiment to a lesser extend than in 3.6.1. Corner tests showed a wide range (Figure 14B) and the results are not statistically significant. The extended neurological score and the Bederson neurological score were again used to evaluate the neurological and motoric outcome after stroke (Figure 14C and D). Motoric functions are almost the same in all mice directly after one day and there are also no significant differences in the following days (Figure 14C). The neurological outcome is the same in all mice after 1 hour of reperfusion and becoming better on the following days in the same extend in *Fpr1*$^{+/+}$ and littermates. For a better evaluation of the results, a Kaplan-Meyer-mortality-graph (Fig. 14F) was created, which shows that two mice of the *Fpr1*$^{+/+}$population died in the period of the experiment compared to no dead mice in the *Fpr1*$^{-/-}$ group. This observation might indicate that mice expressing the Fpr1 receptor are somehow more susceptible to ischemia than mice lacking the Fpr1 receptor. In summary, these results do not change the view on the role of Fpr1 in ischemia compared to the same experiment after 1 day and suggest that Fpr1 might not play a crucial role in sterile inflammation after ischemic stroke.

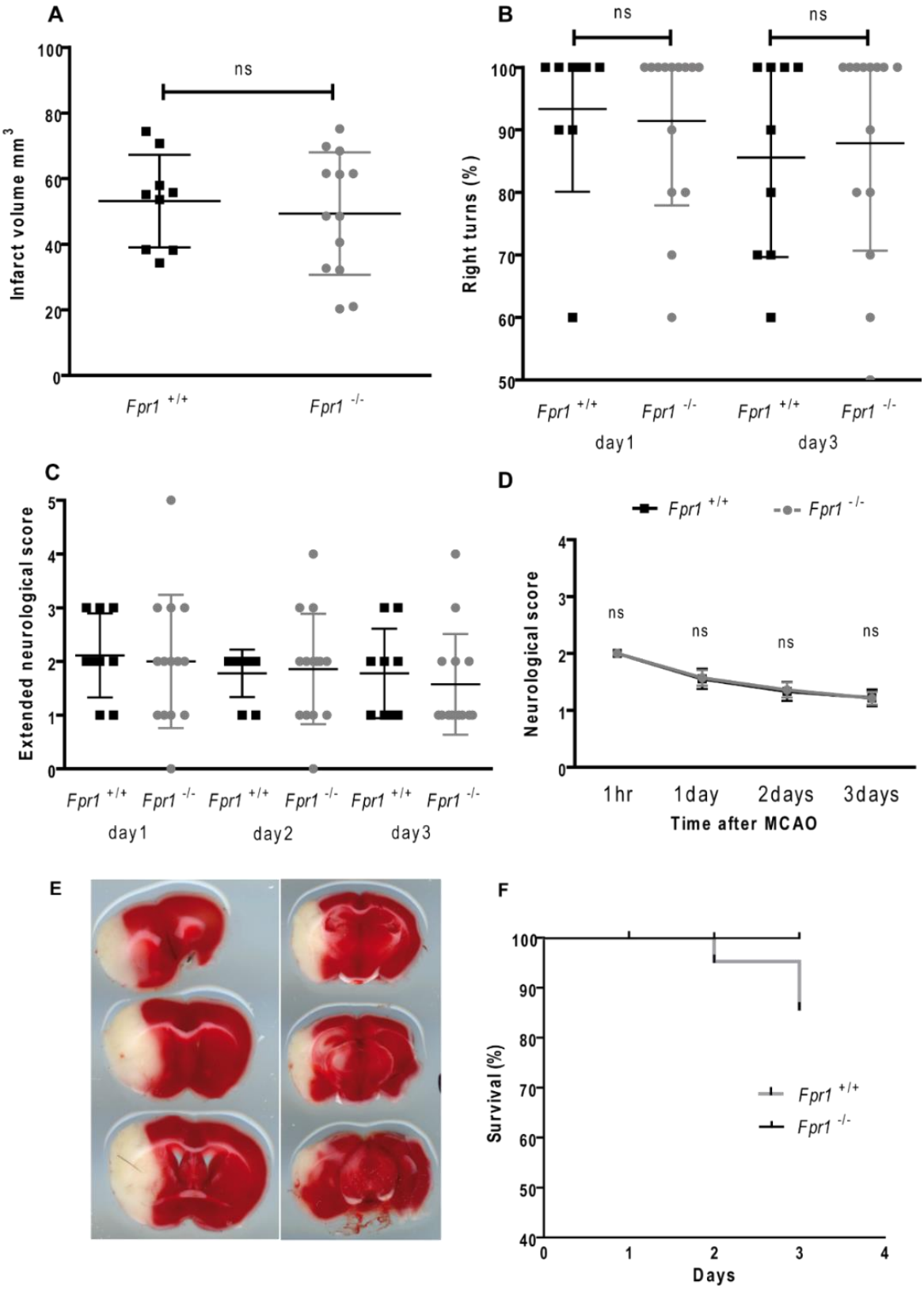

***Figure 14**: Correlation of Fpr1 with infarct sizes in the MCAO model 3 days after reperfusion. **A** infarct volumes of Fpr1$^{+/+}$ mice in comparison to Fpr1$^{-/-}$ mice (n=9/13). **B** right turns in Corner test for sensorimotor function (n=9/13). **C** Extended neurological score for more complex motoric function testing (n=9/13). **D** Neurological score 1 hour, 1 day, 2 days and 3 days after reperfusion (n=8). **E** Representative TTC-stained coronal 1 mm thick brain section of the bregma area 3 days after MCAO. **F** Kaplan-Meyer-mortality-graph for the survival rate of mice, χ^2=1.904.*

4 Discussion

The inflammation process after cerebral ischemia has been of high interest in the last couple of years. Even though it is well accepted, that the inflammatory response leads to neuronal injury and tissue damage, the understanding of the exact mechanisms is still limited. Fpr1 is a receptor mainly presented on neutrophils and involved in systemic inflammatory reactions. The aim of this study was to examine whether Fpr1 is also involved as a trigger of sterile inflammation after stroke. We found that the outcome of animals deficient in Fpr1 after stroke is equivalent to littermate controls.

