

## Spatio-temporal course of macrophage-like cell accumulation after experimental embolic stroke depending on treatment with tissue plasminogen activator and its combination with hyperbaric oxygenation

D. Michalski,<sup>1</sup> M. Heindl,<sup>1,2</sup> J. Kacza,<sup>3</sup>  
F. Laignel,<sup>1,2</sup> L. Küppers-Tiedt,<sup>1</sup>  
D. Schneider,<sup>1</sup> J. Grosche,<sup>2</sup> J. Boltze,<sup>4,5</sup>  
M. Löhr,<sup>6</sup> C. Hobohm,<sup>1</sup> W. Härtig<sup>2</sup>

<sup>1</sup>Department of Neurology, University of Leipzig; <sup>2</sup>Paul Flechsig Institute for Brain Research, University of Leipzig; <sup>3</sup>Department of Anatomy, Histology and Embryology, Faculty of Veterinary Medicine, University of Leipzig; <sup>4</sup>Fraunhofer Institute for Cell Therapy and Immunology, Leipzig; <sup>5</sup>Translational Centre for Regenerative Medicine, University of Leipzig; <sup>6</sup>Department of Neurosurgery, University of Würzburg, Germany

### Abstract

Inflammation following ischaemic stroke attracts high priority in current research, particularly using human-like models and long-term observation periods considering translational aspects. The present study aimed on the spatio-temporal course of macrophage-like cell accumulation after experimental thromboembolic stroke and addressed microglial and astroglial reactions in the ischaemic border zone. Further, effects of tissue plasminogen activator (tPA) as currently best treatment for stroke and the potentially neuroprotective co-administration of hyperbaric oxygen (HBO) were investigated. Rats underwent middle cerebral artery occlusion and were assigned to control, tPA or tPA+HBO. Twenty-four hours, 7, 14 and 28 days were determined as observation time points. The accumulation of macrophage-like cells was semiquantitatively assessed by CD68 staining in the ischaemic area and ischaemic border zone, and linked to the clinical course. CD11b, ionized calcium binding adaptor molecule 1 (Iba), glial fibrillary acidic protein (GFAP) and Neuronal Nuclei (NeuN) were applied to reveal delayed glial and neuronal alterations. In all groups, the accumulation of macrophage-like cells increased distinctly from 24 hours to 7 days post ischaemia. tPA+HBO tended to decrease macrophage-like

cell accumulation at day 14 and 28. Overall, a trend towards an association of increased accumulation and pronounced reduction of the neurological deficit was found. Concerning delayed inflammatory reactions, an activation of microglia and astrocytes with co-occurring neuronal loss was observed on day 28. Thereby, astrogliosis was found circularly in contrast to microglial activation directly in the ischaemic area. This study supports previous data on long-lasting inflammatory processes following experimental stroke, and additionally provides region-specific details on glial reactions. The tendency towards a decreasing macrophage-like cell accumulation after tPA+HBO needs to be discussed critically since neuroprotective properties were recently ascribed to long-term inflammatory processes.

### Introduction

Ischaemic stroke is associated with an enormous socio-economic burden, but unfortunately only restricted treatment strategies are currently available.<sup>1</sup> In recent years, the translation of preclinical successful attempts into the clinical setting has commonly failed, exemplified by the promising drug NXY-059.<sup>2</sup> As one of the main consequences, an extended perspective of tissue salvaging was developed, which involves explicitly more than only neurons.<sup>1,3,4</sup> The neurovascular unit represents a frequently used construct in this regard describing the interactions between neurons, vessels, astrocytes and associated microglia.<sup>5,6</sup> Damaging events after ischaemic stroke caused by reduced cerebral blood flow due to an artery occlusion include mechanisms of hypoxia-related acute excitotoxicity, which overlap with long-lasting inflammatory processes, peaking in delayed stroke phases.<sup>7,8</sup> Thereby, investigating the role of recruited immunoreactive cells in stroke-affected areas has become a major interest in stroke research.<sup>3</sup> Focussing on this topic, several studies have identified invading leukocytes and activated microglial cells as contributors with predominantly deleterious roles in acute stages after cerebral ischaemia.<sup>8-15</sup> Further characterization of these cells also based on immunolabelling with antibodies directed against CD68, CD11b and the ionized calcium binding adaptor molecule 1 (Iba), but the identification of specific subpopulations remains a diagnostic challenge.<sup>16-18</sup> Additionally, the spatio-temporal profile of macrophage infiltration in stroke-related brain tissue has been investigated in vivo by magnetic resonance imaging in rats,<sup>19</sup> mice and humans.<sup>20,21</sup> Taking these time-dependent changes into account, it becomes clear that experimental studies in the field of stroke should rely on long-term observation periods

