Imaging the effects of the transient middle cerebral artery occlusion filament model on the recruitment of neutrophils with non-invasive imaging techniques

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Imaging the effects of the transient middle cerebral artery occlusion filament model on the recruitment of neutrophils with non-invasive imaging techniques.

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH ZURICH)

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
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<tr>
<td>DWI</td>
<td>Diffusion weighted imaging</td>
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<tr>
<td>ECA</td>
<td>External carotid artery</td>
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<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICAM-1null</td>
<td>Transgenic mouse line missing the ICAM-1 coding region</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NIRF</td>
<td>Near-infrared fluorescence</td>
</tr>
<tr>
<td>NTL</td>
<td>None transgenic littermates</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sO₂</td>
<td>Oxygen saturation</td>
</tr>
<tr>
<td>tMCAO</td>
<td>Transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
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Summary

Stroke is one of the leading causes of death in the world and accounts for a significant proportion of the annual expenses incurred by the healthcare system. The predominant forms of treatment for ischemic strokes relies on restoring the blood flow; while only about 5 to 10% of the patients can benefit from this treatment. In the recent years, major advances in stroke research have been made regarding the inflammatory processes occurring after cerebral ischemia. However, the processes and mechanisms involved are not completely understood, namely those involved in immune cell recruitment, the contribution of immune cells to secondary neuronal death, tissue reorganization and repair. The vascular guiding cues which direct immune cells from the periphery to the site of damage in particular seem to be well understood for peripheral organs, but the recruitment mechanisms in the brain seem to be different and are therefore a subject of investigation. Neutrophils are in the focus of research for decades since they appear early in the brain of patients with ischemic stroke and animal models of focal cerebral ischemia, and are present during the time extensive neuronal death occurs. Studies showed beneficial effect in experimental models of cerebral ischemia in which neutrophils were depleted, but in the same time other studies showed no effects or even worse outcome. This has revealed that large gaps still exist in the understanding how neutrophils contribute to the exacerbation of the ischemic injury. This lack of knowledge largely stems from the limitations of currently available methods. Most data was collected in ex vivo studies and lacked specificity. With two-photon microscopy neutrophils can be studied in vivo, but physiological conditions of the animal can be affected through the need of cranial windows accompanied by tissue damage and inflammation, which might affect the observations under study. Current advances in optical imaging in the near-infrared region have enabled to non-invasively study molecular and cellular processes under physiological conditions in small animals. Near-infrared fluorescence (NIRF) imaging has a high sensitivity and is able to detect small quantities of fluorochromes deep in the tissue. The technique has been used to study disease relevant processes in many disease models such as cancer, neurological disorders including stroke and comprised protease activity, adhesion molecule expression, tumour growth, angiogenesis and stem cell migration.

Using NIRF imaging we aimed to study the recruitment of neutrophils in the transient middle cerebral artery occlusion (tMCAO) mouse model of cerebral ischemia. More specifically, we established a protocol to label bone marrow derived neutrophils with a NIRF dye to track them after tMCAO. This approach enabled us to investigate the mediators of the adhesion cascade that are involved in the recruitment of neutrophils to the brain after cerebral ischemia.
For ex vivo labelling of neutrophils, we optimized dye concentration and incubation time to obtain high labelling efficiency while maintaining cell viability. Activation assays were used to test the functionality of labelled cells. Only a minor L-selectin shedding, but no upregulation of neutrophil activation markers was demonstrated, showing that the labelling procedure did not alter neutrophil activation and function. The sensitivity of a planar NIRF system for the detection of labelled cells was assessed using a tissue mimicking silicon phantom. An amount of 1750 fluorescent neutrophils could be detected in a volume of 1 μl placed 2 mm deep. We then tested the approach to detect neutrophil recruitment in tMCAO mice in vivo after adoptive transfer of LIPO-6S-IDCC labelled neutrophils. Increased fluorescent signals over the ipsilateral hemisphere were found in tMCAO compared to sham-operated mice. Immunological staining of ischemic brains demonstrated the presence of adoptively transferred fluorescent labelled neutrophils beside endogenous neutrophils, while in sham operated animals no neutrophils were found in the brain, confirming that labelled neutrophils accumulate at the ischemic lesion. Longitudinal NIRF imaging of adoptively transferred labelled neutrophils into tMCAO mice revealed the time course of neutrophil recruitment with an onset after 2 h and peak values between 18 and 24 h, which were still detected after 48 h after reperfusion.

In a next step, we studied the recruitment cascade to the ischemic brain by using our developed approach together with interventions targeting proposed mediators of neutrophil recruitment. Moreover, we investigate if ablation or pharmacological inhibition of the mediators are neuroprotective. First, we used an antibody against α4-integrin subunit to prevent the interaction of the very late antigen-4 (VLA-4) receptor with vascular cell adhesion molecule 1 (VCAM-1). A single anti-α4-integrin treatment lead to a reduction in neutrophil recruitment after 18 and 24 h seen with NIRF imaging, whereas after 48 h no difference was observed. Histology revealed no differences in neutrophil counts at the study end point and equal lesion volumes between treated and untreated animals. Second, we investigated the role of the intercellular adhesion molecule-1 (ICAM-1) using transgenic mice deficient of ICAM-1 (ICAM-1null mice). In ICAM-1null mice no differences in normalized fluorescence intensities were found compared to non-transgenic controls. Findings were corroborated by immunohistology, which showed no differences in the neutrophil recruitment to the ischemic brain between the two groups. Taken together, these data indicates that VLA-4 is involved in neutrophil recruitment, while ICAM-1 is not required.

During the NIRF imaging studies, we also found elevated fluorescent signals over the temporal muscle of the ipsilateral side. We tested the hypothesis that ligation of the external carotid artery (ECA) leads to damage in the tissue of the ECA territory. Multispectral optoacoustic tomography revealed that the surgery lead to significant lower oxygen saturation (sO₂) values in the temporal muscles in tMCAO and sham operated animals compared to the contralateral side. We assessed microstructural changes with
magnetic resonance imaging (MRI) and observed significantly higher T2-relaxation times at 24 and 48 h after surgery. In the ipsilateral temporal muscle immunohistology demonstrated increased number of neutrophils in the temporal muscle, accompanied by myofiber degeneration and oedema. Taken together, the data shows that ECA ligation leads to damage in the extra-cerebral tissue.
Zusammenfassung


In der vorliegenden Arbeit wurde NIRF Bildgebung verwendet, um die Rekrutierung von neutrophilen Granulozyten zur zerebralen Läsion im ischämischen Schlaganfallmodell der Maus zu untersuchen. In diesem Modell wird die Blutzufuhr der mittleren Zerebralerarterie vorübergehend (transient middle

In einem ersten Schritt entwickelten wir ein Protokoll zur Markierung neutrophiler Granulozyten und optimierten dazu die Konzentration des Farbstoffes, sowie dessen Inkubationszeit, unter Berücksichtigung der Zytotoxizität. Mit Hilfe von Aktivierungssassays wurden die Zellen anschließend auf ihre Funktionalität überprüft. Es wurde ein leichter Rückgang des Adhäsionsmoleküls L-Selektins festgestellt, jedoch keine Hochregulierung der Aktivitätsmarker CD62L, CD11a, CD11b beobachtet. Somit führt die Markierung der neutrophilen Granulozyten nicht zu deren Aktivierung.


Unterschiede in der Anzahl von neutrophilen Granulozyten in Gehirnschnitten. Infarktvolumetrie zeigte signifikant kleinere Läsionsvolumen in anti-α4-Integrin behandelten Tieren in Vergleich zur Kontrollgruppe.


Chapter 1

1. Introduction
1.1. The early inflammatory response after stroke

1.1.1. Stroke

Stroke is the second leading cause of death, accounting for 9% of all deaths worldwide. From these deaths even 12% are under 65 years of age (Murray and Lopez, 1997). The incidence rate of stroke was around 16.9 million worldwide in 2013, while the mortality rate within the first year was at 30% and another 33 million prevalent cases were recorded (Christopher and Murray, 2015; Vos, 2015). During last decades stroke mortality rates in developed countries decreased due to intensive post-stroke care from 50 to 30% within the first year, but the number of incident strokes and disability-adjusted life-years lost due to stroke increased (Bonita, 1992; Feigin et al., 2014; Donnan et al., 2008). Better stroke care together with education of stroke prevention to reduce risk factors of stroke, like smoking and high blood pressure led to a decrease in stroke incidence of 25% e.g. in Australia between 1989 and 1995 (Strong et al., 2007). However, another important risk factor is age, and since the world population is aging stroke is expected to become a highly prevalent disease in the future.

Classification of stroke

Stroke is defined as an acute episode of focal dysfunction of the brain, retina, or spinal cord lasting longer than 24 h, or of any duration if imaging (computed tomography (CT) or MRI or autopsy show focal infarction or hemorrhage relevant to the symptoms (Hankey, 2016). Stroke arises either from blockade (ischemia) or rupture (hemorrhagic incident) of a blood vessel.

In around 20% of patients stroke is caused by hemorrhage. In about two-third of all primary cerebral hemorrhages diagnosed hypertension is the cause (Thrift et al., 1995). Mainly vessels near the base of the brain rupture and cause damage to the deep brain regions like the putamen and the thalamus (Ojemann and Mohr, 1976; Takebayashi and Kaneko, 1983). The other third of hemorrhagic strokes arise from vessel malformation, cerebral amyloid angiopathy, drug abuse, coagulation abnormalities and trauma (Kase, 1991; Caplan, 1988). The tissue damage and neurological deficit caused by the intracerebral hemorrhage develops due to an increased pressure during the formation of a hematoma, followed by edema, necrosis in combination with the release of toxic cell contents, like hemin (Kase, 1991; Dolinskas et al., 1977; Robinson et al., 2009). Secondary brain damage is caused by toxic degradation products, which can occur over weeks after the insult (Robinson et al., 2009).

The majority of stroke cases, ≈80 %, are ischemic strokes. In ischemic stroke the restriction in blood supply due to emboli or blood clots causes an inadequate supply in nutrients (mainly oxygen and glucose), leading to a complex cascade of pathophysiological events (Dirnagl et al., 1999) including
oxidative stress, energy failure and the breakdown of the membrane potential resulting in cellular death (Lodish et al., 2000; Simard et al., 2007).

An exact diagnosis to differentiate between the two subtypes of stroke is the most important step before any treatment can be applied. In addition, time is one of the most critical points after the onset of stroke, since neuronal death occurs within minutes of nutrient restriction, and the lesion grows over time (Dirnagl et al., 1999). Moreover, the approved treatments of ischemic stroke such as thrombolysis with recombinant tissue plasminogen activator (rtPA) have been shown to be effective and safe only within the first hours. Spontaneous recanalization of occluded vessels without treatment occurs only in 25% of the patients within 24 h of clinical symptom onset and in up to 50% within one week (Meves et al., 2002; Rha and Saver, 2007).

Animal models of cerebral ischemia

To study hemorrhagic and ischemic stroke not only observations in patients, but also corresponding animal models are used. For cerebral ischemia focal and global as well as transient and permanent ischemic models are available. Some stroke models create an area with tissue at risk (penumbra) with an outgrowing ischemic core. Using these models many of the underlying mechanisms of cell death and recovery processes after cerebral ischemia have been investigated and putative diagnostic and therapeutic compounds have been tested (Prinz and Endres, 2016).

To mimic the variable phenotypes of human stroke (age, severity, co-morbidities, duration, sex, localization) several animal models of cerebral ischemia have been developed (reviewed in (Dirnagl, 2016). All models require a more or less severe surgical procedure. The most common used stroke model is the intraluminal filament model to induce ischemia in the middle cerebral artery (MCA) territory by occluding the MCA with a filament (Hata et al., 2000a). The advantages of this model are that it do not require a craniectomy, infarct sizes are reproducible, the intervention is rather fast and the occlusion can be transient or permanent. This model allow to study acute and long term outcome in combination with interventions, imaging and treatments.

Symptoms and Diagnosis

After a stroke most of the patients suffer from sudden loss of body functions due to neurological impairment. Examples are unilateral weakness or loss of function of the limbs due to the affection of the corticospinal tract, partial to complete facial paralysis due to a lesion affecting the cranial nerve VII like in the brainstem, blindness when the retina, the optic nerve or the visual cortex are involved (Hankey and Blacker, 2015). Also headache might be an indication for a stroke, sometimes in combination with confusion of the patient. For a fast stroke diagnosis often the Face Arm and Speech Test (so called FAST) or the Recognition of Stroke in the Emergency Room score (called ROSIER) are
used (Hankey and Blacker, 2015; Whiteley et al., 2011). Patients with indication of stroke undergo a cranial CT scan to determine if the patient suffers from an intracranial hemorrhage. CT is a fast, highly sensitive technique to detect fresh intracranial bleedings, but can depict the extent of the ischemic damage poorly. Therefore, CT is mainly used to differentiate patients with hemorrhage and ischemic stroke and for treatment stratification. MRI is increasingly used because it can reveal the vessel occlusion, extent of cerebral ischemia and infarction, and assess microbleeds and hemorrhage (e.g. (Kloska et al., 2010)).

Diffusion weighted (DWI) MRI, T2-weighted, susceptibility weighted and perfusion weighted imaging are a comparatively more expensive and time consuming method to detect lesions and hemorrhage than CT, but provides additional spatial and temporal information. DWI can detect acute ischemia already minutes after onset of the ischemic event with its cytotoxic edema, while susceptibility weighted imaging allows to detect hemorrhages and T2-weighted MRI to visualize lesions older than 6 h (Hankey and Blacker, 2015). Also older asymptomatic infarcts can be detected with MRI. Using these techniques patients with a stroke mimic (e.g. migraine or multiple sclerosis) can be separated from stroke patients. MR angiography can depict bigger occluded vessels and stenosis and help to choose an appropriate recanalization therapy. Perfusion weighted imaging indicates perfusion deficits in the ischemic brain and the mismatch of the overlay with DWI images reflects in parts salvageable brain tissue (Figure 1), also called tissue-at-risk or historically penumbra.

Nevertheless it is difficult to diagnose stroke in the first hours, due to atypical features, patients are unwell or agitated, imaging modalities are not access able or brain imaging is normal (Edlow and Selim, 2011; Fernandes et al., 2013). Patients with a transient cerebral ischemic attack for example, show no changes in DWI, but focal neurological symptoms up to 24 h (Easton et al., 2009).

The pathophysiology of ischemic stroke

Animal models of ischemic stroke have been pivotal to elucidate the pathophysiology. In the ischemic core blood flow is restricted to less than 20% of normal blood flow and the oxygen tension in the brain tissue (PtiO2) decreases to levels below 10 mmHg (normal are around 40 mmHg). Energy failure causes permanent and anoxic cell depolarization, followed by neuronal death within minutes (Hossmann, 1994; Maloney-Wilensky et al., 2009; Hoffman et al., 1996; Scheufler et al., 2002; Lo, 2008).

Oxygen dependent ATP production in the mitochondria during ischemia stagnates causing the failure of the ATP depended Na+/K+-pump (Figure 1). This leads to the depolarization of the cell membrane causing the activation of voltage-dependent Na+ and Ca2+ channels and the release of presynaptic excitatory amino acids into the synaptic cleft. In general amino acids, like neurotransmitters are quickly removed from the extracellular space by neurons and glia, but the reuptake mechanisms are energy
(ATP) dependent, and not functional under severe hypoxia. This increases the amount of excitatory neurotransmitters, like glutamate, in the extracellular space and further activates its receptors (e.g. N-methyl-D-aspartate (NMDA) receptor). High extracellular glutamate concentrations generate repetitive spreading depolarization waves causing additional influx of Na⁺ and Ca²⁺ through further neuronal activation. NMDA and metabotrophic glutamate receptors activate Ca²⁺ channels and mobilize further Ca²⁺ influx, also from intrinsic depots in the cells (Park et al., 1989). The dysregulation of Ca²⁺ homeostasis and the excitotoxicity overload of Ca²⁺ have severe cellular consequences, including neuronal death (Szalay et al., 2016).

Ca²⁺ is a second messenger in cells and activates numerous cascades, from DNA transcription to protein synthesis, degradation and release. Neuronal nitric-oxide synthase is Ca²⁺ dependent and activated by NMDA receptors. The synthesis of NO, a free highly reactive radical, leads in general to the relaxation of blood vessels and improve the blood supply (Huang et al., 1994; Attwell et al., 2010). But if ATP dependent scavenger mechanisms fail to clear NO, it can also react with a superoxide anion to peroxynitrite and promote tissue damage. Beside NO-synthase, Ca²⁺ also can activate phospholipase A and cyclooxygenase-2 (COX2), which further increases the load of free reactive oxygen species (ROS) and promote tissue damage (Dirnagl et al., 1999). The increased production of free radicals damage the cells in multiple ways through membrane or DNA damage, while activating the apoptotic cascades (Leist and Nicotera, 1998; Stoian et al., 1996). ROS production also requires oxygen and therefore increases oxygen consumption. In addition, oxidative stress causes single- or double-strand breaks due to the formation of 8-oxoguanine in the DNA and is considered as lethal damage of DNA through peroxidation, while oxidative stress inhibits at the same time the DNA repair mechanisms (Behnia et al., 2016; Wu et al., 2004; Tsukahara et al., 2000; Ames, 1989).

Oxidative stress leads to the activation of p38 mitogen-activated kinase pathway causing excitotoxic cell death and increased hypoxic brain tissue damage through cell senescence (Menon et al., 2013; Cao et al., 2005; Irving and Bamford, 2002). The NMDA receptor signaling pathway, which leads to an initial phosphorylation of striatal-enriched phosphates inhibiting the p38 mitogen-activated kinase pathway, while striatal-enriched phosphates is degraded after reperfusion and p38 mitogen-activated kinase pathway causes neurotoxicity (Poddar et al., 2010).

In parallel to the above described accumulation of ions in the cells, a passive inwards driven flow of water towards the ion gradient leads to cell swelling and cytotoxic tissue edema (Simard et al., 2007). Chloride ions, which follow the chemical gradient into the cells, increase the ion load further and therefore accelerate cell swelling. This cytotoxic cell edema can be detected already minutes after ischemia onset using DWI (Figure 1). Cell swelling, DNA damage or the breakdown of the membrane
potential contribute significantly to neuronal cell apoptosis or necrosis. Cytotoxic cell edema further produces an ionic gradient from the blood vessels towards the extracellular space and cause a passive flow of Na⁺, Cl⁻ and water into the parenchyma after reperfusion, increasing the cranial pressure, which can result in further blood flow restriction and damage (Simard et al., 2007).

![Diagram](image)

**Figure 1: Using MRI to image acute cerebral ischemia and reflect salvageable tissue.** DWI can show areas of excitotoxic edema, while PWI reflects perfusion deficits in the brain. The mismatch of both MRI sequence images reflects in parts salvageable brain tissue. Without reperfusion of the PWI reflected area most of the tissue at risk dies within hours. Picture from (Moskowitz et al., 2010).

*Penumbra = salvageable tissue?*

In ischemic stroke the tissue surrounding the infarct/lesion core is characterized by a reduced blood supply and partially metabolic energy failure (Hossmann, 1994; Astrup et al., 1981). This area between the ischemic core and normal oxygenated tissue is called penumbra. The ischemic lesion grows continuously out into the penumbra without restoration of the blood flow. The penumbra is well described in animal models of stroke, but it is difficult to assess the penumbra in stroke patients (Furlan et al., 1996; Read et al., 1998; Kaufmann et al., 1999). While the penumbra can be partly reflected using MRI, it gives an indication how much tissue is salvageable with reperfusion (Figure 1). At the
moment recombinant tissue plasminogen activator application and mechanical recanalization are the only clinically approaches approved for the treatment of ischemic stroke. Both can only be used in a narrow time window of 4.5 to 6 h after stroke onset, respectively and the combination of both can improve the outcome further (Hankey, 2016). In combination with neuroprotectants the effect might be further increased. Though, only few patients benefit from these treatments due to the narrow time windows. Patients treated thereafter have an increased risk of hemorrhagic transformation, resulting in worse outcome, neurological deficits and death. In addition, after recanalization therapy in many patients an incomplete restoration of the blood flow especially in the microvasculature is achieved, referred in literature to as the no-reflow phenomenon (Nour et al., 2013).

Therefore, new therapeutic strategies with an extended therapeutic window are highly desired. Inflammation occurs hours and even days after stroke onset and has been implicated to exacerbate the primary ischemic damage (Stoll et al., 1998; Petrovic-Djergovic et al., 2016; Dirnagl et al., 1999). Therefore, inflammation following ischemic stroke might pose a potential therapeutic target.

1.1.2. The sterile inflammation/injury after ischemia

Ischemia causes a sterile injury which induces cellular stress, defense and survival cascades (Behnia et al., 2016). Some of these cascades influence not only the affected cells themselves, but the entire body by upregulation and release of cytokines, chemokines and inflammatory markers. Blood flow increases and a sterile inflammation propagate starting from the affected vasculature (Anrather and Iadecola, 2016).