4.1 Inflammatory mechanisms after cerebral ischemia

Although there are different mechanisms involved in the pathogenesis of stroke, it is well accepted that inflammation is one of the main mediators in post-ischemic brain injury (Dirnagl et al., 1999; Wang et al., 2007). Inflammation is considered a key contributor to the pathophysiology of stroke and the innate, as well as the adaptive immune system, play an important role on the impact of tissue damage and the outcome of patients after stroke (Iadecola and Anrather, 2011). Ischemia in the brain leads to cell death and tissue damage, as well as the breakdown of the blood-brain-barrier. The infiltration of immune cells to the necrotic area and the production of inflammatory mediators, which leads to the recruitment of more immune cells, promote inflammation. The first cells activated after injury in the CNS are microglia and astrocytes (Kim et al., 2016; Yilmaz and Granger, 2010). Following the resident cells of the CNS, circulating leukocytes also migrate into the ischemic brain tissue by binding to adhesion molecules on activated endothelial cells. After ischemic brain injury, a number of cytokines, chemokines and damage-associated-molecular patterns are released and promote further neutrophil activation and recruitment (Kolaczkowska and Kubes, 2013). Those and further findings confirm that neutrophil infiltration initiated by chemokines is important in post-ischemic brain damage. To get a more specific insight into the different immune cells infiltrating into the ischemic hemisphere in mice lacking Fpr1, we analysed the differences in infiltration in *Fpr1*$^{+/+}$ mice compared to *Fpr1*$^{-/-}$ mice with FACS-analysis. In accordance to previous results, we found that on day one, the amount of microglial cells is the highest of immune cells followed by infiltrated monocytes and neutrophils, which for their part have its peak on day 3 (Gelderblom et al., 2009; Jin et al., 2009; Yilmaz and Granger, 2008). The increase of microglia and macrophages in the border zone of the ischemic area has previously been reported (Schroeter et al., 1999), as well as