Correspondence: Dr. Dominik Michalski, Department of Neurology, University of Leipzig, Liebigstr. 20, 04103 Leipzig, Germany. Tel. +49.341.9724256 - Fax: +49.341.9724199. E-mail: dominik.michalski@medizin.uni-leipzig.de

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and should be based on rodent stroke models with pathophysiology comparable to the human condition, such as thromboembolic stroke.<sup>4,22</sup> Unfortunately, these issues have often been neglected by previous studies.

Despite extensive research in the field of acute ischaemic stroke, tissue plasminogen activator (tPA) is currently the only drug-related treatment that has shown conclusive beneficial effects in the clinical setting.<sup>1,4,23</sup> Nevertheless, also toxic effects of tPA have been reported, contributing to vascular disruption with potential cerebral haemorrhage caused by a tPA-mediated activation of matrix metalloproteinases (MMPs).<sup>24-27</sup> In general, MMPs are known for their deleterious effects in acute stroke phases, but also beneficial properties in later phases have been discussed recently.<sup>24,28,29</sup> In this context, invading leukocytes and local microglial cells were found to produce pro-inflammatory mediators,<sup>30-34</sup> and act as primary source for MMP-9.<sup>29,35-38</sup> Considering the deleterious effects of tPA

influencing the immune-related response via MMPs, the idea of combined treatment strategies arose to attenuate harmful effects selectively.<sup>1,24</sup>

Hyperbaric oxygenation (HBO) represents the application of 100% oxygen at an elevated ambient pressure, which increases the rate of physically dissolved oxygen in the blood; hereby, the arterial oxygen pressure enhances from about 90 mmHg at room air to approximately 2000 mmHg while breathing 100% oxygen at 2.5 atmosphere absolute (ata).<sup>39,40</sup> HBO has attracted attention in stroke therapy since pre-clinical studies have shown beneficial effects, e.g., concerning neurobehavioural testing and infarct size,<sup>41-43</sup> as well as the frequency of secondary haemorrhages due to tPA.<sup>44</sup> With regard to inflammatory processes, HBO was found to decrease the infiltration of neutrophils when administered prior to or immediately after permanent experimental cerebral ischaemia,<sup>45</sup> and prior to reversible focal ischaemia.<sup>46</sup> However, also a contrary report exists, which found no effect of HBO on neutrophil accumulation when administered after permanent experimental stroke.<sup>47</sup> In recent years, HBO was discussed as treatment strategy in addition to tPA.<sup>48,49</sup> In a first approach with simultaneous application of tPA and HBO, our group has demonstrated that combined treatment results in early clinical improvement, but did not observe consistent long-term effects following experimental stroke.<sup>50</sup>

The present study aimed on the long-term course of inflammatory processes after focal cerebral ischaemia in rats and considered tPA as widely used treatment as well as simultaneous HBO as a potential neuroprotective strategy in a translational relevant setting. For this purpose, we first addressed different regions of ischaemia with special emphasis on the spatio-temporal accumulation of macrophage-like cells and explored the link to the respective clinical course. Second, delayed cellular reactions, particularly micro- and astrogliosis in ischaemic border regions, were investigated considering the neurovascular unit concept to obtain further insights in long-term post-stroke processes. The emerging results might be useful in adapting future treatment strategies in stroke.