Cytokine release and the stagnated blood flow under ischemia is accompanied by shear stress on endothelial cells and platelets, which activates the inflammatory cascade immediately (Iadecola and Anrather, 2011; De Meyer et al., 2016). These stimuli lead to the upregulation of P-Selectin within minutes through fusion of Weibel-Palade bodies and α-granules with the cell membrane of endothelial cells and platelets, respectively. P-selectin can be recognized from most leucocytes via the expression of P-selectin glycoprotein ligand-1, causing adhesion. This mechanism is also thought to contribute to the no-reflow phenomenon after thrombolyis in stroke. Furthermore, the endothelial activation by its pattern recognition and cytokine receptors leads to the transcription, upregulation and expression of additional adhesion molecules, like ICAM-1 and VCAM-1 both involved in the immune cell recruitment cascade (Petrovic-Djergovic et al., 2016). Targeting these receptors showed a high therapeutic potential in experimental stroke by reducing the ischemic injury in experimental models of cerebral ischemia while at the same time other groups found no beneficial or contrary effects in experimental and clinical studies (Liesz et al., 2011; Lloversa et al., 2015; Connolly et al., 1996;
Enlimomab Acute Stroke Trial Investigators, 2001). Therefore, the effect of blocking adhesion molecules, including the role of ICAM-1 and VCAM-1, have to be investigated further.

In addition, activated endothelial cells downregulate the endothelial protein C thrombomodulin system enabling thrombin during inflammation (Van de Wouwer et al., 2004). Thrombin is highly regulated to prevent extensive spontaneous clot formation and its activation is highly locally confined during inflammation (Delvaeye and Conway, 2009). Free thrombin activates the coagulation cascade, upregulates the transcription of adhesion molecules further via nuclear factor kappa B, activates the complement system, is able to disrupt the blood-brain barrier (BBB) and enhances the inflammatory response (Delvaeye and Conway, 2009; Amara et al., 2008). Furthermore, thrombin attracts monocytes and neutrophils and guides them towards the injured area (Rezaie, 2014).

How thrombin activates the complement system, a part of the innate immune system, is not yet fully understood. It is suspected that free proteases cleave and therefore activate the C3 and C5 complement proteins (Lo, 2008). Some studies indicate that the complement system is activated through the lectin pathway, while mice and humans lacking the mannose-binding lectin have better outcome compared to controls (Cervera et al., 2010). Also microglia seem to be a source of complement system synthesis and activation via the classical component activation way over C1q after ischemia (Schafer et al., 2000). Beside the unknowns in the activation cascade of the complement system, it is also yet not fully understood how the complement system contributes to neuronal damage after cerebral ischemia. It is thought, that immune cells from the myeloid lineage bind to the anaphylatoxins (C3a and C5a), which induce and increase the generation of ROS, the release of pro-inflammatory cytokines and effect degranulation (Anrather and Iadecola, 2016). The only consistent data about the complement system and its activation shows a correlation with unfavorable outcome after stroke in mice and human (Széplaki et al., 2009).

This local metabolic stress, free floating thrombin and damage associated molecular patterns (DAMPS), endothelial and complement activation contributes all to the attraction and activation of the immune system and the breakdown of the BBB. Dying neurons activate microglia, the local immune cells of the brain, which release upon their activation Interleukin (IL)-1β and tumor necrosis factor (TNF)-α, thereby enhancing the inflammatory cascade of endothelial cells. DAMPs can gain access to the blood stream over the leaky BBB or the lymphatic system and mobilize the peripheral immune system. Elevated levels of high mobility group binding protein 1, a DAMP molecule, can be detected in patients after stroke and are correlated with worse outcome, while blocking of high mobility group binding protein 1 improved outcome (Liesz et al., 2015).
It has been shown, that the systemic inflammatory response is characterized by increased neutrophil blood counts, IL-6 and TNF values in experimental and human stroke (Offner et al., 2006; Chapman et al., 2009; Pagram et al., 2016). Increased neutrophil and IL-6 serum levels are correlated with stroke severity in patients (Pagram et al., 2016; Basic Kes et al., 2008), while most other parameters return to baseline within 24 h after ischemia onset (Anrather and Iadecola, 2016). Already decades ago it was postulated that the post-ischemic inflammation causes additional damage to the brain and the resulting lesion can be reduced by targeting the inflammatory cues (Moskowitz et al., 2010; Petrovic-Djergovic et al., 2016; De Meyer et al., 2016; Feuerstein et al., 1998).

Experimental work has shown that the initial activation of the immune system is followed by a systemically immunosuppression starting already 12 h after stroke onset which can persist for weeks. B, T and natural killer cell counts are decreased, predisposing for infections and increase mortality rate (Prass et al., 2003). And indeed around 20 % of stroke patients suffer from pulmonary and urinary tract infections (Langhorne et al., 2000). There are strong indications, that the damaged brain induce the immunosuppression over the activation of the sympathetic nervous system leading to splenocyte apoptosis, regulatory T-cell expansion and a shift towards the myeloid lineage, including in particular neutrophils (Anrather and Iadecola, 2016; Prass et al., 2003). This might increase the potential impact of the innate immune system and of neutrophils after cerebral ischemia and make them to promising targets for stroke treatment.

1.1.3. Neutrophils a potential target of the innate immune system

The immune system is historically divided into two parts: the innate and the adaptive immune system, both take part in the inflammation after ischemic stroke and impact its outcome.

The innate immune system

The innate immune system is also called unspecific immune system, including physical and chemical barriers, free floating molecules (e.g. complement system), different classes of myeloid immune cells, while it protects the body from a broad range of harmful substances and pathogens. Innate immune cells can also recognize DAMPs with their pattern recognition receptors (PRR), which are released from damaged host tissue due to sterile injuries, according to high pressure, extreme temperatures or hypoxia (Matzinger, 2002). Interestingly pathogen and DAMP recognition leads to similar pro-inflammatory responses and may explain collateral damage after neutrophil activation (Kolaczkowska and Kubes, 2013). Recognition of DAMPs leads to a downstream signaling resulting in phagocytosis of damaged cells and cell fragments, granule fusion combined with protease, cytokine and chemokine release. Excessive released proteases degrade cellular structures, the extracellular
matrix and cells, what can cause severe tissue damage. Cytokines released after tissue damage cause inflammation and mobilize myeloid immune cells from reservoirs like the bone marrow and guide them to the inflammatory territory (Bonaventura et al., 2016). Chemokines guide immune cells during migration into the inflamed tissue (Bonaventura et al., 2016).

First neutrophils then macrophages accumulate at the side of inflammation, release their repertoire and phagocyte damaged tissue and pathogens. Also cells of the innate immune system can process digested antigens, express them on their surface in MHC class II receptors and present them to the adaptive immune system to activate it. To intervene during these pro-inflammatory phase may be one of the most potent and promising time points to manipulate inflammation in a favored way.

The adaptive immune system

Once cells of the adaptive immune system get activated, upon binding their specific antigen on the surface of an antigen presenting cells, they multiply, travel to the injury side and attack the potential intruder. The adaptive immune system is highly effective and can clear most of the infections. Activated B-cells produce large amounts of specific antibodies and release them into the blood stream. Antibodies make opsonized structures visible for other immune cells and therefore accelerate their clearance. T-cells can have supportive functions (e.g. CD4+ T-cells) or are able to recognize abnormal host cells (infected cells or mutated cells) and eliminate (CD8+ T-cells) them. After ischemic stroke the adaptive immune system also gets access to the ischemic brain (reviewed in (Anrather and Iadecola, 2016)). Regulatory T-cells might contribute to vessel occlusion in the acute phase (Kleinschnitz et al., 2013), while they secrete in a later stage IL-10 and limit the inflammation (reviewed in (Planas and Chamorro, 2009)). CD8+ T-cells are thought to create also an antigen specific immune reaction against cerebral tissue similar to multiple sclerosis (Gill and Veltkamp, 2016). Due to its relatively long activation time and its late appearance in the ischemic brain the adaptive immune system seems to be another promising target for post stroke treatment.

After inflammation is resolved the involved adaptive immune cells can form memory cells, which are able to survive over years. These cells get reactivated much faster without support of the adaptive immune system in case of another contact with their antigen and create a more potent respond. Therefore, memory cells might play an important role in patients suffering from repetitive episodes of ischemic stroke.

Neutrophil Granulocytes

Generation, trafficking and clearance

Neutrophil derive, from pluripotent hematopoietic stem cells in the bone marrow (Figure 2). After the differentiation to a myeloid progenitor cell, the development towards a neutrophil granulocyte gets
specialized. Paul Ehrlich first classified the three granulocyte subtypes due to their staining characteristics. Neutrophils can be distinguished from basophil and eosinophil granulocytes by their segmented nuclei and the absent to light vesicle reaction to H&E staining (Figure 2). The diameter is 7-10 μm and they have a highly flexible shape after adhesion and can show an amoeboid behavior with pseudopodia after adhesion and extravasation (Carp et al., 1972; Kolaczkowska and Kubes, 2013).

![Schematic development of the immune cells](https://goo.gl/images/u5DEDF)

**Figure 2: Schematic development of the immune cells.** Multipotent hematopoietic stem cells are located in the bone marrow and differentiate into the progenitor cells. From myeloid progenitor cells arise erythrocytes in erythropoiesis and in myelopoiesis granulocytes, monocytes and thrombocytes. Lymphocytes, like T-cells develop from the lymphoid progenitor cells. Adapted from https://goo.gl/images/u5DEDF

Neutrophils can remain in the bone marrow or are released into the blood stream after their differentiation. Once released into the blood stream neutrophils are fully functional, while their life span is up to 12.5 h in mice and 10 h in humans (Pillay et al., 2010; Tofts et al., 2011; Cronkite, 1979). After activation the life span of neutrophils can be extended by cytokines, growth factors and bacterial components (Kim et al., 2011b; Colotta et al., 1992). Circulating neutrophils continuously explore their environment and crawl along blood capillaries. With age they increase the CXC-chemokine receptor 4,
which guides them back to the bone marrow, where neutrophils are eliminated (Eash et al., 2009). Also the liver and the spleen can clear aged neutrophils (Hong et al., 2012).

Neutrophil recruitment

Neutrophils are the first cells arriving at the side of inflammation. In stroke patients it was shown that neutrophils are rapidly recruited into the circulation after stroke onset, and are associated with lesion size and worse functional outcome (Ross et al., 2007; Buck et al., 2008; Kumar et al., 2013). Circulating neutrophils are recruited to the site of inflammation by guiding cues at the vascular endothelium. Neutrophil migration into peripheral tissue seems to be well understood, while immune cell recruitment and migration at the neurovascular unit towards the central nervous systems is not fully determined (Kolaczkowska and Kubes, 2013). The vascular endothelium expresses in the periphery continuously P-selectin, which allows neutrophils to explore the endothelium for any abnormalities. Brain endothelial cells are lacking the continuous expression of P-selectin, which also may contribute to its immune privileged status. Endothelial cells get activated by inflammatory mediators, like cytokines, the detection of pathogens or DAMPs via pattern recognition receptor binding and regulate their adhesion molecules up to recruit immune cells.

During inflammation including cerebral ischemia endothelial cells get activated, express and upregulate P-selectin within minutes followed by other selectins (Ley et al., 2007), while immediate adhesion of neutrophils occur (Barkalow et al., 1996). Neutrophil recruitment peaks somewhere between 18 and 72 hours and declines thereafter rapidly (Enzmann et al., 2013; Neumann et al., 2015; Zhang RL et al., 1994). The recruitment cascaded of neutrophils consists of the following steps: tethering, rolling, adhesion, crawling and transmigration (Figure 3). Neutrophils tether via their P-selectin glycoprotein ligand-1 to the selectins on the endothelium. Human L-selectin expressed on all lymphocytes might promote the binding on already recruited neutrophils on the vessel wall or might be an additional way to bind to vascular selectins (Zarbock et al., 2011; Bargatze et al., 1994; Sperandio et al., 2003). Once neutrophils are bound to the endothelium they have to resist the shear stress of the blood flow. Additional bindings to ICAM-1 through their receptor lymphocyte function-associated antigen 1 (LFA-1, also known as CD11a/CD18) enhance the bond between neutrophil and endothelium (Zarbock et al., 2011). Neutrophils have to be activated further to arrest, crawl and transmigrate towards the inflammation. The close proximity to endothelial cells allow neutrophils to detect their activation state and chemokines like TNF-α or IL-1β (Summers et al., 2010). This primes neutrophils and enables degranulation and oxidative burst (Nicotinamide adenine dinucleotide phosphate oxidase pathway). Priming also induces G-protein coupled changes in the selectin affinity of the VLA-4 receptor and allow firm adhesion by the interaction with VCAM-1 (Montresor et al., 2013). In addition, chemo attractants induce changes in LFA-1 and macrophage-1 antigen receptors to allow crawling and finally...
arrest of neutrophils (see review (Phillipson et al., 2006). After firm adhesion neutrophils can transmigrate through the vascular endothelium either by paracellular or transcellular migration, using the later preferentially in the central nervous system (Ley et al., 2007).

In the last decades it was well accepted, that neutrophils enter the brain parenchyma after recruitment and damage neurons through direct interactions, oxidative burst or protease secretion (Rosenberg, 2009; Zhang RL et al., 1994; Hallenbeck et al., 1986). Pericytes in the neurovascular unit recognize DAMPs, release chemokines, manipulate neutrophil activation and recruitment and guide them towards a sterile injury (Stark et al., 2012). In recent studies it was proposed that neutrophils enter the brain to a far less extent than previously thought, but rather remain at the luminal side and in the perivascular space of cerebral vessels (Enzmann et al., 2013; Perez-de-Puig et al., 2015). These studies were supported by human post-mortem tissue samples showing the same results. This indicates that neutrophils contribute to ischemic tissue damage, while acting on the BBB and compromise vascular function, rather than on neurons and in the brain parenchyma directly.

**Figure 3:** The proposed recruitment cascade of neutrophils to the brain after injury. Neutrophils tether to the endothelial P-selectin via their P-selectin glycoprotein ligand 1 receptor, while they sense chemokines. Additional binding to integrins, like ICAM-1 and VCAM-1 results in a more pronounced binding and rolling along the vasculature. After VLA-4/VCAM-1 mediated firm adhesion neutrophils can crawl towards chemokine gradients and transmigrate through the endothelial layer.

**1.1.4. The role of neutrophils after cerebral ischemia**

**Neutrophil function**

Neutrophils build during their development process secretory vesicles and granules, filled with pro-inflammatory proteins. Since these vesicles contain different enzymes and receptors, which would be degraded from enzymes of other granules, like neutrophil gelatinase-associated lipocalin would be
digested from neutrophil elastase, they are built during different maturation stages (Borregaard, 2010). The vesicles are classified due to their main content and the formation sequence to azurophilic (primary), specific (secondary), gelatinase (tertiary) and secretory granules. Primary granules contain mainly myeloperoxidase, secondary are enriched with lactoferrin and tertiary granules contain matrix metalloproteinase (MMP) 9 (Kolaczkowska and Kubes, 2013). The secretory vesicles contain various receptors and plasma proteins. Upon cell activation they can fuse rapidly with the cell membrane and present the receptors on the surface (Borregaard et al., 1990). Depending on the stimulus neutrophils phagocyte the pathogen and fuse the granule with the phagosome or the granules fuse with the cell membrane and release their toxic content into the extracellular space (Figure 4). With their granule neutrophils have an effective anti-bacterial arsenal, but excessive release, for example after DAMP recognition in stroke also leads to host tissue injury and to the breakdown of barriers (Rosell et al., 2008).

Figure 4: The killing mechanisms of neutrophils. Neutrophils can fight pathogens in three different ways. Neutrophils can phagocytose cell debris and pathogens. Another effective anti-pathogenic mechanism is the degranulation of neutrophils releasing proteases and causing the oxidative burst. Just previously it has been described that neutrophils can trap and kill bacteria while releasing DNA spiked with anti-bacterial proteins during NETosis. These mechanisms meant to fight bacterial infections might lead to excessive tissue damage in sterile injuries, where neutrophils are involved.

Pro-coagulant action of neutrophils

During their recruitment neutrophils are thought to induce intravascular clogging and contribute to the no reflow phenomenon after recombinant tissue plasminogen activator treatment in patients in a
small fraction of capillaries (del Zoppo et al., 1991; Dawson et al., 1996). And indeed neutrophils have been found to arrest and accumulate in small vessels and prevent blood circulation (del Zoppo and Mabuchi, 2003), while in the early recruitment phase neutrophils contribute to thrombus formation by releasing tissue factor (Darbousset et al., 2012). The same authors further reported, that the underlying process of tissue factor release is the neutrophil endothelial interaction via LFA-1/ICAM-1 inducing the extrinsic coagulation pathway. Cathepsin G and elastase released by neutrophils degrade extracellular matrix proteins causing the breakdown of the BBB (Armao et al., 1997) and activation of coagulation factors in rodents (Massberg et al., 2010; von Brühl et al., 2012). In a mouse model of cerebral ischemia the outcome could be improved by inhibition of Cathepsin G, while reduced thrombus formation was observed (Faraday et al., 2013). Neutrophils further build clusters with platelets over P-selectin glycoprotein ligand 1/P-selectin, these clusters are also found in stroke patients (McCabe et al., 2005). This interaction lead to further neutrophil activation, with degranulation and maybe also thrombo-inflammation (Sreeramkumar et al., 2014). In addition, activated neutrophils tend to release DNA to form extracellular traps, which have been shown in mice to partake in deep vein thrombosis (Brill et al., 2012). Other studies showed the presence of extracellular traps in experimental stroke (Perez-de-Puig et al., 2015). Furthermore, DNAse treatment in a rodent model of cerebral ischemia reduced the extent of cerebral injury (De Meyer et al., 2012). Extracellular traps include histones, chromatin and enzymes, which are known to have prothrombic and cytotoxic effects on the surrounding endothelial tissue (De Meyer et al., 2012).

**Proteolysis/BBB damage**

The disruption of the BBB is associated with cerebral oedema and haemorrhagic transformation followed stroke. Neutrophils are the main source of ROS early after cerebral ischemia/reperfusion. Free ROS and reactive nitrogen species contribute to the damage of the BBB, while damaging endothelial cells, smooth muscle cells and astrocytes (Forster et al., 1999; Garcia-Bonilla et al., 2014; Jickling et al., 2015). Edaravone, a ROS scavenger, was shown to reduce the ROS load of neutrophils in blood samples of stroke patients (Aizawa et al., 2006), while patients treated with Edaravone had an increased risk of hemorrhagic transformation (Mishina et al., 2008).

Activated neutrophils release MMPs following stroke, which degrade the extracellular matrix and can cause the breakdown of the BBB, increase the risk of hemorrhage, and induce neuronal death (Cunningham et al., 2005). Neutrophil recruitment and activation starts within 2 hours after cerebral ischemia (Kataoka et al., 2004; Hallenbeck et al., 1986), and is accompanied by elevated MMP plasma values within 2 to 6 h after cerebral ischemia in rodents, primates and humans (Heo et al., 1999; Kelly et al., 2008). Early inhibition of MMP-9 reduces in animal models of cerebral ischemia BBB breakdown and hemorrhagic transformation and improves outcome. The upregulation of MMPs after
recombinant tissue plasminogen activator treatment is thought to be the main cause/mediator of hemorrhagic transformations (Wang et al., 2004).

**Anti-inflammatory roles of neutrophils**

Neutrophils have for a long time been considered to be only pro-inflammatory. In a recent study it was shown that neutrophils can switch from their pro-inflammatory phenotype to a M2 like anti-inflammatory phenotype and contribute to inflammation clearance and neuroprotection (Cuartero et al., 2013). While they phagocyte debris and pathogens they also clear the wound and make space for repair and rebuilding mechanisms. Recently, it have been shown, that neutrophils can secrete large amounts of IL-10 and control the inflammatory response after bacterial infection or in cancer (Zhang et al., 2009; De Santo et al., 2010). The release of MMP-9 is a double-edged sword and can promote inflammation clearance by degrading DAMPs, like actin and high mobility group binding protein 1, beside extra cellular matrix (Cauwe et al., 2009). VEGF released by neutrophils gets activated by MMP-9 under hypoxic conditions and induce angiogenesis in the injured tissue, helping to restore blood flow over time (Christoffersson et al., 2012). During neutrophil degranulation cathepsin G and azurocidin (heparin-binding protein) is released and activates the formyl-peptide receptor on monocytes leading to their recruitment (Soehnlein and Lindbom, 2009; Soehnlein et al., 2008). In addition, neutrophils activate endothelial cells via gp130 and release during apoptosis “find and eat me” signals and further attract monocytes (reviewed in (Soehnlein and Lindbom, 2010).

