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DIRECT COMPARISON OF MICROGLIAL DYNAMICS AND INFLAMMATORY PROFILE IN PHOTOTHROMBOTIC AND ARTERIAL OCCLUSION EVOKED STROKE

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Abstract

Many focal cerebral ischemia models utilize the middle cerebral artery occlusion (MCAO) evoked by coagulation to induce ischemic damage in the cortex and mimic the pathology observed in human patients. A second, increasingly popular model, the photothrombotic stroke, uses a laser beam to irradiate the MCA after administration of a photosensitizing dye. This widely used procedure is slowly replacing the MCAO model because of the easiness of the surgical protocol and the reproducibility of the damage. However, the photochemical reaction also results in wider microvascular injury. In this study, we have evaluated the impact of these two types of stroke in the cell survival and evolution of stroke, focusing on microglial cells, the first responders to cell injury. Two groups of heterozygote Cx3CR1-GFP reporter mice (to follow microglia) were subject to stroke injury either with coagulator-mediated occlusion or photothrombotic MCA damage. Microglial cells dynamics of activation and phagocytosis together with astrocytic response and leukocyte infiltration were characterized at 1, 3 and 7 days after damage. Photothrombotic stroke delayed microglial and astrocytic invasion of the ischemic core and accumulation of phagocytic microglia. It also elicited higher levels of inflammatory cytokines/chemokines and increased infiltration from the periphery. In addition, only the neurons in the MCAO stroke showed phenotype plasticity by downregulating the transcription factor NeuN. These data provide a better understanding of the exact temporal and spatial dynamics of the inflammatory response in these two animal models of stroke and identify more relevant targets for human therapy.

Keywords

stroke; inflammation; photothrombosis; astrocytes; microglia; ischemia

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INTRODUCTION

A stroke is classically defined as an inadequate blood supply to the brain leading to cerebral infarction. In the United States, a stroke occurs every 40 seconds and is one of the leading causes of disability and death in the country, with more than 30 billion dollars in medical costs per year (Mozaffarian et al., 2015). Yet, there exists only one effective medical treatment, thrombolysis with tissue plasminogen activator (tPA), that when applied within the first 4 hours after stroke, reduces stroke damage in a minority of patients (del Zoppo et al., 2009). Manual removal of the blood clot is another option that has recently been incorporated in the treatment of stroke although the patient population that can benefit from this procedure is still under research (Broussalis et al., 2011).

Given that about 87% of all human strokes are ischemic in nature, animal models that use experimental procedures for focal or global artery occlusion have been widely used to study the pathophysiology of stroke and its possible intervention. In the artery occlusion models, a temporary occlusion of the middle cerebral artery induces ischemic damage in cortex and striatum and mimics, for the most part, the pathology provoked by blood clots in stroke patients. Unfortunately, there are substantial obstacles to the use of the MCA occlusion model for the mechanistic analysis of the human brain response to ischemia and for the design of medical interventions. Chief among these obstacles are the variability in infarct volume and in behavioral deficits across experimental subjects (Gladstone et al., 2002).

A second stroke model that is also gaining popularity, the photothrombotic stroke, is slowly replacing the artery ligation stroke because of its small variability. In the photothrombotic stroke, a photosensitive dye is injected to promote damage after laser-irradiation, only requires a small cranial window, preventing unwanted hemorrhage, and results in consistent neocortex infarction but not in deep structures, which is more similar to the damage observed in human stroke (Traystman, 2003).

One important difference between the two modes of stroke is, however, the effect on its microcirculation. Whereas in the artery occlusion model the microcirculation is left intact, the photochemical reaction used in the photothrombotic stroke results in broader microvascular injury and loss of capillary flow (Traystman et al., 2003; Yushmanov et al., 2006). This critical difference between the two types of brain damage has implication for stroke outcome and prompted us to investigate the impact of the two types of injury on cell survival and evolution of the infarct. We found differences not only in stroke progression but also in the cellular response of glial, neuronal and immune infiltrating cells to migration and inflammation.

We here focused on microglial cells, the resident immune cells of the brain, because they are some of the first responder to brain injury and sculpt tissue repair by both phagocytosis and cytokine release (Kettenman et al., 2013). Apart from finding significant differences in the activation state of microglial cells, the inflammatory profile of the two types of strokes clearly differed, too.

EXPERIMENTAL PROCEDURES

Animals

WT C57BL/6 background or Cx3CR1-GFP mice (Jung et al., 2000; Bradfield et al., 2007) were maintained on a 12h light/dark cycle with unlimited access to food and water. All animals used in this study were utilized in accordance with the NIH guidelines for the care and use of laboratory animals in experimental procedures and the protocols approved by the University of Rochester Ethical Committee for Experimental Animals.

2-month old Cx3CR1-GFP animals (3–5 animals per group, males and females) were subject either to photothrombotic stroke or to permanent distal middle cerebral artery stroke and left to recover for 1, 4 or 7 days before processing the brains for immunohistochemistry or cytokine analysis. All animals were fasted overnight before the strokes.