the important role of $CD4^+$ and $CD8^+$ T-cells in experimental stroke in mice (Yilmaz et al., 2006). However, differences in mice expressing Fpr1 and mice lacking Fpr1 were not existent in the relative distribution and the absolute cell number distribution of leucocytes. In our analysis we were not able to show any significant differences in the amount of infiltrating lymphocytes and their subpopulations as well as other immune cells like microglia and macrophages between *Fpr1*$^{+/+}$ mice and *Fpr1*$^{-/-}$ mice. These results are somehow surprising with other studies in mind that suggest that Fpr1 mediates neutrophil recruitment to the site of sterile inflammation (McDonald et al., 2010; Zhang et al., 2010). However, the previous studies were performed in other organ systems than in the brain. It is possible that inflammatory mechanisms after I/R in the brain and other organs differ. Since the distribution of infiltrating cells is in accordance to previous results and the lack of Fpr1 does not provide significantly different results, our results indicate that neutrophil recruitment into the brain is independent of Fpr1 in the setting of stroke. Instead, other mechanisms might be more relevant. Especially CXCL1 and CXCL2 as chemokine ligands are increased after stroke and act as strong neutrophil chemoattractants. Further studies showed that TNFα and IL-1β are increased in experimental stroke and contribute to neutrophil activation (Lambertsen et al., 2012). Recruitment of inflammatory neutrophils contributes to disruption of the blood brain barrier, increased infarct size, hemorrhagic transformation, and worse neurological outcome. Thus, neutrophils are of great interest as treatment targets in ischemic stroke. The recruitment of neutrophils in the ischemic brain occurs between 30 minutes and a few hours and several studies show that blocking of the neutrophil infiltration results in a reduction of infarct sizes and better neurological outcome (Yilmaz and Granger, 2008). Still, the exact mechanisms of the pathogenesis remain under debate. The most important mechanisms that amplify the cerebral inflammatory response contain the production of ROS, the release of pro-inflammatory cytokines, chemokines and MMPs. Interestingly, the infiltration of other inflammatory cells like macrophages, lymphocytes and DCs in the ischemic hemisphere are supposed to precede the neutrophilic influx (Gelderblom et al., 2009). Moreover, several subtypes of lymphocytes have been implicated in the pathogenesis of ischemic stroke (Liesz et al., 2009; Yilmaz et al., 2006). Several studies suggest the infiltration of inflammatory cells as important target in cerebral I/R and therefore it is essential to gain a better understanding of the mechanisms in order to find new therapeutic targets. Our aim was to figure out whether Fpr1, which is involved in sterile inflammation processes after trauma (Zhang et al., 2010) or in sterile liver injury

(McDonald et al., 2010) is also involved as a mediator of sterile inflammation after cerebral ischemia.

4.2 Role of Fpr1 in sterile inflammation

Fpr1 is a G-protein coupled receptor, which is generally activated after infection and plays an antimicrobial role in host defence (Gao et al., 1999). Its ligands are of bacterial origin from invading pathogens. Fpr1 is not only involved in infection, but also activated after tissue trauma as a result of formylated peptides released by dying cells binding to the receptor. This implicates that bacterial-derived and host-derived formylated peptides can lead to inflammation through the binding to Fpr1. Although Fpr1 is mainly expressed on neutrophils and monocytes, it has also been detected on cells of the CNS (Becker et al., 1998; Sozzani et al., 1995; Wang et al., 2016) and might therefore be involved in sterile inflammation processes in the brain. In our studies, neutrophils showed the highest expression of Fpr1 on RNA-level, followed by microglia and macrophages as immune cells of the brain. To a lesser extent, also neurons and astrocytes as resident cells of the CNS express Fpr1. Immunocytochemistry in neuronal stem cells showed the expression of Fpr1 and Fpr2 (Wang et al., 2016) and mediation of the migration of NSCs to lesion sites and differentiation into neurons through Fpr1. In our studies, immunocytochemical stainings of neurons only showed unspecific binding of the Fpr1-antibody and we did not succeed to demonstrate the expression of Fpr1 on neurons. The use of different antibodies might be an explanation for the differing results. Several studies already showed that Fpr1 plays an important role in the activation of neutrophils, followed by systemic inflammation under sterile conditions. The induction of neutrophil chemotaxis through the binding of formylated mitochondrial peptides was first described by Carp (Carp, 1982). Zhang demonstrated that mitochondrial DAMPs, released after aseptic injury or trauma, induce immune responses in the mouse model (Zhang et al., 2010). *In vitro* experiments showed the migration of neutrophils towards mitochondrial DAMPs, important ligands of Fpr1. In addition, with potent inhibitors like Cyclosporin H (CsH) and Anti-Fpr1, neutrophil chemotaxis was inhibited. Besides, injection of liver-derived mitochondrial DAMPs into mouse peritoneum showed neutrophil infiltration and in consequence neutrophilic peritonitis. These findings suggest that Fpr1 is activated through DAMPs and leads to chemotaxis in neutrophils. These results further confirm that formylated mitochondrial peptides released in tissue damage are potent immune activators through binding to Fpr1 (Hauser et al., 2010; Raoof et al., 2010; Zhang et al., 2010). Additional promising observations were made using a mouse model of