Besides the exacerbation of tissue damage neutrophil depletion might pose a therapeutic strategy to ameliorate ischemic damage. However, it has been shown in the last years that this is not straightforward. The anti-inflammatory contribution of neutrophils has been underestimated for a long time and is a possible explanation that clinical stroke trials targeting neutrophils have failed. All these anti-inflammatory effects discovered in animal models might be in humans even more pronounced due to species-specific differences in leucocyte composition. While in human 50-70% of the circulating leucocytes are neutrophils only 10 to 25% of the blood leukocytes are neutrophils in mice (Doeing et al., 2003; Mestas and Hughes, 2004).

Despite a tremendous progress in deciphering the mechanism of inflammation after cerebral ischemia still large gaps exit in how neutrophils contribute to the ischemic tissue damage. Generally, knowledge about leukocyte function relies mainly on cell culture work, and in vivo data that elucidate their function in the brain is till scarce. Immunohistochemistry studies have suffered from a lack of specificity of reagents and cannot capture the immediate dynamics of cell recruitment. Flow cytometry is state-of-the-art for studying function, morphology, composition, proliferation, and protein expression in circulating leukocytes. But the extraction of cells from a living organism may lead to alterations in cell
properties. Moreover, while cells are easily isolated from blood samples, obtaining brain tissue samples requires sacrificing the animal under study. This prevents the long-term study of cells and their interactions in their native biological environment. The feasibility to visualize neutrophils in vivo with two-photon microscopy has been demonstrated (Hasenberg et al., 2015). However, for accessing the brain either a thinned skull region or the preparation of a cranial window are required, which may induce tissue damage and disturb physiology. In addition the method is technically limited to retrieve information from a small field of view and to a depth of ≥500 µm only. Advances in optical imaging instrumentation over the past few years have enabled the macroscopic imaging of physiological and cellular processes by NIRF imaging in the intact animal. While the application of optical imaging in vivo remains challenging, the tools can provide unprecedented pathophysiological insights of the role of neutrophils after cerebral ischemia.
1.2. Using non-invasive optical imaging to study cerebral ischemia in mice

The following introduction paragraph 1.2 is adapted from the book chapter:

Non-invasive optical imaging in rodent models of stroke


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Contribution: generating Pictures, text editing, proof reading
1.2.1. Abstract
With recent advances in optical imaging instrumentation and reconstruction algorithms, preclinical optical imaging has become almost a commodity in biomedical research. Given the availability of specific fluorescent probes and reporter gene technologies, which allow the non-invasive visualization of a variety of pathophysiological processes. The technology might also pose an attractive research tool for preclinical stroke research. This chapter gives a practical overview about NIRF imaging, provides examples about applications of methods in rodent models of stroke and discusses practical aspects and limitations.

1.2.2. Introduction
The development of new optical imaging devices over the past decade has facilitated the translation of optical imaging from microscopic scale to macroscopic level imaging. Novel probes and reporter gene assays have become available, which allow the visualization of a variety of pathophysiological processes in the intact animal. The technology is particularly suited for the use in rodent models. Specific pathophysiological processes can be visualized in vivo, while all regulatory processes are preserved in the live animal, and without impeding animal physiology and welfare. Moreover, the non-invasiveness of the method allows combining it with other readouts, for example, behavior or MRI in the same animal, thus reducing the overall number of animals needed for preclinical studies. In addition, it allows repetitive imaging to establish the dynamics of a process over the disease course, thus improving statistical power, and to monitor the response to therapeutic intervention. In this chapter, the application of NIRF imaging is briefly described in regard to mice models of cerebral ischemia. Furthermore, practical aspects and limitations of the technique will be discussed.

1.2.3. Near-Infrared Fluorescence Imaging
NIRF Instrumentation and Techniques
NIRF imaging makes use of the fact that major absorbers in the tissue like water and hemoglobin have their lowest absorption coefficient in the NIR range (650–900 nm), thus allowing NIR photons to penetrate deep into tissue (Figure 5). In addition, structural components in animal tissue have low autofluorescence emission in the NIR range compared to the visible and mid-infrared (Billinton and Knight, 2001). This gives the unique opportunity of NIR light to detect fluorochrome distribution in biological tissue using sophisticated hardware and reconstruction algorithms.
Figure 5: Absorption spectra of water and hemoglobin, the main absorbers in the tissue. Optical imaging can be performed on the surface of animals almost without restriction to the emission wavelengths of the fluorochrome used. For deep tissue imaging (>500 nm) fluorescent probes within the optical window (650 to 900 nm), where tissue absorption is lowest, have to be used. Adapted from (Joshi and Wang, 2010).

A number of NIRF imaging devices with different setups and modes of data acquisition have been developed and are commercially available. The standard NIRF imaging instrumentation consists of an excitation light source, e.g., a laser diode, and fiber optics to direct the excitation light to the animal under study (Figure 6). The emitted fluorescence is captured using a charge-coupled device camera fitted with adequate emission filters and a lens to adjust the focal plane. By choosing different combinations of excitation sources and emission filters, multichannel imaging in the same animal can be achieved (Kobayashi et al., 2007).
Figure 6: A standard planar NIRF imaging system (a, b, Maestro 500 multispectral imaging system, Cambridge Research & Instruments Inc., Woburn, USA). The system consists of fiber optics (1) which direct the excitation light to the animal under study. The emitted fluorescence is captured using a charge-coupled device camera (2) fitted with adequate emission filters and a lens to adjust the focal plane (3). The mouse is placed onto a heated support to maintain body temperature (4). Volatile anesthesia is provided in an air/oxygen mixture via a nose cone (5). The whole set-up is placed in a light-tight container (6). A white light image of a depilated C57BL6 mouse head taken with a charge-coupled device camera (c).

The most common method for NIRF imaging is to illuminate the object with a plane wave referred to as planar NIRF imaging. Planar NIRF imaging can be applied both in reflectance and transillumination mode. In fluorescence reflectance imaging, the excitation light source and the detector are on the same side of the object. The excitation photons propagate a few millimeters below the surface of the object. Scattered excitation and emission photons travel back to the surface. In transillumination fluorescence imaging, the excitation light source and the detector are on opposite sides. The advantage of transillumination fluorescence imaging is that the entire volume of the object is sampled upon the passage of the excitation light. Furthermore, reflection of excited photons and autofluorescence of the skin is reduced in transillumination fluorescence imaging compared to fluorescence reflectance imaging (Ntziachristos et al., 2005).

Planar NIRF imaging methods have the advantage that they require relatively inexpensive instrumentation (systems are commercially available or can be self-built), are easy to operate, and facilitate high-throughput imaging of animals. However, planar NIRF imaging methods have some limitations: positioning of animals and adjustments of the imaging system should be set reproducibly and checked (e.g. use of reference standards) to obtain comparable data sets. In planar NIRF imaging absorption and scattering of photons in the tissue (which can both change under pathological conditions) make quantification of fluorescence emission challenging and limits the spatial resolution to about 2–3 mm. In addition, tissue autofluorescence might increase as a result of tissue remodeling after injury (e.g. ischemia). The obtained images are two-dimensional and surface-weighted.
Moreover, the detected fluorescence emission depends linearly on the fluorochrome concentration and non-linearly on the depth of the fluorescent object and the optical properties of the surrounding tissue. As a consequence, planar NIRF images do not allow for absolute quantification of the detected fluorescence. Hence, in most studies, the fluorescence emission is expressed in terms of contrast, i.e., as a ratio of the fluorescence intensity measured over the disease affected region to the intensity measured over a non-affected region, termed target-to-background ratio (Klohs et al., 2006).

Quantitative analysis of fluorescence emission can be achieved using fluorescence molecular tomography. In contrast to fluorescence reflectance imaging and transillumination fluorescence imaging, fluorescence molecular tomography uses a point laser source instead of planar ones. By scanning the laser point on the surface, coupled excitation and emission images are sequentially recorded. Based on the diffuse light propagation model, reconstruction leads to an improved image resolution of the order of 1 mm in all three dimensions as compared to planar NIRF techniques (Bourayou et al., 2008; Ntziachristos et al., 2002). The combination of fluorescence molecular tomography with other imaging modalities like MRI and CT can be used to allocate imaging signals on low-resolution NIRF imaging data to a specific anatomical location (Hyde et al., 2009; Stuker et al., 2011). In addition, the use of anatomical information as prior knowledge can improve the reconstruction accuracy of fluorescence molecular tomography data.

**NIRF Imaging Probes and Reporter Genes**

A large number of fluorochromes emitting in the NIR including cyanine dyes, lanthanide chelates, squaraines, tetrapyrrole-based probes, quantum dots, and others are available (Klohs et al., 2008b). The fluorochromes can be used for the generation of NIRF probes by conjugation to ligands such as antibodies, antibody fragments, oligonucleotides, peptides, and enzyme substrates or by labeling cell populations (Figure 7). For the generation of bright fluorescent probes, fluorochromes with both a high extinction coefficient and quantum yield should be chosen. Probe generation might be simple: for example, the labeling of cysteine residues with N-hydroxsuccinimide esterification of NIR dyes or might involve complex chemistry. After labeling of compounds, it should be tested that the labeling procedure has not led to an alteration of ligand properties (binding affinity, solubility, etc.). Cells can be labeled by using membrane-bound NIR dyes or fluorochromes, e.g., nanoparticles, which are internalized by the cells. Alternatively cells can be transfected to express an NIR fluorescent protein as a reporter gene assay (Filonov et al., 2011). The advantage is that transfected cells do not lose signal intensity after cell division as compared to cells, where fluorochromes that are membrane bound or internalized. However, the metabolic stability and toxicity of the fluorochrome (e.g. quantum dots) should be considered. Also labeled or transfected cells need to be tested for their viability and proper function. High amounts of dye, for example, internalized in cells or bound to the cell membrane, might
compromise cell function and lead to cell death and can also lead to a quenching of the dye with a decrease in fluorescence intensity.

**Nonspecific probes**

Nonspecific probes can be used to visualize perfused blood vessels, vascular dysfunction and renal elimination. Prominent examples are indocyanine green or quantum dots.

**Targeted probes**

Targeted probes bind specific molecules expressed on cell surfaces and accumulate on the side of interest. This probes are commonly used to visualize inflammatory processes or the over-expression of proteins. Labeled antibodies, antibody fragments or proteins binding to certain structures are widely used.

**Activatable probes**

Activatable or also called smart probes can resolve proteolytic enzyme activities, while the background of the circulating probe is low through quenching. Once probes get cleaved by its specific enzyme probes emitted light after excitation. Activatable and targeted probes can be combined to improve signal specify and signal-to-noise ratio.

*Figure 7: Available fluorescent probes for in vivo imaging.*

1.2.4. Non-invasive Optical Imaging in Small Animal Models of Stroke

Successful NIRF imaging critically depends on the pharmacokinetics and biodistribution of the injected probe. Cerebral ischemia initiates a plethora of pathophysiological processes which are potential targets for the visualization with optical imaging. Some examples of optical imaging in rodents are presented with focus on cerebral ischemia.
Examples of NIRF Imaging of Stroke Pathophysiology

NIRF Imaging of Hemodynamic Dysfunction and Vascular Thrombosis

NIRF imaging can be used to non-invasively assess hemodynamic parameters in the rodent brain and can hence be employed to assess the perfusion status in experimental stroke. Ku et al. have shown that planar NIRF imaging can be performed dynamically during the intravenous bolus administration of indocyanine green (Ku and Choi, 2012). Hemodynamic parameters are computed from the dynamics of the passage of the bolus of dye through the brain. Using this technique, persisting perfusion deficits were revealed in the ischemic territory of the mouse brain after middle cerebral artery occlusion (MCAO) (Ku and Choi, 2012) and photothrombotic stroke (Kang et al., 2015). However, the low spatial resolution and lack of three-dimensional information of planar NIRF imaging limits considerably the information about the spatial extent of hemodynamic dysfunction that can be obtained with this method. Different approaches might aim to directly visualize molecular mediators underlying the perfusion deficit. For example, Zhang et al. have used an FXIIIa-targeted NIRF probe to visualize secondary thrombosis after inducing a thromboembolic stroke in mice with planar NIRF imaging (Zhang et al., 2012). The coagulation factor FXIIIa and fibrinogen are the key proteins involved in intravascular fibrin formation. NIRF imaging revealed a time-dependent increase in fluorescence over the ischemic hemisphere. Such approaches might be useful to monitor the effects of recanalization therapies in stroke models in vivo.

NIRF Imaging of Inflammation and Tissue Remodeling

Ischemic stroke initiates complex and dynamic inflammatory processes that occur hours to weeks after the onset of ischemia. Inflammation has been implicated in secondary lesion growth but also to partake in repair and recovery of the tissue. A study by Klohs et al. demonstrated that stroke-induced inflammation after MCAO in mice can be visualized after injection of a fluorescently labeled monoclonal antibody against the CD40-receptor (Klohs et al., 2008a). NIRF imaging showed high target-to-background ratio in the brain of MCAO mice injected with the CD40 receptor antibody compared to controls. Histological workup revealed that the fluorescence detected within the ischemic lesion with NIRF imaging was attributed to the presence of blood-derived Iba1+ mononuclear phagocyte that have taken up the labeled antibody. The use of whole-labeled antibodies however requires long washout times of unbound probes, and approaches using antibody fragments might be more suitable to achieve contrast more rapidly.

Several studies have employed NIRF imaging to visualize MMP activity in stroke models. MMPs, in particular MMP-2 and MMP-9, are upregulated after cerebral ischemia and have been implicated in BBB damage and hemorrhagic transformation as well as in tissue regeneration and repair (Wang et al., 2004; Zhao et al., 2006). Klohs et al. have shown that MMP activity after MCAO can be visualized with
planar NIRF imaging using a MMP-activatable probe (Klohs et al., 2009a). The effects of therapeutic interventions on MMP activity using this approach has been investigated in several studies. The administration of recombinant tissue plasminogen activator increases MMP activity in the ischemic brain as detected with NIRF imaging (Takamiya et al., 2012; Tian et al., 2013). The co-administration of a ROS scavenger or the intravenous administration of bone marrow stromal cells can ameliorate this effect. In contrast, hypothermia, which is neuroprotective after ischemia, leads to a reduction of MMP activity (Barber et al., 2012).

Bai et al. have used a $\alpha_\text{v}$$\beta_3$-targeted nanoprobe to investigate angiogenesis after MCAO in mice with NIRF imaging (Bai et al., 2014). They showed that most angiogenesis took place after 10 days after MCAO and that signal increase was not related to unspecific accumulation due to BBB impairment. In contrast, fluorescently labeled bovine serum albumin has been used to visualize BBB impairment after MCAO (Abulrob et al., 2008; Klohs et al., 2009b). The approach can be useful to non-invasively assess the status of the BBB after experimental stroke, which might be relevant for treatment studies. The breakdown of the BBB leads to secondary damage and cell death. Bahmani et al. demonstrated that cell death can be detected in mice after MCAO with NIRF imaging using labeled annexin A5 as probe (Bahmani et al., 2011).

NIRF Imaging the Fate of Cell Transplants

Attempts are made to use cell-based therapies in stroke. Due to its sensitivity, NIRF imaging is ideally suited to track the fate of labeled cells in rodent models of stroke. Examples have demonstrated that marrow stromal cells can be labeled with quantum dots (Sugiyama et al., 2011; Kawabori et al., 2013). Cells were transplanted into the ipsilateral striatum of rats after permanent MCAO, and engraftment of cells into the ischemic territory for up to 8 weeks was monitored. Cell tracking approaches might also be useful to study the role of specific immune cell populations.
2. Objectives and Outline
The aim of this PhD thesis was to characterize the neutrophil dominated inflammatory response after cerebral ischemia. Specifically, I tested whether NIRF imaging can be used:

1. To monitor the recruitment of neutrophils to the ischemic brain territory in a temporal-spatial resolved manner (addressed in Chapter 3.1).
2. To elucidate the roles of the α4-integrin subunit of VLA-4 and of ICAM-1 in neutrophil recruitment to the ischemic brain (addressed in Chapter 3.1 and 3.2).
3. And to clarify when blocking VLA-4 or ICAM-1 had a protective effect with regard to the infarct volume (addressed in Chapter 3.1 and 3.2).

A secondary objective of this thesis was to investigate the effect of ECA ligation on the extra-cerebral tissue in the tMCAO model (addressed in Chapter 3.3).
Chapter 3

3. Results
3.1. Non-invasive near-infrared fluorescence imaging of the neutrophil response in a mouse model of transient cerebral ischemia

Adapted from:

Non-invasive near-infrared fluorescence imaging of the neutrophil response in a mouse model of transient cerebral ischemia

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3.1.1. Abstract

NIRF imaging enables non-invasive monitoring of molecular and cellular processes in live animals. Here we demonstrate the suitability of NIRF imaging to investigate the neutrophil response in the brain after tMCAO. We established procedures for ex vivo fluorescent labeling of neutrophils without affecting their activation status. Adoptive transfer of labeled neutrophils in C57BL/6 mice before surgery resulted in higher fluorescence intensities over the ischemic hemisphere in tMCAO mice with NIRF imaging when compared to controls, corroborated by ex vivo detection of labeled neutrophils using fluorescence microscopy. NIRF imaging showed that neutrophils started to accumulate immediately after tMCAO, peaking at 18h, and were still visible until 48h after reperfusion. Our data revealed accumulation of neutrophils also in extracranial tissue, indicating damage in the ECA territory in the tMCAO model. Antibody mediated inhibition of α4-integrins did reduce fluorescence signals at 18 and 24, but not at 48h after reperfusion, compared to control treatment animals. α-integrin antibody treatment reduced cerebral lesion volumes by 19%. In conclusion the non-invasive nature of NIRF imaging allows studying the dynamic of neutrophil recruitment and its modulation by targeted interventions in mouse brain after transient experimental cerebral ischemia.

3.1.2. Introduction

Transient cerebral ischemia is followed by an inflammatory response that has been implicated to exacerbate ischemic injury, but also to provide the necessary environment for regeneration and repair (Endres et al., 2008). In this regard the role of neutrophils following cerebral ischemia remains controversial. Histological studies have revealed recruitment of neutrophils to the ischemic lesion in experimental models of cerebral ischemia and patients with ischemic stroke at time points when substantial neuronal death occurs (Zhang RL et al., 1994; Ley et al., 2007; Dereski et al., 1992). Treatments that prevented vascular adhesion of neutrophils (Matsuo et al., 1994a; Zhang et al., 1995), as well as neutropenia (Bednar et al., 1991; Chen et al., 1992), were found to result in a reduction of the ischemic damage in preclinical studies of cerebral ischemia. However, clinical trials using ligands or antibodies to prevent neutrophil infiltration into the brain did not show clinical benefits (Enlimomab Acute Stroke Trial Investigators, 2001; Krams et al., 2003), questioning the pathogenic role of neutrophils.

Neutrophils have been implicated in launching and shaping the immune response following injury. Activated neutrophils have been shown to induce superoxide production, degranulation (Berton et al., 1996), and release specific proteases e.g. neutrophil elastase and MMP (Gidday et al., 2005; Allen et al., 2012). In addition, neutrophils interact with platelets, participate in fibrin cross-linkage and trigger
thrombin activation by inducing the extrinsic tissue factor/FVIIa pathway and can thereby predispose the microvasculature to thrombosis (De Meyer et al., 2016). It could therefore be hypothesized that neutrophils play key roles in cerebral ischemia injury, mainly by exerting effects on the cerebral vasculature, but direct in vivo proof is still missing. This lack of knowledge might largely stem from the paucity of techniques that are able to specifically visualize neutrophil trafficking and function in vivo. Histological studies (e.g. myeloperoxidase staining) are often not specific for neutrophils and cannot capture the dynamic and complex (inter-)actions of cells. Attempts to block neutrophil migration in vivo might have suffered from lack of specificity, i.e. other leukocyte subsets might have been blocked as well (Liesz et al., 2011), while neutrophil depletion might have caused confounding immunological side effects (Stephens-Romero et al., 2005; Jaeger et al., 2012).

More recently, the use of two-photon microscopy enabled the intravital tracking of neutrophils in the leptomeninges and superficial parts of the cortex of the mouse with high resolution (Hasenberg et al., 2015), and its application to track neutrophils in a mouse model of tMCAO has been recently shown (Neumann et al., 2015). The technique is capable to monitor local neutrophil dynamics and allows to directly observe the manipulation of cell trafficking. However, two-photon microscopy of the mouse brain requires a thinned skull region or, for assessing deeper structures, the preparation of a cranial window, which may lead to immediate disturbances in local blood perfusion, BBB permeability or mechanical injuries to the cortical surface and bleedings (Holtmaat et al., 2009). In addition the method is technically limited to retrieve information from a small field of view and to a depth of ≤500 µm, and is thus not capable to spatially resolve the neutrophil response in the entire brain.