Experimental Stroke Models

Permanent distal middle cerebral artery occlusion (MCAO)—Anesthetized animals were secured on a stereotaxic frame with a heating pad placed beneath the animal to maintain physiological body temperature. A surgical vertical incision was made from the right eye to the right ear to expose the middle cerebral artery (MCA). Temporal muscle was separated and pushed down to expose the temporal skull. After the small piece of skull was removed, the MCA was exposed and the distal portion of the MCA (before the branches) coagulated by using a small vessel electrocoagulator. The temporal muscle was then placed back into its original position and the incision site closed with surgical nylon sutures. Mice were returned to the cage until fully recovered.

Photothrombotic stroke—Animals were anesthetized and placed on a stereotaxic frame with a heating pad placed beneath the animal to maintain physiological body temperature. A variation of the original protocol for the photothrombotic stroke was performed (Watson et al., 1985; Brown et al., 2007). Briefly, an incision was made in the animals' scalp from the right eye to the right ear and the skull cleaned and dried. Under cold ACSF superfusion, a high speed drill was used to thin a 1 mm diameter region of the skull over MCA. Rose Bengal dye (0.1 mL of 10g/L) was then injected i.v. The thinned skull region was then illuminated with a 0.01 mW green laser for 15 minutes, the scalp sutured together and the animal allowed to recover.

Cytokine Analysis—For cytokine analysis, 2 months old males (6 animals per group) were used. At 1, 4 or 7 days after stroke the animals were anesthetized and their blood flushed out with 20ml cold PBS via transcardial perfusion. Then, the brains were harvested and cut to 1mm thick coronal sections. After a quick TTC staining, infarct core (approximate 2 by 2 mm) was dissected out and frozen with liquid nitrogen. Since we found that the most anterior sections, containing the striatum area, produced the most comparable size between the cortical strokes in each type of model, we limited the cytokine analysis to the first half of the brain. For cytokine analysis, brain tissue was subject to multiplex ELISA analysis through the Quantibody Mouse Cytokine Array for the following cytokines/chemokines: bFGF, BLC, CD30L, eotaxin, eotaxin-2, FasL, G-CSF, GM-CSF, ICAM, IFN-

γ , IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-21, KC, leptin, LIX, MCP-1, MCP-5, M-CSF, MIG, MIP-1a, MIP-1g, PF-4, RANTES, TARC, TCA-3, TNF RI, TNF RII, TNF α (RayBiotech Inc.).

Immunohistochemistry—After stroke, mice were anesthetized and transcardially perfused with 4% paraformaldehyde in PBS. The brains were removed, post-fixed overnight for 24 hr and washed in PBS. After cryoprotection with 30% sucrose, 16 μ m cryostat coronal sections were cut. The following markers were used for immunostaining: GFAP (Rabbit polyclonal 1:500, from Dako), MAP2 (chicken polyclonal 1:5000, from AbCAM), Cd11b (Rat monoclonal 1:200, Serotec), Ly-6G (Rat monoclonal 1:150, Serotec). Sections were counterstained in DAPI-containing Vectashield (Vector). Low magnification images were obtained using a 2x or 10x lens in a BX53 Olympus microscope attached to a DP72 Olympus digital camera. High magnification images were taken with a confocal scanning microscope (Fluoview FV1200, Olympus).

For quantification of immunofluorescence, 2–4 sections per animal cut 160 μ m apart were evaluated (2–3 animals per time point).

For quantification of GFAP immunostaining, we quantified three different regions of fluorescent images taken at 10x: i) at the boundary of the injury, right after the infarcted area, devoid of GFAP immunofluorescence, ii) 50 μ m and iii) 100 μ m farther apart from the injury, on both sides of the stroke. To ensure we quantified comparable regions, we focused on layer 2–4 of cortex, that did not include superficial, deep cortical layers or areas that contained large vessels, where the GFAP staining is much more abundant than in the rest of the cortex, restricting our quantification to the middle cortical layers, where the GFAP staining is more homogeneous.

The mean pixel intensity (optical density), of a fixed area, 256 $\times$ 256 μ m, was quantified with Image-J. Data is represented as average $\pm$ s.e.m of % optical density.

For quantification of phagocytized cells, we counted the number of MAP2+ cells (neurons, red) enclosed inside GFP+ microglia (green) at both sides of stroke boundary, in an 80 $\times$ 80 μ m image collected at 60x. Cells were counted in single planes to distinguish cell boundaries; each plane separated at least 8 microns from each other.

For quantification of NeuN+ cells, we counted the number of NeuN+ cells in three different areas: i) right after the boundary of the stroke, ii) 50 μ m farther apart from the injury, in cortical layer II/III or iii) 50 μ m farther apart from the injury, in cortical layer IV/V. Statistical significance was established using unpaired t-test with $p < 0.05$.