liver inflammation with heat-induced sterile liver injury (McDonald et al., 2010). McDonald showed that neutrophils were guided to the site of sterile inflammation by formyl-peptide signals released by necrotic cells. Further observations in *Fpr1*$^{-/-}$ mice, where neutrophils showed non-directional migration and failed to enter the necrotic zone, confirmed the neutrophil migration in a Fpr1-dependant manner in liver injury (McDonald et al., 2010). In a different mouse model of paracetamol-induced liver injury, increased levels of circulating mtDNA were detected and the blockade of Fpr1 and the chemokine receptor 2 (CXCR2) attenuated local hepatotoxicity and neutrophil migration into the lung (Marques et al., 2012). Recent studies in a mouse model of lung injury demonstrated that mitochondrial formylated peptides are elevated in ARDS (acute respiratory distress syndrome) in accordance with neutrophil influx into the lung leading to sterile lung injury (Dorward et al., 2017). Furthermore, *Fpr1*$^{-/-}$ mice showed a reduction in neutrophil numbers compared to wildtype animals. Those studies revealed the influence of Fpr1 in neutrophil recruitment in sterile inflammation reactions in various organ systems. However, in our studies, deficiency of Fpr1 was not associated with a reduction in inflammatory brain injury. To assess if the existence of Fpr1 has an effect on the rehabilitation of the mice, we examined the neurological outcome and infarct sizes after MCAO in mice expressing Fpr1 and mice lacking Fpr1. Neither after 24 hours, nor after 3 days, infarct sizes were reduced in *Fpr1*$^{-/-}$ mice. There is also no significant difference in the neurological outcome of k.o. mice compared to wildtype littermates, suggesting that the Fpr1 signalling pathway is not directly related to ischemic brain injury induced by I/R. The MCAO is a well established model in our lab and stroke experiments were performed under controlled conditions. The relatively high value of the standard deviation of stroke volumes might be due to a small study population and an increase in animal numbers would have helped to gain more precise results. Considering the importance of the outcome of *in vivo* experiments, these results support the idea that Fpr1 does not play a crucial role in inflammatory processes after ischemic stroke and other mechanisms are more relevant in the infiltration of inflammatory cells.

4.3 Role of Fpr1 in our model

In this study, we were not able to show that Fpr1 plays a relevant role in inflammatory processes after ischemia-reperfusion in the brain. However, several studies suggest that Fpr1, in combination with other mechanisms, promotes neutrophil recruitment (Jickling et al., 2015; Kilic et al., 2008; McDonald et al., 2010). One might have expected that Fpr1 is crucial for triggering inflammation processes in the brain like in other systems such as lung

or liver injury. Yet, there are no previous studies on the role of Fpr1 in ischemic brain injury in the mouse model. In the context of cerebral ischemia, the infiltration of inflammatory cells is associated with the activation of resident cells and pro-inflammatory cytokines and chemokines (Dirnagl et al., 1999). In the present study, we examined the expression of the cytokines and chemokines involved in the pro-inflammatory response in the context of Fpr1. We showed that there is no significant difference in gene expression rates of the relevant cytokine and chemokine mediators (IL-1β, IL-6, TNFα, CXCL1, CXCL2, CCL2) in *Fpr1* knockout and wildtype mice. If the increase of the major cytokines involved in neutrophil migration like IL-1β and TNFα is missing, further neutrophil recruitment is also unlikely. One reason might be that in the case of brain ischemia, other chemokine receptors than Fpr1 are associated with neutrophil recruitment. Our studies suppose that Fpr1 is not directly involved in the neutrophil infiltration after stroke. Instead, other chemokine receptors and the corresponding chemokines are important mediators of neutrophil migration to the lesion site and act therefore as therapeutic targets. The CXC chemokines CXCL1 and CXCL2 and their receptors CXCR1 and CXCR2 are increased after stroke and contribute to neutrophil recruitment to ischemic tissue as well as to the release from bone marrow (Jickling et al., 2015). Yet, inhibition of the receptors has shown different outcomes. Evasin-3 leads to impaired neutrophil activation, but has no effect on stroke outcomes (Copin et al., 2013). On the other hand, inhibition of CXCR1 and CXCR2 with Reparixin showed improved neurological outcomes and a reduction of brain injury in a rat stroke model (Brait et al., 2011; Villa et al., 2007). Another important chemokine in cerebral ischemia is CCL2, which acts on neutrophil and monocyte recruitment. CCL2 knockout mice show decreased infarct sizes (Dimitrijevic et al., 2007). However, it still remains unclear whether the effect is due to neutrophil or monocyte migration, or both. Further mediators of neutrophil migration in experimental stroke are TNFα and IL-1β (Lambertsen et al., 2012). The role of tumor necrosis factor is not fully understood. On the one hand, the administration of neutralizing antibodies to TNFα reduces infarct sizes and improves the neurological outcome (Lavine et al., 1998). On the other hand, studies on mice deficient in TNFα showed neuroprotective effects of TNFα (Lambertsen et al., 2009). IL-1β is one of the most studied cytokines in ischemic stroke and has a clear pathologic effect. Several studies with neutralizing antibodies against IL-1, inhibition of the IL-1 converting enzyme or mice deficient in IL-1 showed reduced infarct sizes (Schielke et al., 1998; Touzani et al., 2002). It still has to be kept in mind that TNFα and IL-1β act on various cell types and it is not fully understood whether the observed effects are mediated through neutrophils or other cell types. Another possible