Recent advances in instrumentation and image reconstruction have led to the emergence of NIRF imaging as a technique that can visualize pathological processes in intact animals using dedicated fluorescent probes and reporter technology (Wunder and Klohs, 2008). NIRF imaging uses light in the spectral range of 700-900 nm, in which absorption of endogenous absorbers like oxy- and deoxygenated hemoglobin and water is lowest and photons can penetrate deeply into living tissue. The technique has been shown to detect fluorochromes in the brain of mice with high sensitivity (Klohs et al., 2006; Bourayou et al., 2008). NIRF imaging constitutes an attractive tool for investigating mouse models of cerebral ischemia. The non-invasiveness of the technique allows to study cellular processes in the live animal with all regulatory processes preserved, without impeding animal physiology and welfare, thereby enabling repetitive assessment of the same animal. NIRF imaging has already been applied to visualize a variety of disease relevant processes (Klohs et al., 2008a, 2009b; a; Kim et al., 2011a; Bahmani et al., 2011; Barber et al., 2012).
Here we present an approach using adoptive transfer of fluorescently labeled neutrophils to monitor the dynamics of neutrophil accumulation in the mouse brain after the tMCAO with planar NIRF imaging. Our data revealed accumulation of neutrophils in the ischemic brain but also in extracranial tissue. Moreover, we employed NIRF imaging to assess the role of α4-integrins in mediating neutrophil accumulation to the ischemic brain in vivo.

3.1.3. Materials and Methods

Animals and Treatment

All procedures conformed to the national guidelines of the Swiss Federal act on animal protection and were approved by the Cantonal Veterinary Office Zurich (Permit Number: 18-2014 and 49-2011). We confirm compliance with the ARRIVE guidelines on reporting animal experiments.

Male C57BL/6J mice (Janvier, France), weighting 20-25g, of 8-10 weeks of age were used, randomly assigned to the experimental groups. Animals were housed in a temperature controlled room in individually ventilated cages, containing up to 5 animals per cage, under a 12-hour dark/light cycle. Paper tissue was given as environmental enrichment. Food (3437PXL15, CARGILL) and water were provided ad libitum.

Experimental protocols

Using G*Power 3.1 software (Heinrich-Heine-Universität, Düsseldorf, Germany; http://www.gpower.hhu.de/), a sample size of n≥10 per group was calculated a priori for the primary end point normalized fluorescence intensities over the ipsilateral brain at 18h after reperfusion, assuming an effect size d=1.7, at α=0.05 and β=0.2. A total of 26 mice received intravenously either rat anti-α4-integrin antibody (n=13) or isotype control (n=13).

VLA-4-mediated adhesion of neutrophils to endothelial VCAM-1 was blocked by i.v. infusion of rat-anti mouse α4-integrin antibodies (clone PS/2, 100 µg/mouse) 15 min prior to surgery, while control mice received rat-anti mouse endoglin (clone MJ7/18, 100 µg/mouse). Both antibodies were generated endotoxin-free in house (Theodor Kocher Institute, Bern). No randomization was applied. Just before antibody injection LIPO-6S-IDCC labeled neutrophils were i.v. injected. Animals were serially imaged at 18, 24, and 48 h after 1 h of tMCAO. At the endpoint of the experiment mice were euthanized and brains were harvested and cryo-embedded for immunohistochemistry.
Focal cerebral ischemia

Transient MCAO was induced using the intraluminal filament technique (Hata et al., 2000b). Anaesthesia was initiated by using 3% isoflurane (Abbott, Cham, Switzerland). Anesthesia was maintained with 1.5-2% isoflurane in a mixture of O₂ (200 ml/min) and air (800 ml/min), supplied via face mask.

Before the surgical procedure, a local analgesic (Lidocaine, 0.5%, 7 mg/kg) was administered subcutaneously (s.c.). Temperature was controlled during the surgery and kept constant at 37±0.5°C with a feedback-heating controlled pad system. The area around the neck was shaved and disinfected.

For tMCAO, a midline neck incision was made and the left common carotid artery was ligated proximal of the bifurcation of the internal carotid artery (ICA) and ECA. Subsequently, the left ECA was isolated and ligated and a suture was placed around the ICA to temporarily restrict blood flow. A small incision was made in the common carotid artery and an 11mm long and 0.19±0.01 mm in diameter silicone coated monofilament (171956PK5Re, Doccol Corporation, USA) was introduced and advanced until it occluded the MCA. A suture around the ICA secured the filament in position. After occlusion of the vessel, animals were transferred to a heated recovery box (30°C) and allowed to wake up. After 57 min animals were reanaesthetized. The filament was withdrawn after 60 min and the ICA was ligated. Sham-operation involved surgical procedures, without occlusion of the MCA. Therefore the filament was inserted into the ICA, pushed up until the MCA and removed immediately.

After surgery buprenorphine was administered as s.c. injection (Temgesic, 0.1 mg/kg b.w. in 500 μl Saline) and animals were placed in the heated recovery box for 2h. Buprenorphine was then given twice s.c. every 6-8 h on the day of surgery and thereafter supplied via drinking water (1 mg/kg) for 36 h. Animals receive softened chow in a petri-dish placed on the floor of their cages to encourage eating. Animals were excluded from the studies when they fulfilled one of the following criteria: prolonged surgery time (>15 min); no reflow after filament withdrawal; larger bleeding during surgery, dead before experimental endpoint. Mortality rate was 0%.

Characterization of the neutrophil response in the blood

Whole blood (≥50 μl) was collected by saphena vein punctuation using a 23G cannula (Braun, Germany) into EDTA-coated tubes (Sarstedt, Germany) at three times prior to tMCAO (baseline), at 0.5, 1.5, 4, 24 or 48 h after reperfusion or corresponding sham surgery in a random fashion (n≥9 per time point). Samples were incubated at room temperature (RT) for 15 min in the dark with both an anti-Ly6G-PE (clone 1A8) and an anti-Ly6G/Ly6C-APC antibody (clone Gr1, both BD Pharmingen, Germany), followed by a red blood cell lysis buffer containing 1.5% Paraformaldehyde (PFA, 500 μl, 10 min, RT, BD Pharmingen, Switzerland). Probes were kept on ice until they were analyzed with a
Gallios™ Flow Cytometer (Beckman Coulter, Switzerland). Up to 10,000 events were acquired and examined for the expression of Ly6C and Ly6G using the Kaluza Analysis Software (Beckman Coulter, USA). The gating strategy is shown in Figure 8.

Figure 8: Gating strategy to analyze neutrophil counts from blood samples before and after tMCAO. First it was gated for Leukozytes according to their forward scatter (FSC) and side scatter (SSC) properties. A region of interest was drawn around cells labeled positive Ly6C and Ly6G (A). This population was further assessed by their FSC and SSC properties (B). Neutrophils are characterized by high granularity, while monocytes/macrophages lack granules.

Isolation of neutrophils from bone marrow

Mice were sacrificed, femur and tibia from both legs were extracted, cleaned and stored in HBSS. The extreme distal tips of each bone were cut off under sterile conditions. Bone marrow was flushed out with HBSS (no calcium and magnesium; applies to all HBSS used) using a 27G cannula (Braun, Germany).

Cell aggregations were dispersed through pipetting, thereafter sample was centrifuged (400 g, 6 min, 4°C). Supernatant was discarded and red blood cells were lysed with 20 ml of a 0.83 % ammonium chloride and Tris-HCl 9:1 (pH 7.5) buffer for 10 min. Lysis was stopped by adding 30 ml HBSS. Sample was filtrated through a 70 µm nylon cell strainer (BD Biosciences, USA) to remove insoluble particles. The suspension was centrifuged (400 g, 6 min, 4°C) and cell pellet was resuspended in 10 ml HBSS. Leukocytes were separated from cell fragments with a first Percoll (GE Healthcare, Uppsala, Sweden) gradient centrifugation. Therefore, 7 ml Percoll was mixed with 700 µl 10x HBSS (life technologies, Scotland). From this solution 7 ml were mixed with 3.8 ml HBSS to a solution containing 64.8% of Percoll. Each 5 ml were placed in a 15 ml Falcon tube and 5 ml of the cell suspension was slowly
overlaid over the gradient (Figure 9). Per gradient only the cell suspension of one animal was used. Falcons were centrifuged at 1000 g, 20 min at 4°C with lowest acceleration and no break at the end. Two clear distinguishable phases have to be seen in the end. Leukocytes build a white interface layer on top of the Percoll gradient. In the cell pellet mainly cell debris were found. The interface was carefully removed, collected in a new Falcon tube and the tube was filled up with HBSS. After centrifugation (400 g, 6 min, 4°C) the pellet was resuspended in 10 ml HBSS.

![Figure 9: Density gradients to obtain neutrophils from bone marrow. Two density gradients were used to isolate neutrophils from the bone marrow. The first gradient, containing 64.8% of Percoll, served to eliminate all kind of cell fragments and remaining red blood cells (A). The obtained leukocytes were resuspended in 5 ml HBSS and layered over the second gradient (61.5% Percoll; B). After centrifugation neutrophils were found in a slightly white cloud at the bottom.](image)

The second Percoll gradient contain 61.5% Percoll and was generated with 6 ml Percoll and 600 µl 10x HBSS. From this solution 6.5 ml were mixed with 4.1 ml HBSS and each 5 ml were transferred into new tubes. Leucocyte solution was pipetted over the gradients and two clear separated phases were visible. Samples were centrifuged at 1000 g, 35 min at 4°C with lowest acceleration and no break at the end. In the interface PBMCs were enriched and neutrophils were found in a slight cloud at the bottom of the falcon. With a pipette 2 to 2.5 ml of the Percoll neutrophil solution were sucked up from the bottom and transferred into a new Falcon. The tube with the neutrophils was filled up to 15 ml with HBSS. To calculate the cell amount 10 µl suspension was transferred into a hemocytometer (Neubauer Chamber, Thermo Fisher Scientific, Switzerland). Tubes were centrifuged (400 g, 6 min, 4°C) to remove Percoll. Cells were resuspended in HBSS to a final concentration of 0.2*10^6 cell/10 µl. We obtained 5±1.5×10^6 neutrophils per mouse.
Fluorescent labeling of neutrophils and viability assay

To identify a suitable NIRF dye for neutrophil labeling, neutrophils were incubated in HBSS (0.2x10^6 cells/10 μl) for 15 min at RT together with either 5, 10, 25 or 50 μg/ml of VivoTrack680 (Perkin Elmer, USA) or LIPO-6S-IDCC (Freie Universität Berlin, Germany). Both dyes have their excitation maximum at around 680 nm and their emission maximum at around 710 nm. To establish the optimal LIPO-6S-IDCC concentration, neutrophils were incubated with either 5, 10, 25, 50, 100, 250 or 500 μg/ml of dye for 15 min. To determine the optimal incubation time neutrophils were incubated with 25 μg/ml of LIPO-6S-IDCC for 5, 10, 15, 20, 25, or 30 min. After incubation all samples were washed twice with HBSS (centrifugation 300 g, 6 min; RT) before measurement. Fluorescence was measured using a Gallios™ Flow Cytometer. To assess cell viability, 10 μl of an aqueous solution of propidium iodide (0.1 mg/ml) was added 10 s before the flow cytometric analysis. Electronic gating was used to differentiate viable cells from apoptotic cells.

Neutrophil activation assays

To assure the labeling procedure does not affect the activation stage of labeled neutrophils, an activation assay was performed. LIPO-6S-IDCC labeled or unlabeled neutrophils isolated from two animals were resuspended in Dulbecco’s phosphate buffered saline (PBS) supplemented with 2.5% calf serum and 0.1% NaN₃. Aliquots of 0.3x10⁶ neutrophils per 200 μl were distributed in a 96-well round bottom microtiter plate and centrifuged at 330 g at 4°C for 3 min. The pellets were dislodged and incubated with the following directly conjugated rat antibodies at 10 μg/ml for 30 min on ice: CD45 APC (BioLegend 103112, clone 30F11); Ly6G APC/Cy7 (BioLegend127624, clone 1A8); LFA-1 FITC (CD11a, BD 01204D, clone 2D7); macrophage-1 antigen PB (CD11b, BioLegend 101224, clone M1/70); CD49d PE (integrin-α4, Southern Biotech 1520-09, clone PS/2); P-selectin glycoprotein ligand 1 AF647 (clone 4RA10, conjugated in house); L-selectin FITC (CD62L, BD 553150, clone Mel-14). In parallel, selected aliquots were incubated with appropriate isotype controls. Lastly, all cells were washed several times with FACS buffer, fixed with 1 % PFA/PBS for 10 min, washed twice with FACS buffer and pellets were resolved in 200 μl FACS buffer. Cells were measured and analyzed using flow cytometry.

NIRF imaging

NIRF imaging was performed with the Maestro 500 multispectral imaging system (Cambridge Research & Instruments Inc, USA). A band pass filter (615-665 nm) was used for excitation. The fluorescence was detected by a charge-coupled device camera fitted with a long pass filter (700 nm). Fluorescence emission images were acquired by automatized incrementally increasing the excitation wavelengths over the indicated range. NIRF images were subjected to spectral unmixing using the CRI Maestro Image software.
**Determination of detection sensitivity in a silicone phantom**

A tissue like silicone phantom was produced as previously described to estimate the detection limit of LIPO-6S-IDCC labeled neutrophils in deep tissue (Stuker et al., 2011). The phantom was based on two components (A and B) of room temperature vulcanizing silicone (Wacker Silicone, Germany). Carbon Black and TiO₂ particles (both Alfa Aesar, Germany) were used to create an absorption coefficient (μₐ) of 0.2 cm⁻¹ and a scattering coefficient (μₛ) of 10 cm⁻¹, which were adapted to match the optical properties of the brain. Silicone mass was filled in a mold and a vacuum was applied to remove bubbles. The silicone was left in the mold for 24 h for hardening. Exchangeable micropipettes (volume 1 μl per 15 mm, Brand GmbH & Co. KG, Germany) were placed in a hole at 2 mm depth parallel to the surface of the phantom (Figure 13). Micropipettes containing either 750, 1000, 1750, 2500, 5000 or 10,000 neutrophils/μl were placed within the phantom for NIRF imaging. Image analysis consisted of drawing a region of interest (ROI) over the area containing the neutrophil suspension, measuring the average fluorescence intensity of all pixels within the ROI divided by the factor of their area.

**Near-infrared fluorescence imaging in vivo**

LIPO-6S-IDCC-labeled neutrophils (7.5x10⁶±0.5x10⁶ cells) were adoptively transferred by intravenous injection 15 min prior to tMCAO or sham surgery. To establish the time course of neutrophil accumulation 31 mice were serially imaged in a staggered design up to 7 times within 48 h (2, 4, 8, 12, 18, 24 and 48 h after tMCAO; sham n=9-15 and tMCAO n=7-10). For the anti-α₄-integrin treatment study mice were serially imaged at 18h (isotype control n=11; rat anti-α₄-integrin n=10), 24 h (isotype control n=12; rat anti-α₄-integrin n=11), and 48 h after tMCAO (isotype control n=12; rat anti-α₄-integrin n=12). In few animals we missed the time point for imaging. Mice were anesthetized with 1.5-2% isoflurane in an oxygen/air mixture (1:4) and the skin overlying the head was depilated. Animals were placed onto an animal support into which a warm-water circuit was integrated to maintain body temperature. The head was always the same way aligned to reduce signal artifacts and to ensure maximum comparison of the data.

ROIs were manually drawn over the right and left hemisphere and temporal muscles (Figure 14A). The person performing the analysis was blinded to the experimental group. The average fluorescence intensity of all pixels within the ROI were measured and divided by the factor of their area. Normalized fluorescence intensities were calculated by dividing the mean fluorescence intensity of the ipsilateral side by the mean fluorescence intensities of the contralateral side×100.

**Tissue processing**

Animals were put under deep anesthesia by intraperitoneal injection of ketamine/xylazine/acepromazine maleate (100/20/3 mg/kg body weight) and the heart was exposed, while animals did
not show any reflexes. The heart was punctured with a 21G butterfly cannula (Venofix Safty Ref: 4056504-01, Braun, Germany) and the vena cava superior was cut with a sharp object to bleed the animal. Mice were transcardially perfused with 20 ml ice cold PBS, followed by 1% ice cold PFA in PBS (pH 7.4; PFA). Liver clearance and muscle tremor was used as perfusion indicator.

For images shown in Figure 11 and Figure 15 paraffin embedding of tissue was performed. Whole heads were removed and fixed in 4% PFA for approximately 48 h (mice sacrificed after 24 h of reperfusion after tMCAO) after removal of the skin, a cut between the eyes and removing of the occipital plates were performed to ensure PFA access to the brain. Skulls were decalcified in Citrate EDTA Buffer pH 7.5 (Quartett, Germany) for 7 days, then sliced (coronary sections, 2mm slices, caudal direction from nose) and routinely paraffin wax embedded.

For images shown in Figure 16 cryo embedding was performed. Brains were immediately removed and sliced (coronary sections at Bregma -1±0.3 mm and -5.1±0.1 mm). Tissue-TEK® O.C.T. (Sysmex Suissie AG, Switzerland) was added in a cryomold® biopsy (Ref: 4565, Sakura, Netherlands) and brain sections were placed in the mold (Figure 10), while samples were snap frozen in a 2-methylbutane (Sigma-Aldrich, Switzerland) dry ice bath and stored at -80°C.

**Figure 10:** Cryo embedding of brain samples. Brains were extracted and cut twice. Sections were placed in a cryomold poured over with Tissue-Tek® O.C.T., snap frozen in a 2-methylbutane dry ice bath and stored at -80°C.

*Histology and immunohistochemistry*

**Cryo embedded probes**

Consecutive 5 µm-thick sections were prepared from frozen brain blocks and were either stained automated with hematoxylin eosin (Sigma-Aldrich) to determine lesion volumes or subjected to
immunohistochemistry for the demonstration of neutrophils as described before (Enzmann et al., 2013). All sections were acetone fixed for 10 min at -20°C and stored at -20°C until further processing. Before staining sections were rehydrated in PBS for 5 min.

Sections for immunohistochemistry were blocked with a mixture of goat and rabbit serum for 15 min. Blocking solution was removed and samples were incubated with the primary antibody at RT for 1 h. The following antibodies were used: anti-Ly6G (clone 1A8, Biolegend, USA) for neutrophils, anti-PECAM-1 (Mec13.3) as positive control and an anti-human complement factor B (9B5; both generated inhouse, kindly provided from B. Engelhardt) as negative control. Slices were washed once with PBS for 5 min and once with PBS/0.1 % Tween 20 (termed as washing step following, Sigma-Aldrich, Switzerland). Subsequently a biotinylated secondary anti-rat antibody (Vectastain ABC-Kit, Vectro Laboratories, USA) was added. After 30 min at RT the samples were washed and thereafter incubated with a preformed streptavidin-biotin complex (RT, 30 min; Vectastain ABC-Kit, Vector Laboratories, USA) followed by another washing step. In a next step to visualize the staining a peroxidase substrate (AEC Kit, Vector Laboratories, USA) was dropped on the sections and samples were observed under the microscope for 12 to 20 min. The reaction was stopped by washing the coverslips in distilled water three times consecutively. A counter staining with hematoxylin (Sigma-Aldrich) was afterwards performed for 1 min and slides were placed in tap water. Water was exchanged until no discoloration was observed. To avoid falling dry of the sections, sections were left in the water until mounting with an aqueous medium (Aquatex, Millipore) was performed.

All slides were acquired with a Pannoramic Digital Slide Scanner at 20x magnification. To assess the effect of α4-integrins blockade on neutrophil recruitment, brain sections were inspected by a person blinded to the treatment groups.

**Paraffin embedded probes**

Consecutive 5 µm-thick sections were prepared and were either automated stained with hematoxylin eosin (Sigma-Aldrich) or subjected to immunohistochemistry for the demonstration of neutrophils. Sections were deparaffinized and incubated with citrate buffer (pH 6.0, 20 min at 98°C) for antigen retrieval. Incubation with the primary rat anti-Ly6G antibody (clone 1A8, Biolegend) was performed at 4°C for 15-18 h. The following staining procedure was the same as described above for “Cryo embedded probes”. All sections were subsequently incubated with a biotinylated secondary anti-rat antibody (RT, 30 min) and thereafter with a preformed streptavidin-biotin complex (RT, 30 min; both Vectastain ABC-Kit, Vector Laboratories, USA). Visualization with diaminobenzidin or peroxidase substrate (AEC Kit, Vector Laboratories) was followed by hematoxylin (Sigma-Aldrich) counterstaining.
All sections were mounted with an aqueous medium (Aquatex, Millipore). Slides were acquired with a Pannoramic Digital Slide Scanner at 20x magnification.