Stroke volume was calculated by measuring the MAP2(–) infarcted area per section in each hemisphere using Image J free hand and multiplying by the depth of the infarcted tissue.

Statistical Analysis of cytokines levels

All statistical analysis was done using SPSS software. Statistical significance was determined by one-way analysis of variance ANOVA with a significant threshold of $p < 0.05$. For analysis including non-parametric samples ($p < 0.05$ by Shapiro-Wilk test for $n < 30$)

Mann-Whitney U test was used. For those samples showing a normal distribution but non-homogeneous variances ($p < 0.05$ by Levene's test), corrected student's t-test was applied. Data are expressed as mean $\pm$ SEM.

RESULTS

Localized cortical damage in the MCAO and the photothrombotic stroke models

For the artery ligation model, we have utilized a variation of the middle cerebral artery occlusion model (MCAO), the distal permanent MCA ligation (Nedergaard and Diemer, 1988; Bacigaluppi et al., 2010; Liesz et al., 2013). In this MCAO model, the tissue injury is limited and mostly restricted to the dorsolateral cortex, with little or no damage to the striatum. These properties make the arterial occlusion model more comparable in size and location to the photothrombotic stroke. Another advantage of comparing this ligation MCAO model to the photothrombotic model is that vascular occlusion is permanent in both (Gonzalez and Kolb, 2003; Yushmanov et al. 2006). At low magnification, both types of stroke exhibited a distinct ischemic core area sharply separated from the surrounding non-ischemic tissue restricted to the somatosensory cortex, and not including the striatum (Figure 1).

Microglial response differ between artery occlusion and photothrombotic stroke models

For both types of stroke, we then followed the evolution of damage over time, with immunostaining against the MAP2 neuronal antigen, and the concomitant microglial response. We used immunolabeling against MAP2, a cytoskeletal protein highly sensitive to neuronal injury (Huh et al., 2003), to outline the ischemic core. In both, the artery occlusion and the photothrombotic stroke model, the injured area was defined by a stroke core, which was devoid of MAP2 signal due to neuronal injury, and of the surrounding tissue, where the MAP2 signal is brighter. A sharp boundary between the MAP2 (–) and MAP2 (+) cells traditionally described in stroke is also observed in both models (Dawson et al., 1996; Schäbitz et al., 2004; Figure 2A). For microglial labeling, heterozygote Cx3CR1-GFP reporter mice, which express EGFP under the microglial chemokine receptor in monocyte-derived cells were utilized. These mice are viable, fertile and normal in size without gross behavioral abnormalities (Jung et al., 2000). Although the homozygote Cx3CR1^{GFP/GFP} results in Cx3CR1 deletion with effects in neurogenesis, neural plasticity and learning (Maggi et al., 2011), there is no interference with monocyte or dendritic cells immune function upon contact with microbial antigens (Jung et al., 2000; Bradfield et al., 2007). For our experiments, we exclusively used the heterozygote mice Cx3CR1^{GFP/+}, which express detectable levels of EGFP.

At 24 hours after stroke, we observed an initial, rapid response of GFP-microglial cells only clearly visible in the MCAO stroke, especially at the boundary of the stroke. Overall, brighter GFP+ green cells, possible indicative of stronger activation of microglia, are observed in the ipsilateral side of the MCAO stroke compared to the photothrombotic stroke, which shows a much weaker signal (Figure 2A (Kozłowski and Weimer, 2012)

At day 4 after injury, when the inflammatory response usually peaks (Wang et al., 2007; Gronberg et al., 2013), there is a clear increase of GFP-microglial cells that start accumulating in the core of the MCAO stroke, although to lesser degree in the photothrombotic stroke (Figure 2B).

At day 7 (Figure 2C), microglial activation is robust in both types of stroke at the time point when stroke volume is no longer expanding and the necrotic area is smaller in size than at day 1 and 4, possibly reflecting the resolution of tissue edema (Orlowski et al., 2013).

At higher magnification, the morphology and activation of microglial cells is better compared between the two models of stroke. At day 1, more ramified but also more hypertrophied cells with thicker ramifications and bigger cells bodies are apparent in the MCAO stroke, thus contributing to the brighter intensity of the GFP signal observed in MCAO vs photothrombotic stroke at lower magnification (Figure 2 and 3).

At day 4, activated microglial cells, as defined by more ameboid morphologies and loss of ramifications, appear around the stroke boundary in both strokes (Figure 3) but accumulate in larger amounts in the MCAO stroke compared to the photothrombotic stroke. Microglial activation persists in the MCAO stroke even several micrometers further away from the stroke with demi-lunar shapes, that resemble phagocytic cell bodies, present far from the stroke boundary, (Figure 3d).

At day 7, the photothrombotic tissue shows very strongly activated GFP-microglia at the stroke boundary and at the peri-infarct tissue. At this stage, the differences in microglial activation between the two types of stroke are less noticeable, both at low and high magnification and in all locations analyzed (Figures 2C and 3).