## Materials and Methods

### Study design and tissue preparation

The study protocol involving animals has been proven by local authorities (Regierungspräsidium Leipzig, Germany) and was conducted according to the European Communities Council Directive (86/609/EEC).

The brain tissue for the present histology-based investigation originated almost exclusively from our previous study.<sup>50</sup> Herein, male Wistar rats – provided by the Medizinisch-Experimentelles Zentrum of the University of Leipzig (Germany) or Charles River (Sulzfeld, Germany) – underwent focal cerebral ischaemia by thromboembolic right-sided middle cerebral artery occlusion (MCAO). The animals were allocated to the following treatment groups: Control, tPA and tPA+HBO. Survival times of the animals after stroke induction were 24 h, 7, 14 and 28 days with subsequent sacrificing in deep narcosis. After perfusion with saline and 4% phosphate-buffered paraformaldehyde, brains were immediately removed from the skull and post-fixed with the same fixative for 24 h. Afterwards, the brains were equilibrated with 30% buffered sucrose and 30 µm-thick serial coronal forebrain sections were cut using a frozen microtome. All sections were collected in 0.1 M Tris-buffered saline, pH 7.4 (TBS) containing sodium azide. To allow semiquantitative analysis in the present study, tissue was selected according to the best-intact structures in combination with clearest infarct presentation, which resulted in a sample size of n = 3 for each treatment group at 24 h, 7, 14 and 28 days.

### Surgical procedure of focal cerebral ischaemia

MCAO was induced according to a thromboembolic model, originally described by Zhang *et al.*<sup>51</sup> with some modifications as described previously.<sup>50</sup> Briefly, after cervical preparation of the right-sided arterial vessels, a polyethylene (PE) tubing with tapered end was inserted into the external carotid artery and moved forward into the internal carotid artery up to the origin of the middle cerebral artery. Here, a blood clot was injected followed by catheter removal and ligation of the external carotid artery stump. A further PE tubing that was inserted into the femoral vein served as secure access path for the future administration of tPA. During the surgical procedure, the animals were anesthetized with 2-2.5% isoflurane in a mixture of 70% N<sub>2</sub>O and 30% O<sub>2</sub>, and the body temperature was adjusted to 37.0°C by using a thermostatically controlled heating pad with a rectal probe (Fine Science Tools, Heidelberg, Germany).

### tPA administration and procedure of HBO

In the control and tPA group, animals breathed room air at normal ambient pressure. Animals received tPA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) intravenously in a dose of 9 mg per kg body weight over 30 min with a total volume of 1 mL - starting 2 h

after initiation of MCAO. Combined treatment with tPA and HBO was performed in a special chamber (Sayers/Hebold, Cuxhaven, Germany) with 100 % O<sub>2</sub> at 2.4 ata for a period of 1 h (excluding compression and decompression time); thereby, the administration of tPA was started simultaneously to HBO 2 h after initiation of MCAO.

### Immunoperoxidase labelling of CD68

Overall, CD68 was used for the identification of macrophage-like cells.<sup>16,18</sup> Thereby, 8 sections per animal (containing the infarct region) were rinsed with TBS and endogenous peroxidase activity of tissues was abolished by treatment with 0.6% H<sub>2</sub>O<sub>2</sub> for 30 min. Following several rinses with TBS, sections were blocked with 5% normal goat serum in TBS (+0.3% Triton X-100) for 1 hour. Subsequently, the tissue was incubated with mouse-anti-CD68 (AbD Serotec, Oxford, UK; 1:400 in the blocking solution) overnight. Next, the sections were extensively rinsed with TBS and processed with highly purified biotinylated goat-anti-mouse IgG (Dianova, Hamburg, Germany; 1:500 in TBS containing 2% bovine serum albumin = TBS-BSA) for one h. Following several rinses with TBS, the sections were applied to preformed streptavidin/biotinyl-peroxidase complexes (12.5+5 µg/mL TBS-BSA) and then stained with diaminobenzidine/ammonium nickel sulphate as chromogen according to Härtig *et al.*<sup>52</sup> Following all histochemical procedures, sections were briefly washed with distilled water, mounted on fluorescence-free glass slides, air-dried and coverslipped with Entellan in toluene (Merck, Darmstadt, Germany).