Detection of LIPO-6S-IDCC labeled neutrophils in brain sections

To differentiate adoptively transferred LIPO-6S-IDCC positive from endogenous neutrophils, cryosections (7 µm) were subjected to a Ly6G immunohistochemistry (described above) and imaged with a LSM710 (Zeiss, Germany) for fluorescence and a Panoramic Digital Slide Scanner (3DHISTEC, Hungary) to obtain bright field images. For excitation at the LSM710 the 633 nm laser with a power of 2 to 13 % and the pre-adjusted emission settings for Cy5.5 were used. Images were co-registered after imaging using landmarks.

Neutrophil count

Absolute numbers of Ly6G+ neutrophils were determined at five levels spanning the entire lesion, i.e. at Bregma +2.8, +1.54, +0.14, -1.94 and -4.6 mm, in both hemispheres. We differentiated between neutrophils located to the meninges and parenchymal cells. The latter term was used for simplification only and does not imply extravasation of neutrophils into the brain parenchyma.

Determination of cerebral lesion volumes

Five 7 µm thick coronal H&E-stained cryosections (taken approximately at Bregma +2.8, +1.54, +0.14, -1.94 and -4.6 mm) were digitized with a Panoramic Digital Slide Scanner (3DHISTEC, Hungary) at 20x magnification. Lesions were determined with NDP.view.4.26 (Hamamatsu Photonics K.K.), while drawing a ROI over the contralateral side, the non-affected ipsilateral and ischemic tissue. The person performing the analysis was blinded with respect to the treatment groups. Cerebral lesion volumes were calculated by summing up the areas of each section and correcting for edema as described in (Gerriets et al., 2004) Lesion volumes were calculated according to:

\[
\text{Lesion volume} = \text{Volume contralateral hemisphere} - (\text{Volume ipsilateral hemisphere} - \text{Volume affected tissue})
\]

Statistical analysis

Data are presented as mean±SD. Comparisons were made by one-way ANOVA or repeated measures ANOVA followed by Holm-Sidak or Tukey’s test, ANOVA on ranks followed by Dunn’s test, or Mann-Whitney rank sum test where applicable. A Spearman’s correlation analysis was performed between the number of neutrophils in the phantom and measured fluorescence intensities. A p-value ≤0.05 was considered significant.
3.1.4. Results

Systemic neutrophil mobilization and neutrophil attraction to the ischemic territory after tMCAO

In order to evaluate effects of the placement of the intraluminal filament on the number of circulating neutrophils we serially quantified blood neutrophils after 1 h of tMCAO in male C57BL/6 mice using flow cytometry (the gating strategy is shown in Supplementary Figure 1. Serial blood samples were taken from C57BL/6 mice, prior to and 0.5, 1.5, 4, 24 and 48 h after 1 h of tMCAO or sham-surgery (Figure 11A). Neutrophil counts were significantly higher in the blood of sham mice at 1.5, 4 and 24 h, while in tMCAO mice significant higher neutrophil counts were detected at 0.5, 1.5, 4 and 24 h after reperfusion (p<0.05 vs baseline). There were no statistical significant differences between sham and tMCAO animals at 1.5, 4 and 24 h after reperfusion (p>0.05 pairwise comparison). This indicates that the systemic neutrophil response was dominated by the surgical intervention required in this model.

We next analyzed the ischemic brain lesion in tMCAO mice for the presence of neutrophils at 24 h after reperfusion (Figure 11C-H), as it has previously been shown that neutrophils are most abundant in the brain between 18 and 24 h after tMCAO (Enzmann et al., 2013). As expected (McColl et al., 2004), the lesion induced by 1 h of tMCAO and 24 h of subsequent reperfusion was represented by extensive necrosis and edema in the striatum and neocortex that extended beyond the MCA territory within the ipsilateral hippocampus and thalamus. Neutrophils (Ly6G+) were found in association with the lesions in low numbers. They were predominantly seen individually within small vessels in the adjacent leptomeninges and the affected parenchyma, occasionally also outside vessels in the affected parenchyma (Figure 11D, F, H), confirming previous reports (Enzmann et al., 2013; Perez-de-Puig et al., 2015). The contralateral hemisphere was generally free from neutrophils (Figure 11C, E, G). In brains of sham-operated mice only few Ly6G+ cells were detected in leptomeningeal vessels (data not shown). This confirms that neutrophils are recruited into the brain as a consequence of the ischemic tissue injury.
Figure 11: Systemic neutrophil mobilization and attraction to the site of ischemic damage following tMCAO. (A) Flow cytometry showed a significant increase in the amount of circulating neutrophils at 1.5 and 4 h of reperfusion after 1 h of tMCAO (grey) or sham surgery (white), but dropped to pre-surgery levels thereafter. Neutrophil levels in sham and tMCAO animals did not differ significantly. These results are indicative of neutrophil mobilization as an immediate response to surgery. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Repeated-measures ANOVA and Holm-Sidak, baseline n=13, sham n=9, MCAO n=9-11 per group for all time points, *p<0.05 vs baseline. (B) Sketch to highlight the location and extent of the ischemic lesion in a coronal brain section (Bregma 0.74mm), and indicating where images C-H were acquired. (C-H) Representative images of a mouse brain after 1 h of tMCAO and 24h after reperfusion to show the presence and distribution of neutrophils (Ly6G+) in paraffin sections. (C, E, G). Contralateral hemisphere showing no neutrophil influx in the leptomeninges (C), cortex (C, E) and striatum (G). (D, F, H) Neutrophils are present in low numbers within leptomeningeal vessels (D, long arrow) and individually within parenchymal vessels (short arrows) in cortex (D, F) and striatum (H). A few individual neutrophils are also seen outside blood vessels, in the parenchyma (arrowheads). Avidin biotin complex method, hematoxylin counterstain. Bars =10 µm.
Plasma membrane labeling of neutrophils for NIRF imaging does not lead to cell activation

NIRF imaging requires labeling of neutrophils, a procedure which should ideally neither alter the phenotype nor the function of neutrophils. We aimed at establishing a protocol for ex vivo labeling of neutrophils isolated from the bone marrow of C57BL/6 mice. Purified neutrophils were incubated with the NIRF dyes VivoTrack680 or LIPO-6S-IDCC at different dye concentrations. Cells showed higher fluorescence intensities when labeled with LIPO-6S-IDCC compared to VivoTrack680 at all concentrations tested (Figure 12, LIPO-6S-IDCC 173±52, 198±54, 332±21, 414±37 vs VivoTrack680 12±2, 29±1, 60±6. 94±12, p<0.05). Confocal microscopy revealed that LIPO-6S-IDCC labels the plasma membranes of Ly6G+ cells (Figure 12). To determine the optimal dye concentration for neutrophil labeling, we incubated equal numbers of harvested neutrophils with different amounts of LIPO-6S-IDCC dye for 15 min and analyzed the samples by flow cytometry (Figure 12C). In addition neutrophils were incubated with propidium iodide to assess adverse effects on cell viability. Fluorescence emission increased with increasing concentrations of LIPO-6S-IDCC with a maximum at 25 μg/ml LIPO-6S-IDCC. Thereafter, fluorescence intensities decreased, likely due to self-quenching. The proportion of non-viable neutrophils was higher with LIPO-6S-IDCC than in unlabeled cells at all concentrations tested (Figure 12C), and concentrations above 50 μg/ml led to a further increase in the number of non-viable cells. Furthermore, to find the optimal incubation time we incubated neutrophils with 25 μg/ml of LIPO-6S-IDCC for different periods of time (Figure 12D). Flow cytometry revealed increasing fluorescence emission with increasing incubation times, but with incubation times longer than 20 min the proportion of non-viable cells increased significantly. Thus, a concentration of 25 μg/ml LIPO-6S-IDCC and an incubation time of 20 min constituted a good compromise between cell viability and fluorescence intensity.

To determine whether in vitro labeling of neutrophils with LIPO-6S-IDCC results in their activation, we performed a neutrophil activation assays. Neutrophils were isolated from the bone marrow of two C57BL/6 mice and pooled. Half of the cells were incubated with 25μg/ml LIPO-6S-IDCC for 20min and the expression of various adhesion molecules was compared to the unlabeled fraction, which served as control. Cells were stained with directly conjugated rat anti-mouse antibodies recognizing CD45, Ly6G, CD62L (L-selectin), CD11a (LFA-1), and CD11b (macrophage-1 antigen). Cell surface expression of the activation markers CD62L, CD11a and CD11b on Ly6G+/CD45+ neutrophils was assessed by flow cytometry (Supplementary Figure 2). Most leukocytes constitutively express L-selectin mediating tethering and rolling along the endothelium. Upon activation of the leukocytes the extracellular domain of L-selectin is generally shed from the surface (Ivetic and Ridley, 2004). LIPO-6S-IDCC labeling induced only negligible shedding (reduction of 8%) of membrane associated L-selectin (CD62L) compared to unlabeled cells. It is widely acknowledged that the surface expression of macrophage-1
antigen (CD11b/CD18, αMβ2) increases due to mobilization of intracellular reservoirs (Jones et al., 1988), whereas surface levels of LFA-1 (CD11a/CD18, αLβ2) are not affected during that process (Lacal et al., 1988). We did not detect an increased expression of CD11a and CD11b on neutrophils in response to the labeling procedure with LIPO-6S-IDCC (Supplementary Figure 2B, C), underscoring that this labeling procedure does not activate the neutrophils.

**NIRF imaging enables monitoring of neutrophil accumulation to the ischemic lesion in vivo**

For the non-invasive whole brain visualization of neutrophil trafficking we have used a planar NIRF imaging system operated in reflectance mode (Figure 13A). Light from a white light source was directed through a band pass filter to the head of the anaesthetized animal. The emitted fluorescence was captured using a charge-coupled device camera fitted with adequate emission filters and a lens to adjust the focal plane. To evaluate the capacity of NIRF imaging to detect fluorescently labeled neutrophils we first used brain tissue mimicking silicon phantoms (Figure 13B). To simulate optical tissue properties of the mouse brain, TiO$_2$ particles and carbon black powder were added as scattering and absorbing agent, respectively. Different numbers of LIPO-6S-IDCC-labeled neutrophils in suspension were filled into a glass capillary, which was inserted at a 2 mm depth below the surface of the phantom. We found a linear correlation between the measured fluorescence intensities and the number of neutrophils in the center of the glass capillary (Figure 13C, $R^2=0.98$, p<0.05). A minimum number of 1750 LIPO-6S-IDCC-labeled neutrophils in a volume of 1.5 µl could be distinguished from the background of the phantom.
Figure 12: Labeling of neutrophils for NIRF imaging. (A) Comparison of the fluorescence intensities measured by flow cytometry after incubation of neutrophils with different concentrations of LIPO-6S-IDCC and VivoTrack 680. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. n=9; *p<0.05 by repeated-measures ANOVA and Tukey’s test. (B) Confocal microscopy images of neutrophils after incubation with LIPO-6S-IDCC and anti-Ly6G-PE antibody. Depicted are cells in the (i) transmitted light, (ii) LIPO-6S-IDCC red channel, (iii) Ly6G green channel and (iv) the overlay. Bar =10µm. (C) Incubation of neutrophils with different concentrations of LIPO-6S-IDCC. The propidium iodide assay was used to assess the effect of labeling on cell viability. Mean±SD; n=3 animals with 3 independent experiments each, *p<0.05 by one-way ANOVA with Tukey’s posttest. (D) Incubation of neutrophils with 25 µg/ml LIPO-6S-IDCC for different time intervals. Mean±SD, n=3 animals with 3 independent experiments each; *p<0.05 by one-way ANOVA with Tukey’s posttest. CTRL = control.
Figure 13: Fluorescence intensity as a function of the number of labeled neutrophils. (A) Set-up for NIRF imaging. Excitation light from a white light source is filtered and directed to the mouse head. Fluorescence is detected using a charged-couple device (CCD) camera fitted with adequate emission filters. (B) Illustration of a silicon phantom mimicking brain tissue. A glass capillary was inserted at a depth of 2 mm and filled with suspensions containing different numbers of LIPO-6S-IDCC-labeled neutrophils. (C) Fluorescence intensities as a function of the number of LIPO-6S-IDCC-labeled neutrophils. Fluorescence intensities were measured in a region of interest of 15 mm x 6 mm over the center of the phantom. Mean±SD of three independent experiments.

For NIRF imaging of neutrophil accumulation in the ischemic brain in vivo we intravenously injected 7.5x10^6±0.5x10^6 LIPO-6S-IDCC-labeled neutrophils into mice 15 min prior to tMCAO. NIRF imaging was performed 24 h after reperfusion (Figure 14A,B). Sham-operated animals injected with LIPO-6S-IDCC-labeled neutrophils served as controls. In tMCAO mice and sham-operated animals injection of fluorescently labeled neutrophils resulted in significant higher fluorescence intensities over the left ischemic hemisphere compared to the contralateral hemisphere (ipsilateral tMCAO 168±42%,...
ipsilateral sham 111±4% versus contralateral 100%, p<0.05). Yet, the fluorescence intensities were significantly higher in tMCAO animals as compared to shams (p<0.05). To establish the time course of neutrophil trafficking to the brain in vivo, we adoptively transferred LIPO-6S-IDCC-labeled neutrophils before tMCAO and recorded fluorescence intensities longitudinally with NIRF imaging (Figure 14C). We observed a steady increase in fluorescence over the ischemic hemisphere in tMCAO mice injected with LIPO-6S-IDCC-labeled neutrophils. Normalized fluorescence intensities were significantly higher in the interval at 18 to 48 h compared to 2 h after reperfusion (p<0.05). In sham-operated mice there was only a minor increase in normalized fluorescence intensities observed over the ipsilateral hemisphere. Normalized fluorescence intensities in the ipsilateral hemisphere was significantly higher in tMCAO at all time points (p<0.05). A subgroup of tMCAO animals that were injected with LIPO-6S-IDCC-labeled neutrophils were sacrificed at 18 h after reperfusion. Fluorescence microscopy and immunohistochemistry demonstrated that both exogenously labeled neutrophils and endogenous neutrophils (Ly6G+) were present in the ischemic territory (Figure 14D). The LIPO-6S-IDCC-labeled neutrophils made up approximately 50% of all neutrophils and appeared morphologically unaltered. Taken together, these finding indicates that labeled neutrophils are recruited to the site of ischemic injury.

Interestingly, we detected significantly higher normalized fluorescence intensities over the ipsilateral temporal muscle compared to the contralateral side in tMCAO mice and sham-operated mice at 24 h of reperfusion (Figure 15A, ipsilateral tMCAO 249±76%, ipsilateral sham 135±16% versus contralateral 100%, p<0.05). There was no significant difference in normalized fluorescence intensities of the ipsilateral muscle between sham and tMCAO animals (p>0.05). This prompted us to further investigate the source of the fluorescence signal. Ly6G immunohistochemistry identified presence of numerous neutrophils in the ipsilateral muscle mainly in interstitial vessels in sham and tMCAO animals, which was not observed in the corresponding contralateral temporal muscles (Figure 15B-E). These findings indicate that the fluorescence signal originated not from the ischemic lesion but is due to neutrophil trafficking into the temporal muscle. To investigate the recruitment of neutrophils to the temporal muscle further, we conducted a subsequent study observing the pathological changes in the temporal muscle using MRI, photoacoustic imaging and histology (chapter 3.3).
Figure 14: Tracking of neutrophils to the ischemic lesion in C57BL/6 mice in vivo. (A) In vivo NIRF images of the mouse head. Depicted are a sham-operated mouse 25 h after adoptive transfer of LIPO-6S-IDCC-labeled neutrophils (left) and a mouse subjected to 1 h tMCAO and 24 h of reperfusion in which LIPO-6S-IDCC-labeled neutrophils were adoptively transferred prior to ischemia (right). Examples of regions of interests drawn over the brain hemispheres and muscle regions are shown (dashed line). (B) Normalized fluorescence intensities over the ipsilateral ischemic and contralateral brain regions. Mean±SD; sham n=15, tMCAO n=10; Kruskal-Wallis one way ANOVA followed Dunn’s pairwise multiple comparison; *p<0.05 versus contralateral, #p<0.05 versus sham. (C) The time course of neutrophil accumulation in the brain was assessed with non-invasive NIRF imaging. Mean±SD, sham n=9-15, tMCAO n=7-10 per group for all time points; repeated-measures ANOVA and Holm-Sidak pairwise multiple comparison; *p<0.05 versus 2 h, #p<0.05 versus sham (D) Fluorescence microscopy of cryosections of a tMCAO mouse brain 18 h after injection of labeled neutrophils. Avidin biotin complex method, hematoxylin counterstain. Shown are LIPO-6S-IDCC-labeled neutrophils (arrows) and endogenous (Ly6G+) neutrophils (arrow head). Bars = 50µm.
Figure 15: Neutrophil accumulation in the temporal muscle after external carotid artery ligation. (A) Fluorescence intensities measured over the left (ipsilateral) and right (contralateral) temporal muscles of sham-operated mice and animals subjected to 1 h of tMCAO and 24 h of reperfusion. LIPO-65-IDCC-labeled neutrophils were intravenously injected 15 min prior surgery and NIF imaging was performed 24 h after the intervention. Mean±SD, sham n=15 and tMCAO n=10; Kruskal-Wallis one way ANOVA followed Dunn’s pairwise multiple comparison; *p<0.05 versus contralateral. (B-E) Ly6G immunohistochemistry with avidin biotin complex method and hematoxylin counterstain of paraffin sections of the temporal muscle. (B) Ipsilateral and (C) contralateral temporal muscle of a sham-operated mouse. (D) Ipsilateral and (E) contralateral temporal muscle of a mouse subjected to tMCAO. (B, D) Individual neutrophils were observed within interstitial vessels (arrows in high magnification insets) in the ipsilateral muscles of both sham-operated and tMCAO mice. In the muscle of the tMCAO mouse, individual degenerate myofibres are observed (arrow heads in high magnification inset). (C, E) The contralateral temporal muscles were unaltered. Bar, 20 µm (overviews) and 10 µm (high magnification insets).

α4-integrins mediate post-ischemic neutrophil accumulation in the CNS in vivo

Intravascular guiding cues direct neutrophils to sites of tissue damage (Ley et al., 2007). In light of a recent study proposing binding of neutrophils via VLA-4 (Neumann et al., 2015), we investigated the effect of a blockade of α4-integrins on neutrophil trafficking with NIRF imaging. The α4-integrin subunit can pair with the β1-integrin subunit to form VLA-4 which binds to VCAM-1 (Vajkoczy et al., 2001), fibronectin and JAM-B on brain endothelial cells in the mouse. Blockade of α4-integrin by the
monoclonal antibody PS/2 significantly reduced the interaction of neutrophils with cerebral blood vessels at the site of ischemic cerebral injury and consecutively reduced the damage in the tMCAO model (Neumann et al., 2015). We serially performed NIRF imaging at 18, 24 and 48 h of reperfusion following antibody mediated inhibition of α4-integrins. In each group one mouse had to be excluded due to skin irritation caused by epilation procedure. Anti-α4-integrin antibody treated animals had significantly reduced fluorescence intensities over the left ischemic hemisphere at 18 and 24 h after tMCAO compared to animals that received a control antibody (Figure 16A, anti-α4-integrin 117±27% and 135±31% versus control 155±19% and 165±23%, p<0.05), indicating substantially reduced neutrophil accumulation in the ischemic hemisphere. At 48h fluorescence intensities were not significantly different between anti-α4-integrin antibody treated animals and controls (anti-α4-integrin antibody 185±51% versus control antibody 186±25%, p>0.05). For cross-validation we counted neutrophils in brain sections of a subgroup of animals euthanized at the endpoint at 48 h after reperfusion (Figure 16B). We found no significant differences in the total number of neutrophils between the two groups (anti-α4-integrin antibody 603±313 versus control antibody 384±123, p>0.05). Moreover, we assessed the effect of anti-α4-integrin antibody on the ischemic damage on HE stained tissue cryosections (Figure 16C). Three brains of the control group were excluded due to damage during tissue processing. Cerebral lesion volumes were reduced by 19% in anti-α4-integrin antibody as compared with control antibody treated animals 48 h after reperfusion (anti-α4-integrin antibody 57.8±14.0 mm³ versus 71.2±15.3 mm³, p<0.05) concomitant with an early reduction of neutrophil accumulation.