Faster appearance of phagocytic activity following MCAO stroke

The appearance of microglial profiles that resemble phagocytic cells in the MCAO stroke earlier than in the PH stroke prompted us to analyze in more detail the phagocytic capability of the microglia in the two stroke models. Single confocal sections of phagocytic cells showed MAP2+ neuronal bodies engulfed by GFP-positive microglia. These cells began to appear in the MCAO model at day 1, increased at day 4 and peaked at day 7 after stroke. In contrast, far fewer phagocytic profiles were observed at early stages in the photothrombotic stroke in all the animals analyzed (Figure 4) confirming the idea that microglial activation and phagocytosis occurs earlier in the MCAO than in the photothrombotic stroke.

Astrocytic gliosis appears earlier in the MCAO stroke

Numerous studies have reported a protective role of reactive astrocytes during stroke and their involvement in damage control and recovery (Otani et al., 2006; Hayakawa et al., 2010; Li et al., 2014; Faulkner et al., 2004), although detrimental effects mediated by astrocytes have also been described (Cotrina et al., 2008; Hines and Haydon, 2013). Therefore, we next studied the time course of astrocytic activation in the two types of stroke and its correlation to microglial activation. Reactive astrocytes, as determined by increased GFAP immunostaining and thickening of cell processes appeared 1 day after stroke in the MCAO

but not in the photothrombotic model (Figure 5A'–B'). At day 4 and 7, the intensity of GFAP immunolabeling was comparable in the two strokes (Figure 5C–D' and 5E'–F').

Thus, similar to what we observed with microglia activation, the appearance of reactive astrocytes was earlier in the MCAO stroke than in the photothrombotic stroke.

Cytokine Profile of MCAO versus photothrombotic stroke

The destruction of the microvasculature in photothrombotic stroke may enhance the endogenous inflammatory response and, in turn, exacerbate brain pathology (Young et al., 2012). Based on the observation that the dynamic responses of both microglia and astrocytes to stroke injury appeared faster in MCAO than in photothrombotic stroke, we next sought to establish the temporal pattern of cytokine/chemokine pathway activation due to their importance in both secondary ischemic injury and tissue repair (Chiba, 2013; Lee, 2014).

To this end, we mapped the cytokine/chemokine inflammatory profile of the ischemic tissue with a quantitative array platform that allows simultaneous detection of 40 cytokines/chemokines involved in inflammation. We found that most of the traditional cytokines were most abundant at day 1 in both types of strokes. However, some molecules showed a larger increase in the photothrombotic stroke than in the MCAO stroke already at day 1: the inflammatory cytokines, IL-6 and IL-17 (two fold increase), and the chemokine platelet factor-4 (PF-4, also known as CXCL4, 3 fold increase), among others.

At day 4, only PF-4 continued to be remarkably higher in the photothrombotic stroke (4 fold increase) than in the MCAO model, together with the intercellular adhesion molecule ICAM-1, which reaches equally the highest levels at day 7 in both types of stroke.

We also observed a trend to increased levels in the photothrombotic stroke in molecules involved with TNF α signaling like the receptors TNFR1 and II, from day 1 onwards, with the highest levels of TNFR2 at day 7 (10 fold increase). The levels of TNF α also showed an increasing trend in the photothrombotic stroke at day 4 and 7 but did not reach statistical significance (table 1).

A few other molecules related to the CC chemokine family were also elevated in the photothrombotic stroke at day 1 (macrophage inflammatory protein, MIP1 α and MIP1 γ), at day 4 (monocyte chemoattractant protein, MCP-5) and day 7 (MCP-1, MCP5 and macrophage colony stimulating factor, MCSF).

These findings confirm previous reports of a higher abundance of inflammatory cytokines within the first 24 hours after stroke (Wang et al., 2007). However, we here are also presenting quantitative and temporal data about the dynamic response of 40 cytokines and chemokines at three different time points and in two models of ischemic damage that are highly comparable, evidencing striking differences in the inflammatory profile between the two models.

In summary, the delayed microglial response observed in the photothrombotic stroke compared to MCAO stroke is not the result of a delayed expression of early inflammatory molecules because the levels of most cytokines/chemokines did not differ between the two

types of stroke. However, several important inflammatory mediators were released at greater extent in the photothrombotic stroke than in the vascular model at day 1 and 4. These molecules include classical pro-inflammatory cytokines like IL-6, IL-17 and FasL but also chemoattractant chemokines like MIP-1a, MIP-1g, ICAM-1 and PF-4. At day 7, there is still elevated production of inflammatory cell recruitment molecules in the photothrombotic stroke (MCP-1, MCP-5, M-CSF, PF-4) but also a concomitant increase of a putative compensatory pro-survival pathway that involves the TNF-RII (McCoy and Tansey, 2008).