explanation could be that cytokines and chemokines are not upregulated in the context of Fpr1 24 hours after the ischemic incident, but in a different time frame that was not examined in the present study. One other explanation could be that Fpr1 is only a co-receptor in addition to others like TLRs and amplifies the inflammatory reaction, but the deficiency of the receptor can be compensated by other receptors or mechanisms. For example, in liver injury, Fpr1 ligands act in combination with CXCL2 in neutrophil migration (McDonald et al., 2010). Previous studies from Kilic showed the importance of TLR4 in combination with Fpr1 in neutrophil recruitment (Kilic et al., 2008). Maybe here, the role of Fpr1 can be compensated by CXCL2 or TLR4, respectively, and its functions. A further cause could be that Fpr1 is important in other models, but not in our stroke model where other mechanisms predominate. Besides, mitochondrial formylated peptides are only a subset of the DAMPs released by trauma and maybe their quantity in the brain is not sufficient to activate Fpr1 in a way that initiates the inflammatory process. Other danger signals may be more important after injury and other immune receptors probably also respond to mitochondrial DAMPs. In necrotic tissue, danger signals like ATP or high mobility group box 1 (HMGB1) are increased. ATP is released as danger associated molecule by necrotic cells and previous studies showed that binding to P2X7 leads to the activation of microglia and macrophages (Kuan et al., 2015; Mezzaroma et al., 2011). Recently, it was shown that HMGB1, which is released after cell injury acts as a cytokine-like mediator in the post-ischemic brain and targeting HMGB1 signalling might be a new therapeutic approach (Gao et al., 2012; Kim et al., 2008). In conclusion, we suggest that Fpr1 is either not relevant in neutrophil migration, because there are other mechanisms including chemokines and chemokine receptors, or plays a minor role and its deficiency can be compensated by other mechanisms.

4.4 Comparison of Fpr1 in humans and the mouse model

Fpr1 has been described in several species and differences in its function in host defence and inflammation have been detected (Ye et al., 2009). Fpr1 is the murine orthologue of human FPR1. They are both expressed on similar cell types and functions like neutrophil chemotaxis, degranulation, cytokine production, and phagocytosis are the same, although the homology is only around 77% (Gao et al., 1998, 1998). Even though intracellular domain structures are highly conserved, the affinity of murine Fpr1 for fMLF is approximately 100-fold less than in humans (He et al., 2013). In humans, three different isoforms of FPR1 have been described (Wenzel-Seifert and Seifert, 2003). Several studies in sterile inflammation in the context of Fpr1 were performed in murine models and can be transferred to humans.

However, the MCAO model used in murine stroke experiments usually results in large areas of infarction, whereas in humans also minor strokes occur more frequently. Thus, it is still unclear if the pathophysiology of post-ischemic inflammation is the same in both species. Yet, it remains important to keep those differences in mind and inferences have to be made carefully. Although it was already reported that FPR1 was detected in human brain and hypoglossal nucleus neurons (Becker et al., 1998) and expressed on neuronal stem cells (Wang et al., 2016; Zhang et al., 2017), we could only detect very low expression levels on neurons in our experiments. Since the receptor in humans and mice is not fully homologue and experiments in human models could show different results. Besides, neutrophil recruitment into the ischemic region occurs in rodents and in human brain, which is accompanied with poor neurological outcome. Tang could show that gene-expression of FPR1 is upregulated 3 hours after stroke in humans and neutrophils accumulate at the site of inflammation (Le Y et al., 2002; Tang et al., 2006). These findings suggest that FPR1 is relevant in neutrophil recruitment and inflammatory reactions after stroke in humans (Akopov et al., 1996; Atochin et al., 2000). In our studies, we did not investigate the alteration of gene-expression of Fpr1 after stroke. But stroke volumes and leucocyte infiltration did not show significant differences in $Fpr1^{+/+}$ and $Fpr1^{-/-}$ mice, which indicates that Fpr1 is not relevant in the recruitment of inflammatory cells to the site of ischemia in mice. Hence, there might be differences between humans and mice in the signalling cascade and function of Fpr1.