Figure 16: α4-integrins mediate neutrophil accumulation to the ischemic lesion in vivo (A) Normalized fluorescence intensities measured over the ischemic hemisphere of mice. Mean±SD, anti-α4-integrin n=10-12, control n=11-12, Mann-Whitney rank sum test, *p<0.05. (B) Absolute numbers of Ly6G+ neutrophils were determined in anti-Ly6G stained brain cryosections at five levels spanning the entire lesion, i.e. at Bregma +2.8, +1.54, +0.14, -1.94 and -4.6 mm, in both hemispheres. Mean±SD, anti-α4-integrin, n=4, control n=5, Mann-Whitney rank sum test, *p<0.05. (C) Edema corrected cerebral lesion volumes at 48 h after reperfusion. Mean±SD, anti-α4-integrin n=13, control antibody, n=10, Mann-Whitney rank sum, *p=0.05 vs control antibody.
3.1.5. Discussion

In the current study we used NIRF imaging to non-invasively track fluorescently labeled and adoptively transferred neutrophils to the ischemic brain in mice. We have isolated neutrophils from the bone marrow of C57BL/6 mice which have been previously shown to be morphologically mature and functionally competent (Boxio et al., 2004). We first established an optimized protocol for labeling neutrophils ex vivo with the NIRF dye LIPO-6S-IDCC minimizing negative effects of the labeling on cell viability with increasing dye concentrations and incubation times. In addition, we examined if the labeling procedure leads to neutrophil activation, which would be undesirable for trafficking studies. Most leukocytes constitutively express L-selectin mediating tethering and rolling along the endothelium. Upon activation of the leukocytes the extracellular domain of L-selectin is generally shed from the surface (Ivetic and Ridley, 2004). In parallel, it is widely acknowledged that the surface expression of macrophage-1 antigen (MAC-1, CD11b/CD18, aMb2) is increased due to mobilization of intracellular reservoirs (Jones et al., 1988). Surface levels of LFA-1 (CD11a/CD18, aLb2) do not seem to be affected in that process (Lacal et al., 1988). We observed only minute shedding of L-selectin in the LIPO-6S-IDCC labeled neutrophil population and surface expression of aMb2 and aLb2 integrins was indistinguishable between labeled and unlabeled neutrophils. This underscores that the labeling does not trigger activation of labeled neutrophils to a significant level.

Non-invasive monitoring of neutrophil recruitment puts high demand on the detection sensitivity of the imaging methods because neutrophil accumulation in the mouse brain after tMCAO was reported to be relatively low (Enzmann et al., 2013; Perez-de-Puig et al., 2015; Gelderblom et al., 2009). We have found that NIRF imaging can detect as few as 1750 LIPO-6S-IDCC-labeled neutrophils in a homogenous phantom mimicking brain tissue. However, when non-invasively imaging the mouse head contributions from all compartments of the head including skin, bone, muscles, meninges, and brain have to be considered. Hence, generation of a detectable contrast does not only depend on the number of accumulated cells in the ischemic territory but also on the optical properties of the various tissue compartments and the biodistribution of the labeled cells after adoptive transfer.

The injection of LIPO-6S-IDCC-labeled cells in sham-operated animals led to an increase in fluorescence of both hemispheres with a minor contrast between the ipsi- and contralateral side. The fluorescence of the contralateral hemisphere is likely due to labeled neutrophil circulating in the blood (5-10% of the total neutrophils) and in the bone marrow of the skull which is a main site of neutrophil clearance (Furze and Rankin, 2008). The slightly higher fluorescence on the ipsilateral side is likely due to a spillover from the fluorescence of accumulated neutrophils in the temporal muscle.
Fluorescence intensities over the ipsilateral hemisphere were significantly higher in tMCAO mice that received LIPO-6S-IDCC-labeled cells than in the corresponding sham-operated animals. Using fluorescence microscopy we found accumulation of LIPO-6S-IDCC-labeled neutrophil at the ischemic territory, confirming that the detected NIRF contrast is due to the accumulation of adoptively transferred neutrophils.

Longitudinal NIRF imaging in ischemic mice revealed an early presence of neutrophils starting immediately after onset of reperfusion, which shows that neutrophils are among the first immune cells that respond to an ischemic insult. Neutrophil accumulation peaked at 18 h after reperfusion. Previous studies assessing neutrophil accumulation in the brain of C57BL/6 mice after 1 h of tMCAO using flow cytometry and immunohistochemistry showed considerably variability in dynamics i.e. to peak at 18-24 h (Enzmann et al., 2013), at 48 h (Stevens et al., 2002), and at 3 days after reperfusion (Gelderblom et al., 2009). Fluorescence signals from neutrophils in the ischemic hemisphere remained elevated up to 48 h after reperfusion, indicating that neutrophils might have a prolonged lifespan, compared to 11 h under homeostatic conditions (Lord et al., 1991), which is mediated by pro-survival cytokines and chemokines in the ischemic lesion (Coxon et al., 1999). Taken together, the findings show that NIRF imaging is capable to non-invasively monitor the dynamics of the neutrophil response after cerebral ischemia in vivo and its modulation by interfering with the underlying regulatory mechanism. A general disadvantage of the presented approach is that the adoptive transfer of labeled neutrophils alters the neutrophil count in the recipient mouse significantly (Peters et al., 2002).

The dynamics of the neutrophil response in the peripheral circulation was different from that in the ischemic territory. Significantly increased levels of circulating neutrophil were detected at 0.5 h after reperfusion, which was likely due to a rapid egress of neutrophils from the bone marrow reserve into the circulation (Furze and Rankin, 2008). However, a similar extent of neutrophil mobilisation was also observed in sham-operated animals indicating that the effect on circulating neutrophils is mainly due to surgery and not to ischemia. In contrast, the growing ischemic lesion did not seem to (further) mobilize neutrophils within the circulation, which returned to baseline within 24 h after reperfusion, at which high levels of neutrophils were detected in the ischemic brain region. Using blood samples the loss of blood might be crucial in small animals, and have to be taken carefully into account, while we restricted the sampling to a maximum of 3 samples and a total volume of 100 µl per mouse before the terminal collection.

To our surprise we detected fluorescence signals in tMCAO and sham-operated mice after adoptive transfer of LIPO-6S-IDCC-labeled neutrophils on the extracranial ipsilateral regions. Immunohistochemistry demonstrated accumulation of neutrophils in the temporal muscles. The tMCAO model
requires ligation of the ECA and thereby restricts perfusion to the extracerebral ECA territory. For rats the tMCAO procedure has been reported to cause tissue damage in muscles of the ECA territory (including muscles for mastication and swallowing) with negative consequences on outcome (Dittmar et al., 2003). It is conceivable that ECA ligation has also functional implications in the widely used tMCAO mouse model. However, to our knowledge this has not yet been investigated. Beside functional implications, neutrophil accumulation in the ipsilateral muscle might have also affected quantification of fluorescence signals. ROIs were drawn carefully to measure only over the brain regions excluding extracerebral tissue (Figure 14A). In sham-operated animals, the normalized fluorescence intensity was found to be about 10% higher over the ipsilateral hemisphere compared to the contralateral side, despite no detectable neutrophil accumulation in the brain. This indicates that there is spillover from fluorescence of labeled neutrophils from the ipsilateral muscle into the brain region.

During inflammation circulating neutrophils are recruited from the vessels via rolling, arrest and diapedesis, guided by endothelial adhesion molecules and chemokines (Ley et al., 2007). However, if mediators of the multistep adhesion cascade regulate neutrophil recruitment at the cerebral vasculature following transient ischemia is hitherto unknown. We have used NIRF imaging to study the role of α4-integrins, that has been implicated in neutrophil arrest to the endothelium (Ley et al., 2007), but is also expressed on other leukocyte subpopulations (Liesz et al., 2011). We observed a significant reduction in normalized fluorescence intensities in mice that received an antibody against α4-integrin at 18 and 24 h after reperfusion compared to control, while at 48 h no differences between the groups was detected. Immunohistochemistry, though performed in only a few animals, confirmed our NIRF data at 48 h after reperfusion. However, we did not investigate earlier time points. This findings support a role of VLA-4 with any of its endothelial ligands, VCAM-1, fibronectin or JAM-B in mediating neutrophil trafficking to the brain after cerebral ischemia.

The neutrophil response clearly occurs during the acute stage when substantial secondary lesion growth occurs (Hata et al., 2000b). With immunohistochemistry we found only low numbers of neutrophils around the ischemic lesion. Recent studies indicate that these neutrophils do not infiltrate the brain parenchyma, but rather remain restricted to luminal surfaces or perivascular spaces of cerebral vessels including the leptomeninges (Enzmann et al., 2013; Perez-de-Puig et al., 2015). Neutrophils might still exert their deleterious action via interaction with the vasculature. It has been shown that neutrophils can release cytokines, proteases and ROS during an oxidative burst and are involved in microvascular disturbances (del Zoppo et al., 1991), and BBB damage (Gidday et al., 2005). In addition, neutrophils might contribute to secondary thrombus formation after cerebral ischemia as they interact with platelets, participate in fibrin cross-linkage and trigger thrombin activation by inducing the extrinsic tissue factor/FVIIa pathway (De Meyer et al., 2016). In our study blocking of α4-
integrin led to a reduction of the ischemic lesion at 48 h after reperfusion. Anti-α4-integrin antibody mediated treatment, has so far yielded contradictory results for its neuroprotective effects following cerebral ischemia in rodents. While several previous studies have demonstrated a reduction of the ischemic damage and improved functional deficits after that anti-α4-integrin antibody treatment (Liesz et al., 2011; Neumann et al., 2015; Becker et al., 2001; Relton et al., 2001), two studies reported no effect of treatment on the ischemic lesion (Langhauser et al., 2014; Llovera et al., 2015). The difference in the neuroprotective efficacy might be attributed to different batches of antibodies used, or differences in housing and experimental conditions that might affect the inflammatory response following cerebral ischemia (Zhou et al., 2013; Layer et al., 2016). However, we also calculated the group size according to normalized fluorescence intensities as a primary end point. For the cerebral lesion the present study was underpowered (power =0.67). Preclinical multi-center studies are more suited to address this question and to conduct treatment studies with sufficient statistical power. Indeed such a trial with large pooled data samples demonstrated that anti-α4-integrin antibody did not reduce lesion size after filament tMCAO in the mouse (Llovera et al., 2015).

In future studies we want to refine our current approach and use molecular probes that can directly visualize neutrophils function to elucidate how neutrophils partake in vascular and tissue injury. NIRF imaging might constitutes an attractive tool to study the neutrophil response in experimental models of cerebral ischemia and potentially in other inflammatory diseases.
3.1.6. Supplementary material

Supplementary Figure 1: Gating strategy for flow cytometry analysis of neutrophils in blood. (A) Leukocytes obtained from whole blood were first analyzed according to their forward scatter (FSC) and side scatter (SSC) profiles and are displayed in a dot blot. (B) The gated leukocyte population was further analyzed for their neutrophil subpopulation (Ly6C+ cells).

**Supplementary Figure 2**  
To determine whether *in vitro* labeling of neutrophils with LIPO-6S-IDCC induces activation of that cell population we performed neutrophil activation assays. Neutrophils were isolated from the bone marrow of four C57BL/6 wildtype mice. Half of the cells were labeled with LIPO-6S-IDCC and the expression of various adhesion molecules was compared to unlabeled neutrophils. Briefly, both cell populations were stained with antibodies recognizing mouse CD45, Ly6G, CD11a (LFA-1), CD11b (macrophage-1 antigen), CD49d (α4-integrin), P-selectin glycoprotein ligand 1 and CD62L (L-selectin), and surface expression was assessed by flow cytometry of the Ly6G+/CD45+ population. Most leukocytes constitutively express L-selectin mediating tethering and rolling along the endothelium. Upon activation of the leukocytes the extracellular domain of L-selectin is generally shed from the surface (Ivetic and Ridley, 2004). In our hands only minute shedding of L-selectin in the LIPO-6S-IDCC labeled neutrophil population was detected. It is widely acknowledged that the surface expression of macrophage-1 antigen increases due to mobilization of intracellular reservoirs (Jones et al., 1988), whereas surface levels of LFA-1 (CD11a/CD18, αLβ2) are not affected during that process (Lacal et al., 1988). Upon labeling of neutrophils with LIPO-6S-IDCC surface levels of αM and αL integrins were indistinguishable between both populations underscoring that the labeling procedure does not activate neutrophils.
Supplementary Figure 2: LIPO-6S-IDCC labeling of bone marrow-derived neutrophils does not activate neutrophils. Neutrophils were isolated from the bone marrow of two C57BL/6 mice, pooled and half of the cells were incubated with 25 μg/ml LIPO-6S-IDCC for 20 min, while the unlabeled fraction served as control. Both labeled and unlabeled neutrophils were stained with antibodies recognizing CD62L (L-selectin), CD11a (LFA-1) and CD11b (macrophage-1 antigen). (A) Isolated neutrophils are shown in a forward side scatter plot. (B) The histogram depicts the expression of CD62L by LIPO-6S-IDCC labeled neutrophils (yellow) in relation to the unlabeled (blue) population and the appropriate isotype control (red). Hence, LIPO-6S-IDCC labeling induced only minor shedding of membrane associated CD62L. (C, D) Compared to the unlabeled population (blue), LIPO-6S-IDCC labeled neutrophils (yellow) do not increase the surface expression of CD11a (C) or CD11b (D) in response to the labeling procedure.
3.2. ICAM-1null C57BL/6 mice are not protected from experimental ischemic stroke

Adapted from submitted manuscript:

ICAM-1null C57BL/6 mice are not protected from experimental ischemic stroke

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3.2.1. Abstract
Cerebral ischemia is followed by an inflammatory response headed by the recruitment of neutrophils to the side of injury. Neutrophils are considered to cause additional tissue injury to the brain after stroke. In the ischemic brain ICAM-1 is upregulated and thought to be crucial for neutrophil recruitment. Several studies in ICAM-1 deficient mice showed protective effects in experimental stroke models. However, translation of pharmacological targeting ICAM-1 into the clinics failed to show efficacy and caused severe side effects. While in preclinical trials incomplete ICAM-1 knockout mice were used, we questioned us if this might be the underlying reason and investigated thereof stroke pathology in complete ICAM-1null mice. Transient MCAO did not ameliorate cerebral ischemia outcome in the acute phase. Neutrophil accumulation was not different in wildtype and ICAM-1null mice using NIRF imaging. Immunohsitochemistry revealed that neutrophils accumulated mainly in the leptomeninges and the parenchymal microvessels, similarly in both groups. The lesion volumes were comparable in wildtype and ICAM-1null mice at 48 h of reperfusion. Our results indicate that ICAM-1null mice have no cerebral protection after stroke and ICAM-1 have no effect on neutrophil accumulation at the side of injury.

3.2.2. Introduction
Under healthy conditions the migration of immune cells towards the brain is limited through the BBB (Engelhardt and Sorokin, 2009). Under cerebral ischemia and other neurological disorders adhesion molecules on the vascular endothelium are upregulated and the BBB gets leaky (reviewed in (Stanimirovic and Friedman, 2012)). These changes lead to the recruitment of inflammatory immune cells to the brain over a multistep recruitment cascade in the postcapillary venules, promoting cerebral injury (Engelhardt and Ransohoff, 2012; Owens et al., 2008; Ley et al., 2007).

Cellular death after stroke causes pro-inflammatory cytokine (e.g. TNF-α and IL-1β) release by astrocytes, microglia, pericytes and endothelial cells (reviewed in (Barone and Feuerstein, 1999)) followed by the up regulation of the adhesion molecules P-selection and ICAM-1 (Okada et al., 1994). Neutrophils get recruited via tethering and rolling to the ischemic brain and cause further damage via granule content release and increased oxygen consumption in combination with ROS production (reviewed in (Kolaczkowska and Kubes, 2013). In recent studies with ischemic stroke material of patients and mice it was shown that neutrophils accumulate in the neurovascular unit and barely migrate to the brain parenchyma (Enzmann et al., 2013; Perez-de-Puig et al., 2015). Together with in-vitro data showing that neutrophils cross the BBB to lipopolysaccharide stimulation via ICAM-1/LFA-1 interaction, but fail to cross the BBB under hypoxia (Gorina et al., 2014), it is questionable if neutrophils get recruited over this mechanism.
However, ICAM-1 was found to be a potential therapeutic target after experimental stroke. ICAM-1 deficient mice were protected from cerebral ischemia, while less neutrophil invasion was observed (Connolly et al., 1996; Soriano et al., 1996). Breaking this observation down to blocking ICAM-1 regulated neutrophil recruitment is neuroprotective after stroke, the approach was translated into a clinical trial. The monoclonal murine anti-human ICAM-1 antibody (R6.5) administered to patients after stroke increased neurological deficits and even the mortality rate (Enlimomab Acute Stroke Trial Investigators, 2001). The failure of the clinical trial might be explained by the non-humanized antibody used or the fact, that in the preclinical trials incomplete ICAM-1 knockout mice were used and the results were miss interpreted.

To investigate if complete ICAM-1 deletion protects from ischemic stroke by preventing neutrophil recruitment we used ICAM-1tm1Alb (ICAM-1null) mice in a mouse model of tMCAO (Dunne et al., 2003). After tMCAO we observed no difference in neutrophil accumulation in none transgenic littermates (NTL) compared with ICAM-1null mice. In both groups neutrophils were similarly distributed over the ischemic hemispheres. In ICAM-1null mice infarct extension did not show differences compared to NTL, indicating no beneficial effects on ICAM-1 deletion after cerebral ischemia in mice.

Here are only data shown which were acquired in my work. However, the results of the entire study will be used in the discussion section.

3.2.3. Material and Methods

General procedures are described in detail in the material and method part in the chapter “Non-invasive NIRF imaging of the neutrophil response in a mouse model of transient cerebral ischemia” (3.1.3).

Animals and Treatment

All procedures conformed to the national guidelines of the Swiss Federal act on animal protection and were approved by the Cantonal Veterinary Office Zurich (Permit Number: 18-2014 and 49-2011). Male ICAM-1null mice and male NTL (both provided by Britta Engelhardt group Theodor Kocher Institut, Bern), weighting 20-25g, of 8-10 weeks of age were used. ICAM-1null mice do not express any functional ICAM-1 molecules, while lacking the ICAM-1 coding region in the genome (Dunne et al., 2003). Animals were housed in a temperature controlled room in individually ventilated cages, containing up to 5 animals per cage, under a 12-hour dark/light cycle. Paper tissue was given as environmental enrichment. Food (3437PXL15, CARGILL) and water were provided ad libitum.
**Experimental protocols**

For accessing the role of ICAM-1 after focal cerebral ischemia on neutrophil recruitment ICAM-1-null mice (n=12) and NTLs (n=10) were used. Neutrophils were isolated from the bone marrow of wild type donor mice and labeled with LIPO-6S-IDCC. Prior to 1 h of tMCAO mice received $7.5\times 10^6 \pm 0.5\times 10^6$ labeled neutrophils. Animals with a Bederson score =0 were excluded from the studies (n=2 NTL, n=2 ICAM-1-null). At 18 h of reperfusion animals were imaged with a Maestro 500 multispectral imaging system (Cambrige Research & Instruments Inc., USA). Due to skin irritations two NTLs had to be excluded. After NIRF imaging animals were transcardially perfused with 20 ml of PBS followed by 20 ml of a 1% PFA/PBS solution. Brains were extracted and snap frozen in Tissue-TEK® O.C.T. (Sysmex Swissee AG, Switzerland). Five μm thick cryo-sections at the positions at Bregma +2.8, +1.54, +0.14, -1.94 and -4.6 mm were made. Problems during sectioning lead to the exclusion of one NTL and two ICAM-1-null mice. H&E stained sections were used to assess infract volume, while Ly6G-Immunohistology was used to investigate neutrophil recruitment and distribution in the brain after tMCAO.

**Data analysis**

NIRF-images were spectrally unmixed and ROIs were drawn over the brain, excluding extra-cerebral tissue. Normalized fluorescence intensities were calculated and the intensities over the ischemic hemispheres of NTL and ICAM-1-null mice were compared. To calculate edema-corrected infarct volumes ROIs were drawn over the lesion, contralateral hemisphere and the ipsilateral non-ischemic area, and the percentage of affected brain tissue was calculated. For statistics Student’s-t-test was performed. Differences in neutrophil accumulation patterns after tMCAO in NTL and ICAM-1-null mice were estimated on Ly6G-immunohistological sections.

**3.2.4. Results**

*ICAM-1 seems not to be involved in neutrophil recruitment*

We tracked LIPO-6S-IDCC labeled and adoptively transferred neutrophils after 1 h of tMCAO followed by 18 h of reperfusion by NRIF-imaging (Figure 17). Both groups showed ipsilateral an increased signal compared with the contralateral side (p<0.01; T-test), indicating neutrophil accumulation at the lesion site. However, we did not find a significant difference in the normalized fluorescence intensities over the ischemic hemispheres of ICAM-1-null and NTL (178.4±43.6% versus 171.2±42.5%, respectively while contralateral side 100%).
**Figure 17**: In vivo tracking of NIRF labeled neutrophils after tMCAO. Representative in vivo NIRF images of epilated mouse heads (A). Mice were imaged 19 h after adoptive transfer of LIPO-6S-IDCC labeled neutrophils followed by 1 h of tMCAO and 18 h of RP. Upper row shows the acquired images, lower row the fluorescence signals after spectral unmixing and the regions of interest drawn over the brain hemispheres. Normalized fluorescence intensities over the ischemic ipsilateral hemispheres and the contralateral hemisphere (B). Mean±SD; NTL n=6, ICAM-1null n=10; Student’s T-test; *p<0.05 versus contralateral.