### Multiple immunofluorescence labelling

Considering that microglial and astrocytic activation are closely connected with inflammatory processes like neutrophil infiltration,<sup>10,13,32,53-56</sup> multiple fluorescence labelling was performed for further characterization (see also Härtig *et al.*<sup>57</sup>). Generally, CD68 and CD11b were used for the identification of macrophage-like cells, whereas CD11b accents more granulocytes and neutrophils but also microglia; Iba predominantly labels microglia, while glial fibrillary acidic protein (GFAP) stains a large subset of astrocytes.<sup>16,18,32,58-60</sup> According to the neurovascular unit concept, which describes amongst others the interaction of astrocytes and associated microglia with neurons,<sup>6,10</sup> antibodies directed against neuronal nuclei (NeuN) were additionally used as neuronal marker.<sup>61</sup> Table 1 provides an overview of the applied antibodies and triple

immunofluorescence staining methods.

The omission of primary antibodies in control experiments led to the expected absence of any cellular staining. Switching the fluorophores related to the relevant markers resulted in unaltered staining patterns of CD68, CD11b, Iba, GFAP and NeuN.

### Primary microscopy and imaging

An Axioplan microscope (Zeiss, Jena, Germany) was utilised for initial light and fluorescence microscopic analyses. Fluorescently labelled sections were also analysed with a confocal laser-scanning microscope (LSM 510 Meta (Zeiss), equipped with an argon laser (488 nm) for the excitation of FITC and Cy2, and two helium-neon lasers for the excitation of Cy3 (543 nm), as well as Cy5 (633 nm). The following band-pass (BP) filters were used: BP 500-530 nm (Cy2, FITC, green), BP 565-615 nm (Cy3, red) and BP 654-718 nm (Cy5, infrared, colour-coded in blue). Original images were processed with Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA, USA). Thereby, brightness, contrast and sharpness of some final pictures were slightly enhanced.

### Semiquantitative evaluation of macrophage-like cells and its interrelation to the clinical course

Sections of CD68-immunoperoxidase staining were used for semiquantitative analysis. Thereby, the section presenting the largest ischaemic infarct as well as the neighbouring rostral and frontal section (3 in total) were processed by using an Axioplan 2 imaging microscope (Zeiss) with digital camera (ORCA-ER, Hamamatsu, Hamamatsu City, Japan) and evaluation software (Volocity 4.3.2, PerkinElmer, Waltham, MA, USA). After orientation on an overview scan, one region of interest (ROI; dimensions 327.4×249.4 µm) was inserted into the ischaemic area, and a

further ROI was placed in the ischaemic border zone. Using a 25x objective (numerical aperture 0.8), the CD68-immunoreactivity was analysed by intensity measurements of the whole area in each ROI, resulting in an amount of CD68-positive pixels per optic field as surrogate for the accumulation of macrophage-like cells. Thereby, the following settings were applied: *find objects by standard deviation intensity* (lower limit -100, upper limit -0.5) and *exclude objects by size* (<5 µm). Figure 1 shows a representative overview scan with exemplarily inserted ROIs. This overview was created by automatic scanning single images (Openlab 5.5.0, PerkinElmer) with a 10x objective and subsequent image stitching (Autopano Giga 2.0, Kano, France). Measurements were performed for each of both ROIs per section resulting in 6 values in total. Finally, means were calculated for both ROIs, which led to 2 values per study subject.

To explore the interrelation between the accumulation of macrophage-like cells and the clinical course, a linear regression model was calculated by using IBM SPSS Statistics (Vers. 20; IBM Corp., New York, NY, USA). Thereby, the ratio of CD68-positive pixel of the ischaemic area vs ischaemic border zone - representing the amount of cell accumulation - served as independent factor. In parallel, the change of the neurological deficit during the overall observation period served as dependent factor. The respective data on neurological impairment were extracted from our previous work,<sup>50</sup> restricted to the subjects used in the present study.