Infiltration from periphery is increased in PH compared with MCAO stroke

Because both PF4 and the CC family of chemokines have been associated with chemotaxis for peripheral cells to the site of injury and ICAM with leukocyte-endothelial adhesion events, we next examined the degree of cellular infiltration from the periphery to the stroke core. Although the Cx3CR1-GFP mouse labels monocyte-derived cells, it is possible to distinguish resident microglia from invading immune cells by adding a second marker like Cd11b or Ly6G. Cd11b (or Mac1) is an alpha M integrin involved in adhesion interactions of monocytes, macrophages and granulocytes whereas Ly6G (GR-1) is a marker for granulocytes, mostly, although it is also present in macrophages and differentiating monocytes.

At day 1, numerous Cd11b+/GFP- cells are observed in the photothrombotic stroke but not in the cortical model (Figure 7). This difference continues at day 4 post-stroke. These Cd11b + cells could be neutrophils that invade the stroke core earlier in the photothrombotic model, as they can be clearly distinguished (red) from the resident microglia (green) but it is also possible that the signal could correspond to Cd11b+/GFP^{low} monocytes/macrophages as MIP-1a can also attract this cell type (Benakis et al., 2015). In contrast, the MCAO brain shows more abundant Cd11b+/GFP+ cells, which could correspond to penetrating periphery macrophages that express both markers. At day 7, both models show comparable immunoreactivity for Cd11b.

Another marker, Ly6G, confirms a more robust neutrophil infiltration in the photothrombotic stroke at days 1 and 4 but not at day 7.

Widespread changes in neuronal phenotype only occur in the MCAO but not in the photothrombotic stroke

So far, we analyzed the effects that differences in the microvasculature may cause in the inflammatory environment and reactive gliosis of the stroke. A key question in this study is, however, what happens to the neurons in the recovery stroke core and in the adjacent penumbra. Therefore, we next investigated whether there were differences not only in the neurons of the stroke core and penumbra (positive for the neuron-specific transcription factor NeuN) but also in the newly born neurons migrating to the stroke area (positive for doublecortin – DCX+) (Figure 8).

Analysis of the DCX+ cells at day 7 post-stroke did not reveal obvious differences in the pattern of staining of these cells in the two stroke types as both models showed strong presence of migrating DCX+ cells from the ventricular zone. The pattern of migration, however, seemed to diverge between the two strokes as cells in the MCAO stroke migrate in

a more dispersal mode than cells in the photothrombotic stroke, which tend to migrate in tighter clusters.

One striking difference we found, however, was that the number of NeuN+ cells in the penumbra and core of the vascular stroke was significantly lower in the MCAO than in the photothrombotic stroke. This reduction in NeuN immunoreactivity was not due to increased cell death in the MCAO stroke over the PH stroke because both MAP2 and nuclear DAPI staining remained comparable in both stroke models.

This observation may indicate that the MCAO stroke allowed more neuronal plasticity than the photothrombotic stroke by down-regulating the expression of the transcription factor NeuN (Kim et al., 2013)

DISCUSSION

This study directly compared stroke evoked by arterial occlusion with photothrombotic stroke induced by exposing cortex to UV light after intravascular administration of rose bengal, a photoactive dye that generates oxidative damage. We find that photothrombotic stroke, that produces more microvasculature damage, delayed microglial and astroglial activation, delayed microglial invasion of the ischemic core and delayed accumulation of activated and phagocytic microglia, especially within the first 4 days after stroke compared to the MCAO stroke. Interestingly, the delayed morphological signs of microglial activation were contrasted by an enhanced inflammatory response defined by higher level of cytokines/chemokines after photothrombotic stroke.

Photothrombotic stroke also displayed increased infiltration from the periphery of circulating leukocytes. Perhaps the most important observation was that only the neurons in the MCAO stroke showed phenotypic plasticity by downregulating the transcription factor NeuN. The data presented here is critical in understanding the exact temporal and spatial dynamics of the inflammatory response in two widely used animal models of stroke and most relevant for identifying targets with a wider therapeutic window.

Activation of microglial cells is one of the first steps after an ischemic damage (Wang et al., 2007). Ameboid, activated microglia transform then into phagocytic cells that invade the damaged area to remove cellular debris, a temporal sequence of events widely described after MCAO (Benakis et al., 2015). The CX3CR1-GFP reporter mice allowed us to monitor the detailed responses of microglia and we found also a rapid and strong microglial response from day 1 to day 4 after stroke but only in the MCAO model. The photothrombotic stroke, however, showed a delayed activation and phagocytic response. Rapid phagocytosis after trauma would allow not only the elimination of neuronal debris but also a faster recovery and enhanced plasticity of neural cells after injury (Kettenman et al., 2013). Inhibition of phagocytosis, on the other hand, has proven beneficial in preventing neuronal death after focal brain ischemia (Neher et al., 2013). Since this study was not designed to study stroke outcome but, rather, to evaluate the innate inflammatory response to MCAO and PT stroke, this issue needs further investigation in the two stroke models presented here.