**ICAM-1 deletion does not alter the recruitment pattern of neutrophils**

Immunohistochemistry revealed that the distribution patterns of recruited neutrophils did not differ between NTL and ICAM-1null mice after 1 h of tMCAO and 18 h of reperfusion (Figure 18). Ly6G+ neutrophils were found predominantly in the leptomeninges and in parenchymal blood vessels in both groups. Less neutrophils were present in the striatum, where the ischemic core is located.
**ICAM-1**null** mice are not protected from ischemic stroke**

Previous studies showed a reduction in infarct volume in incomplete ICAM-1 knockout and anti-ICAM-1 treated animals. To investigate if complete deletion of ICAM-1 reduces the infarct volume we used computer-assisted lesion volumetry to measure the extent of the ischemic lesion in ICAM-1null mice and NTL on HE stained brain sections (Figure 19). Irrespective of the investigated genotype, 1 h of tMCAO caused comparable damage to the ischemic hemispheres (ICAM1null 65.1±14.6 versus NTL 67.7±16.7 mm³, P>0.05). The lesion incorporated the basal ganglia, the somatosensory and motor cortex, various thalamic nuclei, and occasionally the hippocampus.
Figure 19: ICAM-1 deficiency is not neuroprotective after cerebral ischemia. Representative H&E sections at the Bregma +0.14 after 1 h of tMCAO followed by 18 h of reperfusion (A). Infarcted area is outlined. Scale bar equals 2 mm. ICAM-1null mice are not protected from cerebral ischemia (B). The scatter plot shows the percentage of the affected hemisphere after edema correction of n=7 NTL and n=8 ICAM-1null mice. Mean±SD; Student’s T-test; p>0.05.

3.2.5. Discussion

It is well accepted that ICAM-1 in the periphery is involved in neutrophil adhesion and crawling after injury and in inflammation, while neutrophils recruited to the ischemic brain have been implicated to exacerbate primary ischemic damage (del Zoppo et al., 1991). Studies investigating the role of ICAM-1 in the recruitment after cerebral ischemia are controversial, and it is yet not fully understood if ICAM-1 is involved and needed. ICAM-1null mice seemed to be the perfect tool to investigate the need of ICAM-1 in the recruitment of neutrophils after focal cerebral ischemia.

ICAM-1 is known to interact with the LFA-1 receptor on neutrophils and other leucocytes, leading to their arrest and crawling on endothelial cells. In an in-vitro model using bacterial endotoxin to activate cerebral endothelial cells it was shown, that neutrophils use primarily LFA-1/ICAM-1 binding to interact with the inflamed BBB (Gorina et al., 2014). In addition ICAM-1 deficiency (Soriano et al., 1996) and the
blockage of ICAM-1 using antibodies against ICAM-1 (Matsuo et al., 1994b) or its counterpart were shown to be protective after tMCAO in rodents (Prestigiacomo et al., 1999). This indicates an important mechanism in post stroke inflammation. However, a clinical trial blocking neutrophil recruitment via an anti-ICAM-1 antibody failed (Enlimomab Acute Stroke Trial Investigators, 2001). Also two other clinical trials targeting CD18 (Hu23F2G, LeukArrest)(Becker, 2002) and CD11b (ASTIN trial)(Krams et al., 2003) both subunits of adhesion receptors on neutrophils failed to show beneficial effects. Here further knowledge is required to understand why these trials targeting the adhesion cascade had failed. To explore the endothelial adhesion cascade further we investigated the effect of complete ICAM-1 deletion on neutrophil recruitment after focal cerebral ischemia in mice.

Our results showed, contrary to our expectations, no quantifiable discrepancy of NIFR labeled neutrophils in NTLs and ICAM-1null mice after 18 h of reperfusion. This suggest that ICAM-1 does not play an essential role in neutrophil recruitment after cerebral ischemia reperfusion injury in this model. It is possible that the deletion of ICAM-1 is compensated by other adhesion molecules like VCAM-1. We recently showed the importance of VLA-4/VCAM-1 interaction in the recruitment of neutrophils (Vaas et al., 2016). In addition our collaborators showed that VCAM-1 is upregulated after cerebral ischemia in ICAM-1null mice, supporting this hypothesis (Enzmann et. al submitted). Since the accumulation patterns of neutrophils did not differ between NTLs and ICAM-1null mice we also can exclude that ICAM-1 is important for the adhesion under milder (e.g. outer penumbral regions) or sever (core regions) ischemia.

Neuroprotective effects by either blocking or deleting ICAM-1 was shown in previous studies (Connolly et al., 1996; Soriano et al., 1996; Matsuo et al., 1994b). In our study using ICAM1null mice we did not find a neuroprotective effect. This might be explained by a (functional) compensation in the case of ICAM-1 deletion, in contrast to pharmacological blockage (Rossi et al., 2015; De Souza et al., 2006). In addition, the ICAM-1 deficient mice used in previous studies were not complete knockout mice. They still were able to generate functional alternatively spliced soluble ICAM-1 isoforms, which are biologically active and can block leuckocyte recruitment (Rieckmann et al., 1995; van Den Engel et al., 2000). Also these soluble ICAM-1 levels are elevated in patients and animals after stroke (Siniscalchi et al., 2014). But yet soluble ICAM-1 isoforms are poorly understood and have different impacts on neurological diseases (Hu et al., 2010). Together with our results this might indicate an neuroprotective role of soluable ICAM-1, by influencing neutrophil recruitment and might explain the contradictory results obtained in our study.

While stroke treatment options are limited and only a small population of patients profit from the available recanalisation therapy the understanding of the immune system especially of neutrophils and
their contribution to secondary injury is essential. Studying the recruitment mechanism of neutrophils might yield new therapeutic targets. Taken together ICAM-1 is not required for neutrophil recruitment and is not neuroprotective in an ICAM-1null mouse model of 1 h tMCAO injury.
3.3. Extracerebral tissue damage in the intraluminal filament mouse model of middle cerebral artery occlusion

Adapted from:

Extracerebral tissue damage in the intraluminal filament model of middle cerebral artery occlusion

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Contribution: Planning, performance and analysis of all experiments
3.3.1. Abstract:

Background and Purpose: MCAO is the most common model of focal cerebral ischemia in the mouse. In the surgical procedure the ECA is ligated, however, its effect on the tissue supplied by the ECA has so far not been described.

Methods: C57BL/6 mice underwent 1 h of tMCAO or sham surgery. Multi-spectral optoacoustic tomography was employed at 30 min after surgery to assess oxygenation in the temporal muscles. Microstructural changes were assessed with MRI and histological examination at 24 h and 48 h after surgery.

Results: Ligation of the ECA resulted in decreased oxygenation of the left temporal muscle in most sham-operated and tMCAO animals. Susceptible mice of both groups exhibited increased T2 relaxation times in the affected muscle with histological evidence of myofibre degeneration, interstitial edema and neutrophil influx. Ligatures had induced an extensive neutrophil-dominated inflammatory response.

Conclusions: ECA ligation leads to distinct hypoxic degenerative changes in the tissue of the ECA territory, and to ligature-induced inflammatory processes. An impact on outcome needs to be considered in this stroke model.

3.3.2. Introduction:

Rodent models of cerebral ischemia are employed for studying pathophysiological mechanisms and for preclinical drug testing (Carmichael, 2005). Among them, MCAO with an intraluminal filament is a well-characterized and widely accepted model of focal cerebral ischemia. In MCAO, the common carotid artery is surgically exposed, and a filament is inserted into the common carotid artery, advanced into the internal carotid artery (ICA) until the branch of the MCA, to transiently or permanently interrupt blood flow to the MCA territory. The procedure requires ligation of the ECA and thereby also restricts perfusion of the extracerebral ECA territory. In rats hyperintensities in T2-weighted MRI and histological evidence of myofibre degeneration and regeneration were reported on day 5 after MCAO (Dittmar et al., 2003). ECA ligation was associated with delayed body weight recovery and impaired motor recovery compared to procedures where the ECA was not ligated (Dittmar et al., 2003; Trueman et al., 2011).

Despite the wide usage of the mouse MCAO model the effects of ECA ligation have so far not been investigated in this species. In a recent study we observed neutrophil influx into the ipsilateral temporal muscles in mice after 1 h of tMCAO or sham surgery (Vaas et al., 2016). This has prompted
us to further investigate the effects of ECA ligation on tributary tissues in the mouse MCAO model (Figure 20).

Figure 20: Schematic drawing of a mouse cranium including the muscles supplied by the ECA. Adapted from (Jeffery and Mendias, 2014)

3.3.3. Methods

Animals and procedures

All procedures conformed to the national guidelines of the Swiss Federal act on animal protection and were approved by the Cantonal Veterinary Office Zurich (Permit Number: 18-2014). All procedures fulfill the ARRIVE guidelines on reporting animal experiments. Male C57BL/6J mice (Janvier, France), weighing 20-25 g, of 8-10 weeks of age were used. Animals were housed in a temperature controlled room in individually ventilated cages, under a 12 h dark/light cycle. Paper tissue was given as environmental enrichment. Access to pelleted food (3437PXL15, CARGILL) and water were provided ad libitum. In total, 37 mice were randomly allocated to tMCAO (n=16) or sham surgery (n=21). Surgical procedures were performed as described above (Focal cerebral ischemia).

Study design

Using G*Power 3.1 software (Heinrich-Heine-University, Düsseldorf, Germany; http://www.gpower.hhu.de/), a group size of n=4 was determined a priori for the primary end point T2 relaxation time, with an effect size d=2.0, α=0.05 and β=0.2. Consequently, group sizes of n>4 were used.

Standardly only male C57BL/6J mice are used for stroke models in mice. For randomization the web tool www.randomizer.org was used. Animals received randomized numbers and were randomly allocated to their cages and groups (sham or tMCAO). Surgeries were performed in a random order,
while the experimenter was blinded to the group until the insertion of the filament during the surgery. One group of animals underwent assessment of muscle tissue oxygenation with MSOT at 30 min after induction of tMCAO or sham surgery. A second group of animals underwent MRI examination at 24 or 48 h after reperfusion. Due to technical reasons fewer animals were measured at the 24h than at the 48h time point. Afterwards mice were transcardially perfused with 20ml saline and 20ml of 4% PFA solution in PBS for histological analysis. We performed evaluation of all read-out parameters while blinded to the experimental groups. The following criteria were used to exclude animals from the endpoint analysis: (i) subarachnoidal or intracerebral hemorrhage; (ii) no reflow after filament withdrawal, (iii) Bederson score = 0 (only in MCAO groups) and (iv) dead before experimental endpoint.

**Multi-spectral optoacoustic tomography**

For MSOT mice were anesthetized with 1.5-2% isoflurane in an oxygen/air mixture (1:4). The fur overlying the head was first trimmed with an electrical shaver and then hair was removed by using a depilation cream (Nair). Mice were placed in a mouse holder in supine position. Isoflurane anesthesia (1.5%) in oxygen/air mixture was supplied via a nose cone. Ultrasound gel (Diagramma, Switzerland) was applied to the mouse head for ultrasonic coupling and the animals were wrapped in a polyethylene membrane. The mouse holder was placed in an acquisition chamber filled with water for acoustic coupling. Water was kept 34°C to maintain body temperature while imaging.

The MSOT inVision 128 small animal imaging system (iThera Medical GmbH, Munich, Germany) was previously described (Morscher et al., 2014). Briefly, a tunable optical parametric oscillator pumped by an Nd:YAG laser provides excitation pulses with a duration of 9 ns at wavelengths from 680 to 980 nm at a repetition rate of 10Hz with a wavelength tuning speed of 10 ms and a peak pulse energy of 100 mJ at 730 nm. Ten arms of a fiber bundle provide even illumination of a ring-shaped light strip of approximately 8 mm width. For ultrasound detection, 128 cylindrically focused ultrasound transducers with a center frequency of 5MHz (60% bandwidth), organized in a concave array of 270 degree angular coverage and a radius of curvature of 4 cm, are used.

**Magnetic resonance imaging**

Data was acquired on a 4.7T Bruker PharmaScan 47/16 (Bruker BioSpin GmbH), equipped with a volume resonator operating in quadrature mode for excitation and a four element phased array surface coil for signal reception. During MRI acquisition, mice were kept under isoflurane anesthesia 1.5% in a 4:1 air/oxygen mixture. Body temperature was monitored with a rectal temperature probe (MLT415, ADInstruments) and kept at 36±0.5°C using a warm water circuit integrated into the animal support (Bruker BioSpin GmbH). First, one sagittal 2D slice was acquired with a true fast imaging with steady state precession (TrueFISP) sequence to reproducibly position the image slices for the $T_2$ map.
between animals and different days of assessment. The TrueFISP sequence was applied with the following parameters: echo time (TE) = 3 ms, repetition time (TR) = 6 ms, 100 averages, slice thickness (ST) = 1 mm, field of view (FOV) = 2.56x1.28 cm², matrix size (MS) = 256x128, giving an in-plane resolution (IR) = 100x100 µm². For time-of-flight MR angiography (TOF-MRA) a 3D FLASH sequence with the parameters TE = 3 ms, TR = 12, α = 20°, 3 averages, FOV = 20x20x20 mm³ and a matrix = 256x256x256 were acquired. To assess the T₂ relaxation time of muscle tissue a 2D Carr-Purcell-Meiboom-Gill multislice-multi-echo (MSME) sequence was performed with 14 echoes, TE₁ = 12 ms, an inter-echo time = 12 ms, TR = 2783 ms, averages=4, FOV = 20x20 mm², matrix = 100x100.

**MSOT image reconstruction and spectral unmixing**

Images were reconstructed using a backprojection algorithm with a non-negativity constraint imposed during inversion (Kruger et al., 2003). Linear unmixing was applied to resolve signals from deoxygenated and oxygenated hemoglobin (Burton et al., 2013). For each pixel in the image, the method fits the total measured optoacoustic spectrum to the known absorption spectra of oxy- (HbO₂) and deoxy-hemoglobin (Hb). On axial cross-sections of the mouse head regions-of-interest were drawn over the left ischemic (ipsi) and right (contra) temporal muscle. Oxygen saturation of each region was calculated according to:

\[ sO₂ = \frac{HbO₂}{Hb + HbO₂} \]  

The ratio of the oxygen saturation (sO₂) values between the ipsi- and contralateral side were calculated.

**MRI data processing and analysis**

T₂ maps were computed using Paravision software (Bruker) by mono-exponential fitting of the spin-echo magnitude signal decay, S,

\[ S = S₀ \exp \left( -\frac{TE}{T₂} \right), \]

where both S₀, the signal at TE=0, and T₂, the irreversible relaxation time, are fitted.

**Histology**

Animals were put under deep anesthesia by intraperitoneal injection of ketamine/xylazine/acepromazine maleate (100/20/3 mg/kg body weight) and transcardially perfused with 20 ml PBS, followed by 1% PFA in PBS (pH 7.4; PFA). Whole heads were postfixed in 4% PFA for approximately 48 h. After fixation, skulls were decalcified in Citrate EDTA Buffer pH 7.5 (Quartett, Germany) for 7 days, then sliced (coronary sections, 2 mm slices, caudal direction from nose) and routinely embedded.
in paraffin wax. Five µm-thick coronary consecutive sections were prepared and stained with hematoxylin eosin (Sigma-Aldrich).

Statistical analysis

Comparisons between groups were made by a Mann-Whitney rank sum test and Student’s t-test (SigmaPlot 12.5). A p-value p-value<0.05 was considered significant.

3.3.4. Results

Arrest of blood flow in ECA territory caused reduced tissue oxygenation in temporal muscle

The ligation of the common carotid artery and ECA led to an arrest of blood flow in the corresponding vessels as depicted with TOF-MRI (Figure 21). We investigated the impact of blood restriction on tissue oxygenation levels in the temporal muscles after tMCAO and corresponding sham surgery using a MSOT system. The tissue oxygen saturation levels of the ipsilateral muscle were significantly decreased in 7 out of 10 sham and 7 out of 9 tMCAO animals compared to contralateral side (Mean±CI, sO₂ sham 66±22%; tMCAO 59±14%, vs. 100%, excluding non-responders; p<0.05, respectively). There were no significant differences observed between the ipsilateral muscle of sham and tMCAO operated animals (p>0.05).

Figure 21: In vivo assessment of temporal muscle oxygenation with multi-spectral optoacoustic tomography. (A) Axial maximum-intensity projection of a TOF-angiogram 24 h after tMCAO. Arrow points to non-perfused common carotid artery and ECA. (B) Depicted are axial cross-section MSOT images of mice after sham surgery and tMCAO (approximately at Bregma -1.7±0.3 mm). Spectral unmixing reveals signal from oxygenated and deoxygenated hemoglobin in blue and red, respectively. Signals are overlaid on the single wavelength image (900 nm). Examples of ROI are shown (white lines). (C) Ratio of sO₂ between the left ischemic (ipsi) and right contralateral (contra) temporal muscles. sO₂ are decreased in the ipsilateral muscle in both tMCAO and sham-operated animals. Mean with 95% confidence intervals, sham n=7, tMCAO n=7; Mann-Whitney Rank sum test; p>0.05 tMCAO versus sham.
**Tissue damage in the ECA territory**

Parametric maps of tMCAO and sham operated animals at 24 h and 48 h after surgery were inspected (Figure 22). At the final time point 10 out of 11 sham and 6 out of 7 tMCAO animals increased T2 relaxation times in the temporal muscle ipsilateral to ECA ligation. The signal changes in the temporal muscle were not homogenous across the muscle, where central parts had higher T2 values than the muscle periphery. Statistical analysis (excluding mice with no differences in T2 values) showed significantly higher T2 values between the ipsilateral temporal muscle and contralateral side, both at 24 h and 48 h after surgery (Mean±CI, T2 sham 40.9±3.1 ms vs. 34.5±4.7 ms and 40.7±1.3 ms vs. 34.7±1.7 ms; tMCAO 44.2±7.1 ms vs. 34.3±5.8 ms and 46.6±9.3 ms vs. 35.3±1.2 ms, at 24 h and 48 h after surgery respectively, p<0.05) with no differences between the sham and tMCAO animals.

![Figure 22: Serial assessment of temporal muscle degeneration with MRI. T2 mapping was performed at 24 and 48 h after reperfusion. (A) Representative T2 maps of a mouse head acquired in axial orientation at 48 h after sham surgery. White arrows point to areas of increased T2 values. ROI analysis of (B) T2 values of the ipsilateral and contralateral temporal muscle of tMCAO and sham-operated animals. Mean with 95% confidence intervals; sham 24 h n=5, sham 48 h n=10; tMCAO 24 h n=5, tMCAO 48 h n=6; Kruskal-Wallis one way ANOVA followed Dunn’s pairwise multiple comparison; *p<0.05 versus contralateral.](image)

To assess tissue damage induced by the ligation of the ECA, we generated 24 h after surgery whole head slices of sham and tMCAO animals and performed H&E stainings (Figure 23). Examinations of the ipsilateral temporal muscle at a higher magnification identified that increased T2-relaxation times correspond to mild to moderate myofibre degeneration in tMCAO animals, whereas the contralateral temporal muscle was histologically unaltered. Muscle degeneration was generally associated with evidence of neutrophil recruitment into the muscle and mild interstitial edema. Rare individual
degenerated fibers were also seen in the ipsilateral temporal muscle of sham-operated mice, but without evidence of neutrophil recruitment.

However, myofiber degeneration was also observed in other muscles of the ligated ECA territory, in particular the masseter muscle bulks, where it was generally more intense than in the temporal muscle, and associated with neutrophil influx.

Figure 23: Histological findings in tMCAO mouse, 24 h after reperfusion. Hematoxylin eosin stains. Coronal section of the head at Bregma 1.1 (A–E). (A) Overview, indicating the cerebral lesion (*), the affected temporal muscle (a) and masseter muscle (b). Bar=2.5 mm. (B) Ipsilateral temporal muscle with myofibres undergoing degeneration (arrows) and mild interstitial edema of a sham animal. (D) Ipsilateral temporal muscle of a tMCAO animal with myofibres degeneration (arrows). There is interstitial edema with several neutrophils in interstitial capillaries. (C and E) Corresponding contralateral muscles to B and D without any alterations. (B–E) Scale bar indicates 40 μm.
3.3.5. Discussion

We demonstrated that ECA ligation required for tMCAO/sham procedure results in acute reduction of tissue oxygenation and in subsequent tissue damage. Generally, a high variability in read-outs was observed between animals. This might be likely due to differences in vascular architecture of individual animals (Froud, 1959). Given the differences in vascular architecture between different mouse strains the effect of ECA ligation may differ in strains other than C57BL/6.