## Results

The used embolic stroke model expectedly resulted in ischaemic infarctions with different morphologies primarily located in the mid-

dle cerebral artery territory involving the striatum, and partially affected the thalamus and hypothalamus. In general, macrophage-like cells were easily identified in ischaemic areas based on sufficient CD68-immunoperoxidase staining, which allowed a semiquantitative evaluation. Based on the observed accumulation of macrophage-like cells, an ischaemic core with maximal tissue damage and in later phases nearly complete loss of cellular staining could be distinguished from an ischaemia-related area with significant macrophage-like accumulation and from a surrounding ischaemic border zone with less macrophage-like cell accumulation (Figure 1).

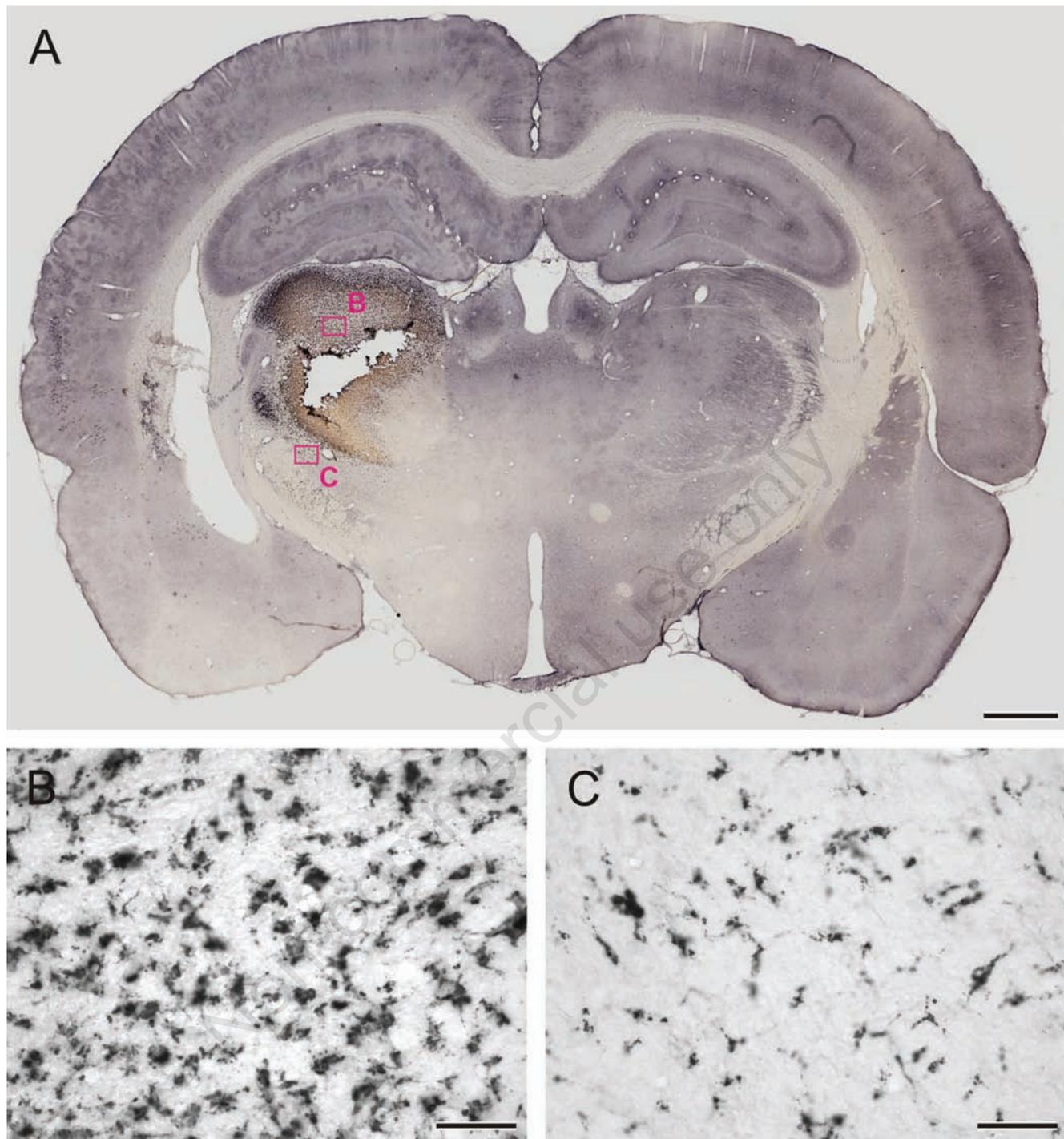
### Spatio-temporal course of macrophage-like cell accumulation after focal cerebral ischaemia