4.5 Inflammation as therapeutic target

Stroke is the third leading cause of death in industrialized countries. This study showed that Fpr1 is not a crucial mediator in this inflammatory process in mice. However, inflammation is still one main factor that contributes to the stroke volume. In the following, we discuss possible other mechanisms involved in post-ischemic inflammation. The ischemic brain tissue releases pro-inflammatory factors such as cytokines which results in the recruitment of further immune cells that mediate inflammation. One important hallmark of the prolonged inflammatory response is the recruitment of neutrophils. Neutrophils are among the first systemic immune cells that arrive at the ischemic region and reach their peak on day 3 (Gelderblom et al., 2009). In this regard, the upregulation of various cytokines including IL-1, IL-6, IL-17A, IFN-γ and TNF-α and the increase of intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin, which promote leukocyte adherence and accumulation, lead to further inflammation. Especially elevated levels of IL-1β after MCAO are related to

an increase in infarct size and influx of neutrophils (Yamasaki et al., 1995). Several studies have shown that the upregulation of various adhesion molecules after cerebral ischemia guide the invasion of neutrophils (Goussev et al., 1998; Lakhan et al., 2009; Lindsberg et al., 1996). One possibility to prevent neutrophils from migrating to the lesion site might be the blocking of pro-inflammatory cytokines or adhesion molecules that function as trigger of neutrophil recruitment. Lymphocytes are key cells in innate and adaptive immune responses and might therefore be important targets in therapeutic approaches after cerebral ischemia. $CD4^+$ T-cells are responsible for the production of IFN-γ, which leads to the invasion of macrophages (Gelderblom et al., 2012). Furthermore, activated macrophages produce TNF-α and the increase is associated with cell death and inflammation (Wang et al., 2004). These findings suggest that $CD4^+$ T-cells, macrophages and concomitant pathways are important mediators in stroke pathophysiology. Moreover, the infiltration of γδ T-cells after ischemia resulted in the secretion of IL-17A and the amplification of the inflammatory cascade (Gelderblom et al., 2012; Shichita et al., 2009). The secretion of IL-17A leads to an upregulation of CXCL-1, which is a potent neutrophil chemoattractant. As a consequence, neutralization of IL-17A by an antibody resulted in decreased neutrophil infiltration and a better neurological outcome in mice. Regulatory T-cells (T_{reg}) were shown to initiate mechanisms that are neuroprotective in a mouse model for stroke (Liesz et al., 2009). In conclusion, other potential therapeutic targets include the various types of T-cells that are involved in inflammation in cerebral ischemia. One way might be to suppress γδ T-cells and the production of IL-17A as crucial effector of tissue damage as previously described by Gelderblom (Gelderblom et al., 2012). A different strategy includes the expansion of T_{reg} cells which play a protective role and might therefore be beneficial in stroke. Toll-like receptors (TLR) are further components of the inflammatory signalling and recent data showed that TLRs play a relevant role in ischemic tissue damage after stroke. TLR2 and TLR4 have extensively been studied in the context of cerebral ischemia. It was reported that both receptors are expressed in neurons and levels of TLR2 and TLR4 are increased in the cortex after ischemic brain injury (Tang et al., 2007). Other studies involving TLR4 knockout mice also resulted in reduced damage compared to controls (Cao et al., 2007; Kilic et al., 2008). Although Zhang showed that TLR9, which is activated by mitochondrial DNA, plays a crucial role in sterile inflammation and stimulation of neutrophils after tissue trauma, its role in ischemic brain injury could not be confirmed (Hyakkoku et al., 2010; Zhang et al., 2010). We tried to investigate whether Fpr1 also has a direct effect on neurons like TLRs. In our studies, mitochondrial DAMPs did not cause increased cell death of neurons in *Fpr1*$^{+/+}$