We also observed a delayed reactive astrocytic response in the photothrombotic stroke compared to the MCAO model. The exact role of reactive astrocytes in neuroregeneration may depend on time and place of action. For example, some studies have shown that ablation of reactive astrocytes impedes recovery as they provide barriers for entry of inflammatory cells into the CNS (Faulkner et al., 2004; Sofroniew, 2015). On the other hand, Wilhelmsson et al. (2004) have shown that preventing hypertrophy of reactive astrocytes by eliminating the intermediate filaments allows for considerable synaptic regeneration in the hippocampus. Albeit reactive astrocytes may not ideally support surrounding neurons, their presence is a prerequisite for recovery in the spinal cord (Sofroniew, 2015). The faster activating response we observe in the MCAO astrocytes could thus contribute to a diminished inflammatory response in this model, at least, in the initial phases of tissue damage when synaptogenesis has not yet started.

Activated microglia and astroglia release a variety of cytokines and chemokines that initiate both cytotoxic or neuroprotective processes and thereby modulate the tissue response to injury (Lakhan et al., 2009). A number of pro-inflammatory molecules released after ischemic brain damage has been extensively described for both experimental and human stroke, like cytokines (IL-1 β , IL6, TNF α), chemokines (IL-8, IL-10, MCP-1/CCL2) and adhesion molecules (ICAM-1) (Mojsilovic et al., 2012; Lambertsen et al., 2012). Most of these molecules appear as early as 3 hours after stroke, depending of the model, and peak in the first 24 hours of stroke. In coherence with this, our quantitative/qualitative analysis revealed many cytokines/chemokines released mostly at day 1 in the two types of stroke. Of these, six molecules were significantly increased in the photothrombotic stroke compared to the MCAO stroke, most of them associated with chemotaxis for peripheral cells after stroke (Vindegard et al, 2013). We also observed an increase in leukocyte infiltration in the photothrombotic stroke compared to the MCAO model, especially at day 1 and 4. Although the identity of the infiltrating cells needs to be determined, our data is consistent with the peak of neutrophils infiltration at day 3 to be later engulfed by macrophages/microglia (Weston et al., 2007). In relation to this, Gelderblom et al. (2012) described an IL-17-mediated increase in neutrophil extravasation in the ischemic brain in response to increased CXCL1 and CXCL2 ligands. Although these two chemokines were not present in our inflammatory array, we did find much higher levels of the related chemokine PF-4/CXCL4 and also a significant increase in the levels of the IL-17 cytokine in the photothrombotic stroke. Our data is thus consistent with a more relevant involvement of the IL-17-CXCL pathway in the photothrombotic ischemic model than in the MCAO model.

The involvement of neutrophil infiltration in stroke damage is complicated and involves activation of both deleterious as well as beneficial pathways. On one hand, neutrophils can release inflammatory molecules and reactive oxygen species that could amplify the ischemic damage (Weston et al., 2007) and Wen et al. (1995) also claimed that CXCL4 after stroke increases the severity of the trauma. On the other hand, therapies designed to inhibit neutrophil infiltration have failed to improve stroke outcome (Easton, 2013). In our study, there was a remarkable difference in the levels of CXCL4 and neutrophil infiltration in the first 4 days of stroke accompanied by less neuronal plasticity in the photothrombotic stroke, suggesting the possibility that this type of injury may promote slower recovery than the MCAO model.

Interestingly, as the stroke recovery process progressed, we did observe increasing levels of molecules involved with the TNF α signaling system, the receptors TNF-RI and TNF-RII, from day 1 to day 4, reaching significantly higher levels in the photothrombotic compared to the ligation stroke at day 7. The role of TNF α in stroke is not clear, though, as its presence has been associated with increased damage but also with neuroprotection, depending on the model used and stage of damage (McCoy and Tansey, 2008). It is plausible to think that an increase in the TNF-RII pathway in the photothrombotic stroke would act as pro-survival to reduce neuronal death due to excessive production of pro-inflammatory molecules at an earlier stage. TNF-RII does not contain a death domain like TNF-RI and an increase in the soluble form of this receptor could act as neuroprotectant by buffering TNF α (McCoy et al., 2015). Indeed, this system has been also described as essential for regulating neuronal death and microglial uptake in a model of Alzheimer's disease (Combs et al., 2001) and an engineered form of the extracellular domain of human TNF-RII was able to reduce stroke volume and neural deficit in a mouse model of stroke (Sumbria et al., 2012). At this moment, the exact significance of such an increase in TNF-RII after photothrombotic damage is unclear.

A very striking observation we made in this study is the disappearance of the NeuN transcription factor only in neurons of the cortical stroke. Although this protein has been widely used as a marker for mature neurons, a reduction in the NeuN antigen without overall loss of neurons has been described after brain injury, including MCAO (Ünal-Çevik et al., 2004; Duan et al., 2015). A reduction in the NeuN protein could thus allow changes in neuronal specific genes. At this moment, it is not known whether NeuN expression regulates genes involved in neuronal survival after damage.