Skeletal muscle is more tolerant to ischemia than the brain, and requires several hours of ischemia before damage manifests (Steinau, 1988). In the MCAO model the ECA ligation is not reversed and we have shown that this leads to increased T2 values. Histological examination revealed myofiber degeneration, edema and neutrophil influx in areas of increased T2. In addition to the ischemic muscle damage, ECA ligation induces local inflammatory processes in the vessel wall and surrounding tissues, where the inflammatory processes can even involve the cervical ganglion.

In the current study, we have not investigated functional consequences of ECA ligation. However, it was previously reported that tMCAO leads to weight loss, ischemia-induced muscle wasting and reduced food intake which is correlated with lesion volume (Steinau, 1988). Based on the results of the present study one could speculate that the tMCAO and even the surgery alone can be responsible for some of the decreased food intake, both due to muscle degeneration in the ECA territory and due to surgery associated local inflammatory processes which are associated with pain and/or affect the sympathetic nervous system. Extensive muscle damage can lead to impaired function of muscles involved in mastication and swallowing, and myalgia. Studies have previously described the impairment of motor function in the intraluminal tMCAO model in rats (Dittmar et al., 2003; Trueman et al., 2011). Moreover, ischemia of the skeletal muscles leads to a systemic inflammatory response (Blaisdell, 2002), which might affect the dynamics and distribution of immune cells in the tMCAO model. Indeed, we reported an increase in the proportion of circulating neutrophils in the blood of sham-operated animals, similar to tMCAO mice (Vaas et al., 2016). Also, muscle ischemia leads to factor XII-driven contact-induced activation of the coagulation system and can induce coagulopathy (Cafferata et al., 1968). Though, also the type of anesthesia and analgesia, the execution and duration of the surgery might affect these described parameters, the effects of ECA tissue damage on the read-outs need to be considered. A possible solution to prevent muscle damage in the ECA territory is to reperfuse also the common carotid artery and the ECA after retraction of the filament. This would require closing the incision in the common carotid artery and might thus be technically very challenging.
3.3.6. Summary/Conclusions

ECA ligation leads to variable tissue damage. Its confounding impact on study parameters in experimental stroke studies needs to be further investigated.
Chapter 4

4. General discussion and outlook
4.1. General discussion

It is commonly accepted that neutrophils are the first immune cells attracted to injured tissue, yet their role after cerebral ischemia remains controversial. Experimental models of cerebral ischemia have been employed to study neutrophil function but still many questions remain, e.g. to what extend and how neutrophils are recruited to the brain, and how they partake in ischemic tissue injury (reviewed in (Jickling et al., 2015; Famakin, 2014)). This gap of knowledge stems mainly from limitations in currently available technology. Generally, knowledge about neutrophils function relies mainly on cell culture work, and ex vivo data. In vivo evidence that elucidate their function in the brain is still scarce. Immunohistochemistry studies have suffered from a lack of specificity of reagents and cannot capture the immediate dynamics of cell recruitment (Fleming et al., 1993; Breckwoldt et al., 2008; Garau et al., 2005). Flow cytometry is state-of-the-art for studying function, morphology, composition, proliferation, and protein expression in circulating leukocytes. But the extraction of cells from a living organism may lead to alterations in cell properties (Lyons et al., 2007). Moreover, while cells are easily isolated from blood samples, obtaining brain tissue samples requires sacrificing the animal under study. This prevents the long-term study of cells and their interactions in their native biological environment. Intravital microscopy has been shown to enable direct monitoring of leukocyte trafficking to the ischemic brain in vivo. However, for accessing the brain either a thinned skull region or the preparation of a cranial window are required, which may induce tissue damage and disturb physiology.

Advances in optical imaging instrumentation over the past few years have enabled the macroscopic imaging of physiological and cellular processes by NIRF imaging in the intact animal. Non-invasive optical imaging techniques have a high sensitivity, use stable labels and are particularly suited to study cell trafficking, in animal models of cerebral ischemia in vivo. The non-invasiveness of these methods enable to characterize the dynamics of processes without impeding animal physiology and welfare. We have contributed to the understanding of neutrophil trafficking by developing a NIRF imaging-based technique to track neutrophils to the mouse brain in the tMCAO model of focal cerebral ischemia. Using this tool we were able to show the dynamics of the acute response in neutrophil recruitment and we assessed the role of two important mediators of neutrophil recruitment. We detected fluorescence in the ischemic lesion at subacute time points after tMCAO. This might indicate an extended life span which is known to occur in a pro-inflammatory environment. However, caution should be exerted when assessing the long term fate of labeled neutrophils as they might be taken up by phagocytosing cells, like macrophages together with the fluorescent dye. Here, verification experiments at later time points are warranted.
After severe hypoxia, occurring also after ischemia, endothelial cells release pro-inflammatory cytokines and upregulate adhesion molecules to enable immune cell recruitment to the affected region (reviewed in (Androulakis et al., 2016). Vascular guiding cues have been defined in the periphery, but importantly they seem to behave differently in distinct tissue including the brain. ICAM-1 has been identified as one of the key vascular adhesion molecules of neutrophils in the vessel of the periphery, for the recruitment to the liver (Ochietti et al., 2002), to the lung (Kamochi et al., 1999), to the colon (Bennett et al., 1997), or to the heart (Feeley et al., 2000). We showed that ICAM-1 is not required for the recruitment of neutrophils to the brain after transient cerebral ischemia in mice (manuscript submitted). Moreover, we did not find a neuroprotective effect in ICAM-1null mice compared to wild-typ mice subjected to tMCAO. However, these results are not in agreement with previous works, which showed that mice lacking the ICAM-1 receptors are neuroprotected after ischemia/reperfusion injury and have a lower number of recruited neutrophils (Connolly et al., 1996; Soriano et al., 1996). In both studies ICAM-1 mutants employed have been shown to still express functional soluble splice variants of ICAM-1 (van Den Engel et al., 2000). These functional splice variants influence leukocyte adhesion other than neutrophils and migration via LFA-1/ICAM-1 interaction (van Den Engel et al., 2000; Rieckmann et al., 1995). Therefore, the protective effect might be due to a reduced recruitment of cells of other leukocyte subgroups.

In light of a recent publication by Neumann and colleagues which proposed that neutrophil recruitment can be influenced via VLA-4/VCAM-1 binding (Neumann et al., 2015). Therefore, we investigated the blockage of the α4-integrin subunit of the VLA-4 receptor after tMCAO. NIRF imaging revealed reduced neutrophil recruitment up to 24 h after tMCAO and immunohistochemistry indicated neuroprotective effects at 48 h. The neuroprotective findings are in line with several previous studies (Liesz et al., 2011; Neumann et al., 2015; Becker et al., 2001; Relton et al., 2001), but are controversial discussed since other studies including a powerful multicentre study could not find this treatment effect (Langhauser et al., 2014; Llovera et al., 2015). The differences in the readouts might result from the injection time points of the antibodies chosen. We injected the antibody before tMCAO, while in the multicentre study it was applied 3 h after the filament was removed and reperfusion was allowed. Here the multicentre trial reflects a more clinical relevant situation. Since we and others have shown that neutrophil recruitment already starts within the first hours (Neumann et al., 2015), this might explain the differences. Additionally, the antibody treatment might delay the lesion growth. In our study the lesion volume was measured after 48 h, whereas in the multicentre trial lesion volumes were evaluated after four days. Our results suggest a pathogenic role of neutrophils after cerebral ischemia in their early recruitment phase. It still needs to be investigated over which period neutrophils express a pro-inflammatory phenotype after stroke and when they switch to an anti-inflammatory phenotype.
Indeed, studies showed a high plasticity with phenotype changes in neutrophils. In an early study from Johnston and colleagues a change in the expression of chemokine receptors and adhesion molecules in a model of chronic autoimmunity was shown for neutrophils (Johnston et al., 1999). Some pathogens can prime neutrophils towards an anti-inflammatory response (Tosello Boari et al., 2012; Tsuda et al., 2004), while invariant natural killer T-cells can invert this switch (De Santo et al., 2010). In addition neutrophils were shown to shape the immune response by crosstalk with macrophages (Tsuda et al., 2004; Mantovani et al., 2011). There are also reports on pro-angiogenic neutrophils, which promote neovascularisation under hypoxia in transplants (Christoffersson et al., 2012) and cancer (Jablonska et al., 2010). These neutrophils were recruited by the release of vascular endothelial growth factor (VEGF), a downstream product of hypoxia-inducible factor-1α, from endothelial cells due to hypoxia (Christoffersson et al., 2012). VEGF levels in patients are increased after stroke within 24 h, where substantial neutrophil recruitment occurs (Talwar and Srivastava, 2014; Lee et al., 2010; Enzmann et al., 2013). Interestingly, Christoffersson and colleagues reported, that these cells had a high load of MMP-9 (Christoffersson et al., 2012), which significantly contributes to BBB impairment, while at later stages MMP-9 promotes angiogenesis, tissue clearance and repair. It might be further investigated how specific neutrophil subsets partake in ischemic tissue injury and repair, and might thus enable a more specific therapeutic intervention.

We showed the feasibility to monitor fluorescently labelled neutrophils over time in the tMCAO model. A disadvantage of our approach is that neutrophils need to be generated, labelled and adoptively transferred. While in mice bone marrow derived neutrophils are functional (Boxio et al., 2004), we have shown that ex vivo labelling of neutrophils with a membrane dye does not lead to their activation. However, the ex vivo handling of cells might lead to changes in the phenotype of cells, which might also influence their behaviour in the recipient mice. Moreover, by adoptively transferring labelled cells the number of neutrophils was significantly increased in the recipient animal which might ultimately lead to alterations in the magnitude or dynamics of the inflammatory response. Alternative strategies that might be explored are the labelling of cells in the blood stream using labelled neutrophil-targeting antibodies (will be discussed further below (von Brühl et al., 2012). Or the use of transgenic mice where endogenous neutrophils express a fluorescent protein (Hasenberg et al., 2015). However, mice generating neutrophils with proteins that are fluorescent in the NIR range have not yet been created.

In recent decades, several experimental therapies have been developed and tested for their ability to ameliorate stroke-related brain injury, but unfortunately, none of the tested drugs that show promising effects in animal models of stroke have shown clinical benefits in stroke patients (Enlimomab Acute Stroke Trial Investigators, 2001; Krams et al., 2003). Many reasons for failed translation have been identified including low statistical power, lack of quality-control mechanisms,
deficiencies in reporting, and negative publication bias (Dirnagl, 2006). Despite this shortcomings of experimental stroke research rodent models of stroke are considered useful to investigate pathophysiological mechanism and for preclinical drug testing. There are many similarities in the immune response between animals after experimental stroke and stroke patients. Neutrophils are released after injury in human and mice from the bone marrow, which function as reservoir, into the blood stream (Devi et al., 2013). After their activation their life-span extends in both human and mice from several hours up to days (Cronkite, 1979; Tofts et al., 2011; Kim et al., 2011b). Also after cerebral ischemia neutrophil behaviour reflects the situation in patients over large scales: recruitment patterns (Enzmann et al., 2013; del Zoppo, 2010); MMP-9 regulation (reviewed in (Turner and Sharp, 2016); NO release (Forster et al., 1999; ladecola et al., 1996). However, there are also several important differences. The inflammatory response on the genomic level differ between mice and man significantly (Seok et al., 2013). Also the composition of leukocytes is different between humans and rodents where humans have a higher neutrophil content than mice which might also lead to differences in the outcome of neutrophil targeted therapies in patients and animals (Doeing et al., 2003; Mestas and Hughes, 2004). Moreover, human stroke occurs spontaneously mainly due to cardiovascular disease, while cerebral ischemia in models needs to be induced experimentally with surgery.

We showed in the filament model of tMCAO in mice, that the ECA territory is severely affected by the ligation of the ECA. Using MSOT, MRI and histology we demonstrated, that the affected muscles have reduced tissue oxygenation, show signs of myofiber degeneration, edema and neutrophil infiltration. Muscle damage, like cerebral ischemia leads to a systemic inflammatory response (Anrather and ladecola, 2016; Blaisdell, 2002). In addition, muscles supported by the ECA are involved in chewing and swallowing and damage might thus affect food uptake and animal welfare (Dittmar et al., 2003), which in turn might have an impact on lesion growth (Springer et al., 2014). Furthermore, Denes and colleagues showed in the MCAO mouse model that the immune response is affected by the surgical intervention and anaesthesia (Denes et al., 2013). We showed in blood samples analysed by FACS increased neutrophil counts in circulation in both sham and tMCAO mice, suggesting that the systemic immune response is dominated by the surgical intervention in the tMCAO mouse model. In addition, studies have shown that surgeries on muscles in patients caused coagulopathy followed by the activation of the factor XII-driven contact coagulation system with thrombus formations (Cafferata et al., 1969). Together with the systemic inflammation the activated coagulation system has an impact on the development of the ischemic lesion (Mccoll et al., 2007; Kleinschnitz et al., 2006). Therefore, the effects of surgery and ECA ligation need to be considered in studies using the tMCAO mouse model, a widely used and accepted model, in particular when investigating inflammatory processes.
NIRF imaging has been shown to be a suitable tool to study in vivo processes after cerebral ischemia in mice (Ku and Choi, 2012; Kang et al., 2015; Zhang et al., 2012). For the first time we monitored neutrophil recruitment to the ischemic brain in mice over time in intact animals and with large coverage and depth. For all cell tracking approaches e.g. antibodies, membrane intercalating dyes or by transfection in general several factors need to be considered. For example, Ly6G antibodies have been used to visualize neutrophils with two-photon microscopy (von Brühl et al., 2012). But antibodies can block specific cell epitopes and at higher concentrations specifically deplete cells from circulation (von Brühl et al., 2012; McDonald et al., 2010). Another disadvantage using antibodies to label structures is the resulting immune response against the labelled cells. McDonald and colleagues managed to mark neutrophils in vivo with a low concentration of fluorescent labelled antibodies and following these cells over a short time using spinning disc confocal intravital microscopy (McDonald et al., 2010). An additional disadvantage of antibody-based approaches is their long half-life when injected into the circulation. Unbound circulating antibody creates a background and prevents detection of bound probes. Hence, such approaches require long wash-out times (Klohs et al., 2008a). Nanobodies with their short plasma half-life, reduced immune reactivity and good tissue penetration might be a future choice. Transgenic animal that expresses the fluorescent protein under a neutrophil specific promoter indicating their activation might also be employed for neutrophil tracking. Recently, a transgenic reporter mouse line, where endogenous neutrophils expressed tdTomato under the Ly6G promoter, was presented (Hasenberg et al., 2015). The tdTomato protein has its excitation maximum at 554 nm and its emission maximum at 581 nm (Shaner et al., 2005). While this renders the protein suitable for flow cytometry and intravital two-photon microscopy, the absorption of photons in that spectral range would prevent photons from reaching and emitting from deeper tissue. Therefore, it cannot be used for non-invasive NIRF fluorescence imaging. In the future transgenic reporter animals expressing one or more fluorochromes in the NIRF range might become available (Genevois et al., 2016).

However, despite the advantages NIRF imaging provides, like stable labels and high detection sensitivity, for tracking neutrophils, it faces also several limitations. The optical properties of the tissue govern the ability to perform NIRF imaging in the rodent brain. Absorption and scattering of photons in the tissue (which can both change under pathological conditions) make absolute quantification of fluorescence emission challenging. Hence, in most studies the fluorescence emission is expressed in terms of contrast, i.e. as a ratio of the fluorescence intensity measured over the disease-affected region to the intensity measured over a non-affected region. Quantitative analysis of fluorescence emission can be achieved using fluorescence molecular tomography. Such fluorescence molecular tomography systems have been built and are even commercially available. The technique relies on
algorithms to reconstruct fluorochrome distribution. However, these algorithms still need to be improved before they can be reliably applied in in vivo studies. Moreover, scattering of photons limits the spatial resolution of planar NIRF imaging system to 2-3 mm and to 0.45 mm for fluorescence molecular tomography, and are thus not suitable to follow neutrophils at the microscopic scale. Recent advances have shown to use NIRF imaging in the NIR window 1300-1400 nm which reduces scattering significantly and enabled a resolution of sub-10 µm resolution (Hong et al., 2014). Thus, in the future NIRF imaging will bridge to microscopic imaging.

In conclusion, NIRF imaging is a versatile tool to study neutrophil recruitment in vivo. Using the technique we have described the dynamics of neutrophil recruitment to the ischemic brain and have revealed that the VLA-4, but not ICAM-1 is crucial for neutrophil recruitment.
4.2. Outlook

We developed a new NIRF-imaging tool to spatiotemporally resolve the neutrophil recruitment in a model of transient cerebral ischemia. In the next steps of the project we will try to overcome the limitations faced in the studies and direct the project into different directions observing the inflammatory processes after cerebral ischemia. Our cell labelling approach as such could be used to study the role of neutrophils in other disease models but can also be used to monitor other immune cell types, such as monocytes or macrophages in models of cerebral ischemia. Monocytes are recruited by neutrophils after stroke and thought to further accelerate the inflammation-driven damage. However, they also have been implicated to restrict the inflammation, help clear the affected tissue from cell debris as well as induce tissue remodelling and repair (reviewed in (Kim and Cho, 2016). Studying the contribution of monocytes/macrophages is an important step in understanding the pathophysiology of stroke.

In the current project the dynamics and mediators of neutrophil recruitment were described, however the role of neutrophils in the pathogenesis of the disease has not been addressed. In a next step the cell labelling approach together with targeted and activatable probes will enable us to visualize neutrophil function, e.g. ROS generation, protease release etc. This could be used to expose new therapeutic targets.

In the current approach the adoptive transfer of labelled neutrophils leads to an increase in neutrophil numbers in the recipient animal. Moreover, the need for extraction of cells from the bone marrow of a donor mouse may lead to alterations in cell properties. Therefore, it would be highly desirable to generate transgenic animals which express fluorescent proteins in the near infrared optical window in subclasses of immune cells or in other cell types under the regulation of a proinflammatory promoter. The possibility of such approaches was already demonstrated in multiple ways. But so far only one group managed to generate a transgenic mouse line, in which only neutrophils express a fluorescent protein (Hasenberg et al., 2015), unfortunately with unfavourable optical properties for deep tissue imaging. The generation of such animals would replace the time consuming adoptive transfer of labelled cells with all its limitations, but yet it might be time and resource consuming to generate such transgenic animals. An alternative would be the use of targeted fluorescent probes e.g. labelled antibodies targeted against receptors of immune cells subsets.

During our studies we discovered an effect of the ECA ligation that is required in the tMCAO model. Here the model have to be improved further. The best option would be to close the incision in the CCA with a suture and reopen all arteries to allow complete reflow. Another good option would be to insert the filament over the ICA and only ligate the ICA twice. This option would cause no ischemia to the
ECA territory, while the ICA territory would be still affected by a reduced blood flow. The feasibility of this approaches have to be investigated.

Planar NIRF imaging has a spatial resolution of 2-3 mm and provides two-dimensional images with no depth information (Vaas and Klohs, 2016). Fluorescence molecular tomography, which employ more complex data acquisition schemes but can resolve the signal in a 3D manner, enable fluorescence quantification, and increase spatial resolution to 1 mm (Bourayou et al., 2008). Using both fluorescence reflectance imaging and transillumination fluorescence imaging will further increase the accuracy of the measurements. In addition transillumination fluorescence imaging allows to sample the entire volume and benefits from a reduced autofluorescence (Vaas and Klohs, 2016). Combining fluorescence molecular tomography with MRI adds structural information and can increase the resolution of the fluorescence signal, maybe giving us the possibility to separate the extracranial from the cranial signal obtained from the two sites of neutrophil accumulation (Vaas et al., 2016). Hybrid systems also might be used to co-localize cellular information with physiological information of the tissue and in turn help to understand how cells partake in ischemic tissue remodelling and repair. This would provide an opportunity to use therapeutic approaches to more specifically modulate the immune response. Such strategies might pose a better way to abrogate the detrimental aspects of inflammation and promote processes required for functional recovery, without causing undesired side effects. However, currently the application fluorescence molecular tomography in experimental studies is hampered by the long acquisition times and computation times, and the need to improve reconstruction algorithms.

Taken together there is sufficient space to optimize and follow up research questions that have been addressed during the work of this PhD thesis. Addressing the above mentioned points will help to understand the role of neutrophils and of inflammatory processes more in general after cerebral ischemia and this help to improve the diagnosis of the disease and to develop and evaluate novel therapies.
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List of Publications

Journal articles


G. Enzmann, S. Pavlidou, M. Vaas, J. Klohs and B. Engelhardt, "ICAM-1null C57BL/6 mice are not protected from experimental ischemic stroke" in preparation.

Book chapters

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