mice compared to *Fpr1*$^{-/-}$ mice. The simultaneous addition of DAMPs and CpG-ODN which is a ligand binding to TLR9 did not show any differences in cell death either. One possible explanation might be that the expression rates detected on neurons were only minimal and markedly lower than in neutrophils, which strengthens the fact that Fpr1 has no direct effect on neurons after cerebral ischemia. Altogether, these findings report a detrimental role of TLR2 and TLR4 in ischemic brain injury and suggest them as potential targets in therapeutic manipulation. Yet, other TLRs like TLR9 and Fpr1 seem to play no critical role. Overall, this study suggests that *Fpr1*-deficient mice are not protected in cerebral ischemia and Fpr1 does not play a critical role in the inflammatory signalling triggered by ischemia and reperfusion. Although Fpr1 is not relevant, interventions, which target the inflammatory signalling cascade, still remain a promising therapeutic target. Other mechanisms including lymphocytes and pro-inflammatory cytokines are potential therapeutic targets in stroke, which have to be validated in further studies.

5 Summary

Ischemic stroke is still considered one main cause of death in the western world. Despite intensive research, the knowledge on post-ischemic inflammation as part of secondary tissue damage after stroke is still limited. For this reason it is very important to gain a better insight in the pathophysiology in order to find new therapeutic strategies. The objective of this study was to define the role of Fpr1 in sterile inflammation after ischemic stroke. Fpr1 is a G-protein coupled receptor mainly expressed on neutrophils acting as part of the innate immune system. Activation results in different cell responses like chemotaxis and phagocytosis of tissue debris or invading pathogens.

First of all, expression of Fpr1 could be determined in cells of the peripheral immune system and cells of the CNS, although expression rates on neurons were considerably low compared to neutrophils and macrophages. In our *in vivo* experiments in a model of experimental stroke, we found evidence for an early strong pro-inflammatory immune response in ischemic hemispheres of WT mice, with an upregulation of cytokines, chemokines and a massive infiltration of pro-inflammatory immune cells. The most abundant immune cells encompassed microglia, neutrophils and macrophages. However, in our experimental stroke model we were not able to show significant differences in the infiltration of immune cells between *Fpr1*$^{-/-}$ mice and *Fpr1*$^{+/+}$. We further extended our studies to *in vitro* experiments on neurons. We were able to show that brain lysate from ischemic hemispheres lead to increased cell death in WT neurons, but similar to our results from experiments on the infiltration of immune cells, the expression of Fpr1 did not further affect the neuronal outcome. These results indicate that Fpr1 has neither a direct effect on the neuronal cell survival nor on the chemotaxis of immune cells into ischemic hemispheres. Finally, we compared stroke volumes and neurological outcome of *Fpr1*$^{-/-}$ mice and their littermates in the MCAO stroke model. In accordance with our results from mechanistic studies, we were neither able to detect any significant effects on infarct sizes nor the neurological outcome in both genotypes. These results indicate that Fpr1 does not play a pivotal role in the induction of the immune mediated tissue damage following stroke. In order to find new targets for therapeutic strategies it would be crucial to identify alternative signalling pathways, which are crucial for the activation of the detrimental early ischemia/reperfusion injury in stroke.

6 Zusammenfassung

Ein ischämischer Schlaganfall ist immer noch die häufigste Todesursache in der westlichen Welt. Trotz aufwändiger Forschungsarbeiten ist das Wissen über die post-ischämische Entzündungsreaktion als Teil der sekundären Schädigung des Hirngewebes immer noch limitiert. Deshalb ist es umso wichtiger, ein besseres Verständnis der Pathophysiologie zu erlangen, um neue therapeutische Ansätze zu entwickeln. Das Ziel dieser Arbeit war es, die Rolle von Fpr1 als Teil steriler Entzündung nach einem Schlaganfall klarer zu definieren. Fpr1 ist ein G-Protein gekoppelter Rezeptor, der als Teil des angeborenen Immunsystems hauptsächlich auf neutrophilen Granulozyten exprimiert wird. Dessen Aktivierung führt zu Zellreaktionen wie Chemotaxis und Phagozytose von nekrotischem Gewebe oder Erregern.