Figure 2 shows the time course of CD68-immunoreactivity in relation to the respective treatment and region of stroke-affected tissue. In the ischaemic area (Figure 2A), only a few macrophage-like cells were seen in all 3 groups at 24 hours after MCAO, followed by a drastic accumulation up to 7 days. At this time point, subjects of the control and tPA+HBO group displayed a tendency to a slightly higher macrophage-like cell accumulation when compared with tPA. Interestingly, the tPA+HBO group tended to less CD68-immunoreactivity in the ischaemic area during the following 3 weeks, whereas subjects of the control and tPA group provided similar courses. Markedly different findings were obtained in the ischaemic border zone (Figure 2B). During the first 14 days after MCAO, the accumulation of macrophage-like cells was found here at an almost constant low level. At 28 days, only a slight increase of CD68-immunoreactivity was observed in tPA-treated animals. In contrast, subjects of the tPA+HBO group tended to the lowest rate of macrophage-like cells in the ischaemic border zone.

**Table 1. Triple fluorescence labelling of CD68, glial and neuronal markers.**

Primary antibodies	Source	Dilution	Secondary immunoreagents*
<b>Method 1</b>			
Fluoresceinated mouse-anti-CD68 IgG	AbD Serotec, Oxford, UK	1:50	Cy2-anti-fluorescein
Biotinylated mouse-anti-CD11b IgG	AbD Serotec	1:50	Cy3-streptavidin
Rabbit-anti-Iba 1	Wako, Neuss, Germany	1:200	Cy5-donkey-anti-rabbit IgG
<b>Method 2</b>			
Biotinylated mouse-anti-CD68 IgG	AbD Serotec	1:50	Cy2-streptavidin
Cy3-tagged mouse-anti-GFAP	Sigma, Taufkirchen, Germany	1:200	-
Rabbit-anti-Iba 1	Wako	1:200	Cy5-donkey-anti-rabbit IgG
<b>Method 3</b>			
Mouse-anti-NeuN	Invitrogen, Karlsruhe, Germany	1:50	Cy2-donkey-anti-mouse IgG
Guinea pig-anti-GFAP	Synaptic Systems, Göttingen, Germany	1:400	Cy3-donkey-anti-guinea pig IgG
Rabbit-anti-Iba 1	Wako	1:200	Cy5-donkey-anti-rabbit IgG

\*All fluorescent immunoreagents were obtained from Dianova (Hamburg, Germany) as supplier for Jackson ImmunoResearch (West Grove, PA, USA).



**Figure 1.** Overview scan (A) of a coronal brain section in a rat with focal cerebral ischaemia, located in the right middle cerebral artery territory affecting the thalamus, with accumulation of macrophage-like cells, revealed by immunoperoxidase staining of CD68. Scans with 25x magnification in the ischaemic area and in the ischaemic border zone – exemplarily shown in (B) and (C) – were used for semi-quantitative analysis of CD68-immunopositive cells. Scale bars: A), 1 mm; B-C), 50  $\mu$ m.