We also observed a clustered mode of migration of newly born DCX+ neuroblasts in the photothrombotic stroke, compared to a more dispersed migration in the MCAO stroke. It is possible that a higher concentration of chemoattracting molecules in the ischemic core is responsible for this variation. SVZ-derived neuroblasts express chemokine receptors, like CCR2 and CCR5 (Trans et al., 2007; Kizil et al., 2012) and several CCL-family of chemokines have been implicated in regulation of migration of neural progenitors (Belmadani et al., 2006) and neurogenesis and cognitive function (Villeda et al., 2011).

Of the 40 molecules analyzed, we found two of the CCR2 ligands, MCP1/CCL2 and MCP5/CCL12, significantly increased in the photothrombotic stroke at day 7, the time of initial neurogenesis. Thus, variations in chemokines concentrations could be responsible not only for establishing a different inflammatory microenvironment between the two strokes but also for differentially regulating the pattern of neuroblast migration and differentiation in the two models of stroke.

CONCLUSIONS

In summary, we conclude that photothrombotic and MCAO stroke damage is not comparable between day 1 and 4, in terms of glial response, inflammatory/chemotactic molecules and infiltration of leucocytes. In addition, a differential environment of chemokines involved in migration could also regulate neuroblasts migration and

differentiation at day 7, as we see changes in migration and neuronal phenotype between the two strokes.

Thus, the photothrombotic stroke, although less variable and more convenient, does not replicate the inflammatory and protective milieu of the MCAO model.

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Figure 1. Schematic diagram of a vascular stroke (MCAO) and a photothrombotic stroke (PH) Left: (A) Diagram of a vascular stroke via coagulation of the distal MCA; (B) Diagram of a photothrombotic stroke via illumination with a green laser. The pictures on the right show comparable damage restricted to the cortex in both types of stroke, as seen with TTC staining at 1 day after stroke. The graph at the bottom is a comparison of infarct volume as percentage of total volume of the stroked hemisphere. $P < 0.05$, t-test.

Figure 2. Progression of damage 1, 4 and 7 days after vascular (MCAO) or Photothrombotic stroke (PH)

Serial sections at different levels of Cx3CR1-GFP mouse brain subject to cortical or photothrombotic stroke and stained with MAP2 antibody (red) to visualize the extent of stroke damage. **(A)** At day 1, robust activation of microglial cells (GFP, green) is observed in the ipsilateral side of the cortical stroke compared to the photothrombotic stroke. **(B)** At 4 days after injury, the activation of microglia (GFP, green) still looks more robust in the MCAO than in the PH stroke, with more patches of green cells accumulating in the stroke

area. **(C)** At 7 days after injury, the degree of microglial activation in the cortical stroke more closely resembles the one in the photothrombotic stroke (GFP, green cells). Scale bar, 500 μ m.

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Figure 3. Higher magnification images of microglial activation in cortical (MCAO) and Photothrombotic stroke (PH) at 1, 4 and 7 days after injury

Confocal images of brain slices stained with anti-MAP2 antibody (red). Nuclei are counterstained with DAPI (blue). Microglial cells (green) accumulate in the core of the stroke more readily in the cortical than in the photothrombotic stroke and exhibit more ramified morphology both at boundary (b) and in areas farther from the stroke (c, d). At day 4, microglial cells in the MCAO still exhibit stronger level of activation than the ones in the PH stroke with very rounded, phagocytic morphology inside the damaged area and

progressive ramification farther from the stroke. Farther from the boundary, half-moon shapes (presumably, phagocytic cells, arrows) are observed only in the vascular stroke (c). At day 7, the difference between the two types of stroke is less noticeable and microglial morphology and activation looks similar in all areas of stroke analyzed. Asterisk indicates core of the stroke. Nuclei are counterstained with DAPI. Scale bar, 150 μm (a and a'), 20 μm (b, c, d and b', c', d'). Dotted line indicates the boundary of the stroke.

Figure 4. Phagocytic microglia appear earlier in the CS than in the PH stroke

Single plane confocal images of GFP-microglia (green) at 1, 4 or 7 days after stroke (arrowheads). Profiles of MAP2+ cells engulfed inside microglial cells can be observed from day 1, increase at day 4 and peaked at they 7 after CS stroke. Only at day 7 phagocytic profiles can be seen in the PH stroke (arrow). Scale bar 20 μ m. Graph at the bottom shows a statistical significant increase of phagocytic microglial profiles in the CS stroke at day 7 compared to the PH stroke. $P < 0.05$, t-test. Scale bar, 20 μ m.

Figure 5. Reactive astrocytes appear earlier in MCAO than in PH stroke
 GFAP+ cells (blue) are already present at day 1 in the MCAO stroke (A') but not in the PH stroke (B'). At day 4, there is still more gliosis (thicker GFAP+ processes) in the MCAO (C') than in the PH stroke (D'). At day 7, no obvious differences can be observed in the reactive astrocytes between the two types of stroke (E'-F'). A'-F' are high magnification images of the boxes in A-F. Scale bar 150 μ m (A-F), 20 μ m (A'-F'). Dotted lines indicate the boundary of the stroke.