Genexpression von Fpr1 konnte auf Zellen des peripheren Immunsystems und Zellen des zentralen Nervensystems nachgewiesen werden. Allerdings waren die Expressionsraten von Neuronen deutlich geringer als von neutrophilen Granulozyten und Makrophagen. In den *in vivo* Schlaganfall-Experimenten konnten wir eine deutliche, frühe pro-inflammatorische Immunantwort in ischämischen Gehirnhälften von Wildtyp Mäusen, sowie eine Erhöhung von Zytokinen, Chemokinen und die Infiltration pro-inflammatorischer Immunzellen nachweisen. Die häufigsten Immunzellen waren Mikroglia, neutrophile Granulozyten und Makrophagen. Allerdings konnten in unserem Model keine signifikanten Unterschiede in der Infiltration von Immunzellen zwischen *Fpr1*$^{-/-}$ und *Fpr1*$^{+/+}$ Mäusen gezeigt werden. Desweiteren haben wir *in vitro* Experimente mit Neuronen durchgeführt. Die Ergebnisse zeigten, dass Hirnlysat aus ischämischen Hirnhälften zu erhöhten Raten an Zelltod in Neuronen führte. Vergleichbar mit unseren bisherigen Ergebnissen zur Infiltration von Immunzellen, konnten wir allerdings zeigen, dass die Expression von Fpr1 keinen Unterschied bezüglich des Zelltods von Neuronen machte. Diese Ergebnisse weisen darauf hin, dass Fpr1 weder einen direkten Effekt auf das Überleben von Neuronen, noch auf die Chemotaxis von Immunzellen in die ischämische Hirnhälfte hat. Schließlich verglichen wir die Schlaganfallgrößen und den neurologischen Status der verschiedenen Nachkommen im MCAO-Modell miteinander. In Übereinstimmung mit anderen Studien konnten wir weder in den Infarktgrößen, noch im neurologischen Zustand der beiden Genotypen Unterschiede aufzeigen. Diese Resultate deuten darauf hin, dass Fpr1 keine entscheidende Rolle in der durch das Immunsystem vermittelten Gewebeschädigung in Folge eines Schlaganfalls spielt. Um neue therapeutische Angriffspunkte zu finden, wäre es erforderlich, alternative Signalwege, die für den Schaden nach einer Ischämie verantwortlich sind, zu identifizieren.

7 Abbreviations

AF	Alexa fluor
APC	Allophycocyanin
ATP	Adenosine triphosphate
BME	Beta-mercapto-ethanol
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CCL	CC chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonuclease
CSF	Cerebrospinal Fluid
CNS	Central nervous system
CpG-ODN	Oligodeoxynucleotide with high amount of CpG-motifs
CsH	Cyclosporin H
Ct	Cycle threshold
CXCL	Chemokine C-X-C motif ligand
DAG	Diacylglycerol
DAMPs	Danger associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DMEM	Dulbecco modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
fMLP	N-formyl-peptide-formyl-methionine-leucyl-phenylalanine
Fpr1	Formyl-peptide-receptor 1
GFAP	Glial fibrillary acidic protein
HBSS	Ca^{2+} M^{+} free Hank's balanced salt solution
HMGB1	High mobility group box 1 protein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
Iba1	Ionized calcium binding adapter molecule 1
IL	Interleukin
IP3	Inositol-triphosphate
I/R	Ischemia-reperfusion
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharid
Ly-6g	Lymphocyte antigen 6 complex locus G6D
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MEM	Minimum essential media
Mg^{2+}	Magnesium
MMP	Matrix metalloproteinase
mtDNA	Mitochondrial deoxyribonuclease
NFκB	Nuclear factor 'kappa light chain enhancer' of activated B-cells
NGS	Normal goat serum
NKcells	Natural killer cells

NKT	Natural killer like T-cells
NO	Nitric oxide
NSC	Neuronal stem cell
OGD	Oxygen glucose deprivation
P/S	Penicillin streptomycin solution
P2X7	P2X purinoreceptor 7
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PFA	Paraformaledehyde
PI3K	Phosphatidyl-inositol-3-kinase
PIP2	Phosphatidyl-inositol-4,5-bisphosphate
PIP3	Phophatidyl-inositol-3,4,5-triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rounds per minute
RT	Room temperature
Rt-PA	Recombinant tissue plasminogen activator
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
Sdha	Succinate Dehydrogenase subunit A
SSC	Side-scattered light
TBST	mixture of tris-bufferd saline (TBS) and polysorbate 20 (Tween 20)
TLR	Toll-like-receptor
TNF	Tumor necrosis factor
TTC	Tetrazolium chloride
UTP	Uridine triphosphate
UV	Ultraviolett

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10 Curriculum vitae

Entfällt aus datenschutzrechtlichen Gründen.

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