Graph shows a quantification of the relative intensity of GFAP+ cells in three different areas of the stroke penumbra. Only at day 1 there is a statistical difference in astroglial reactivity in all regions analyzed. $P < 0.05$, t-test.

Figure 6. Inflammatory profile of MCAO or Photothrombotic strokes at 1, 4 and 7 days after stroke

Bar diagrams of the levels of cytokines/chemokines in both types of strokes. Statistical significance is shown as $p<0.05$ (*) or $p<0.005$ (**). N=6

Figure 7. Low and high magnification images of infiltrating cells in MCAO and PH models at 1,4 and 7 days after stroke

Cd11b+/GFP- macrophages (red cells) appear earlier in the PH stroke than in the MCAO model at day 1 after stroke (A). Similarly, Ly6G+ neutrophils are abundant in the PH (arrowheads) at days 1 and 4 compared to the MCAO stroke (arrowheads) in B. At day 7, very few neutrophils remain in the core of the stroke although many cells are still observed around and inside blood vessels (arrow). Scale bar, 150 μ m, 50 μ m (insets).

Figure 8. Marked differences in neuronal responses to MCAO and photothrombotic stroke at day 7 after stroke

Coronal brain sections stained against doublecortin (DCX) show migrating newborn neurons from the subventricular zone to the stroke area (red). Cells tend to aggregate more in the migratory pathways of the PH stroke. GFP-microglial is shown in green (A-B'). Dotted lines indicate the migratory route from the SVZ along the white matter. A dramatic reduction in NeuN immunoreactivity is observed in the MCAO but not in the photothrombotic stroke (red). C-D is higher magnification of the boxes outlined in C'-D'. Insets represent zoomed areas of C and D. Quantification of this effect is shown in the graph. ***P<0.005, *p<0.05,

t-test. Scale bars, 350 μm (A, B), 50 μm (A', B'), 500 μm (C', D'), 150 μm (C, D), 30 μm (insets in C, D).

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Table 1
Summary of the statistical significance of cytokine profile in the two models of stroke

P-values of comparison between the significantly different cytokines in the two stroke types at 1, 4 and 7 days after stroke.

(pg/ml)	DAY 1	DAY 4	DAY 7
Fas-L	0.045	0.618	0.199
ICAM-1	0.547	0.042	0.575
IL-1a	0.113	0.032	0.685
IL-6	0.006	0.155	0.101
IL-17	0.037	0.618	0.607
MCP-1	0.142	0.153	0.016
MCP-5	0.749	0.037	0.023
M-CSF	0.278	0.442	0.045
MIP-1a	0.037	0.932	0.158
MIP-1g	0.044	0.363	0.391
PF-4	0.002	0.016	0.037
TNF RI	0.2	0.15	0.021
TNF RII	0.232	0.284	0.01

For day 1, one-way ANOVA/Posterior Ttest calculated for: Eotaxin; Eotaxin2; ICAM1; IFNg; IL1a; IL2; IL6; IL7; IL15; MCP1; MCSF; MIP1g; TARC.

- T-Test for equal variances not assumed: TCA3; PF4.

- Mann-Whitney U Test for: bFGF; BLC; CD30L; FasL; GCSF; GMCSF; IL1b; IL3; IL4; IL5; IL10; IL12p40; IL13; IL17; IL21; KC; Leptin; LIX; MCP5; MIG; MIP1a; RNATES; TNFR1; TNFR2; TNFα.

For day 4, one-way ANOVA/Posterior Ttest calculated for: Eotaxin; ICAM1; IL1a; IL2; IL6; IL10; IL15; IL21; LIX; MCP5; MIP1g.

- T-test for equal variances not assumed: IL4; KC.

- Mann-Whitney U Test: bFGF; BLC; CD30L; Eotaxin2; FasL; GCSF; GMCSF; IFNg; IL1b; IL3; IL5; IL7; IL12p40; IL13; IL17; Leptin; MCP1; MCSF; MIG; MIP1a; PF4; RNATES; TARC; TCA3; TNFR1; TNFR2; TNFα.

For day 7, one-way ANOVA/Posterior Ttest calculated for: Eotaxin; Eotaxin2; ICAM1; IFNg; IL1a; IL4; IL6; IL17; MCP5; MCSF; MIG; MIG; MIP1a; MIP1g; TARC; TNFR1.

- Mann-Whitney U Test: bFGF; BLC; CD30L; FasL; GCSF; GMCSF; IL1b; IL2; IL3; IL5; IL7; IL10; IL12p40; IL13; IL15; IL21; KC; Leptin; LIX; MCP1; PF4; RNATES; TCA3; TNFR2; TNFα.