## Clinical relevance of macrophage-like cell accumulation

Based on the fact that CD68-immunoreactivity was markedly enhanced in the ischaemic area compared with its border zone (Figure 2), a pixel ratio between both areas was calculated as a surrogate for the accumulation of macrophage-like cells, and its association to the overall clinical course was further investigated. Figure 3 displays the relationship between both variables in terms of scatter plots addressing the overall sample (Figure 3A) and treatment-related populations, respectively (Figure 3B-D). Although statistical significance is generally lacking, we found a trend towards an association of accumulating macrophage-like cells and pronounced reduction of the neurological deficit during the observation period, indicated by negative Beta coefficients. This effect was most evident in the control group (Figure 3B), barely missing statistical significance.

## Delayed changes of markers for macrophage-like cells, microglia, astrocytes and neurons after focal cerebral ischaemia

To obtain further insights into long-term reactions following acute focal cerebral ischaemia, triple immunofluorescence labelling was performed to address the co-occurrence of different inflammatory cell types. This approach was focussed on control animals at day 28, the group with most CD68-immunopositive cells in the present study (Figure 2A).

Figure 4 shows an ischaemic area with accumulation of macrophage-like cells (expressing CD68 and CD11b) and microglia (Iba). This region was clearly dominated by Iba- and CD11b-immunopositive cells appearing activated in the merged Figure 4D. Moreover, a few cells were stained by all three markers. Focussing on the border zone of focal cerebral ischaemia and taking astroglial reactions into account, Figure 5 demonstrates the concomitant staining of macrophage-like CD68-immunoreactive and Iba-positive microglial cells combined with the detection of GFAP-containing astroglia. In general, microglia and astroglia appeared activated. The boundary of accumulated macrophage-like cells was clearly pronounced, stretched from the left lower to the right upper corner in Figure 5A. Beyond this line, a strong astrogliosis was found in the ischaemic core-averted area, whereas a loss of astroglia occurred predominantly in the area of predominant macrophage-like accumulation. The regions of strongest Iba-labelling and highest accumulation of macrophage-like cells were largely

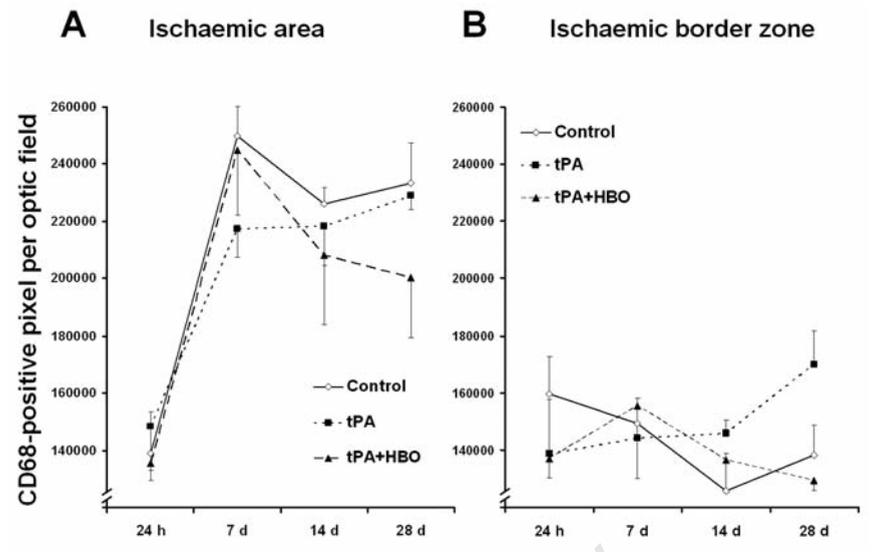


Figure 2. Spatio-temporal course of pixel-based calculations per optic field detecting the accumulation of CD68-positive cell in two different ischaemia-related areas depending on different treatment regimes. Symbols represent means and added bars represent standard errors. Sample size: Three sections were analysed in each case, 3 subjects served for each treatment group at each time point.

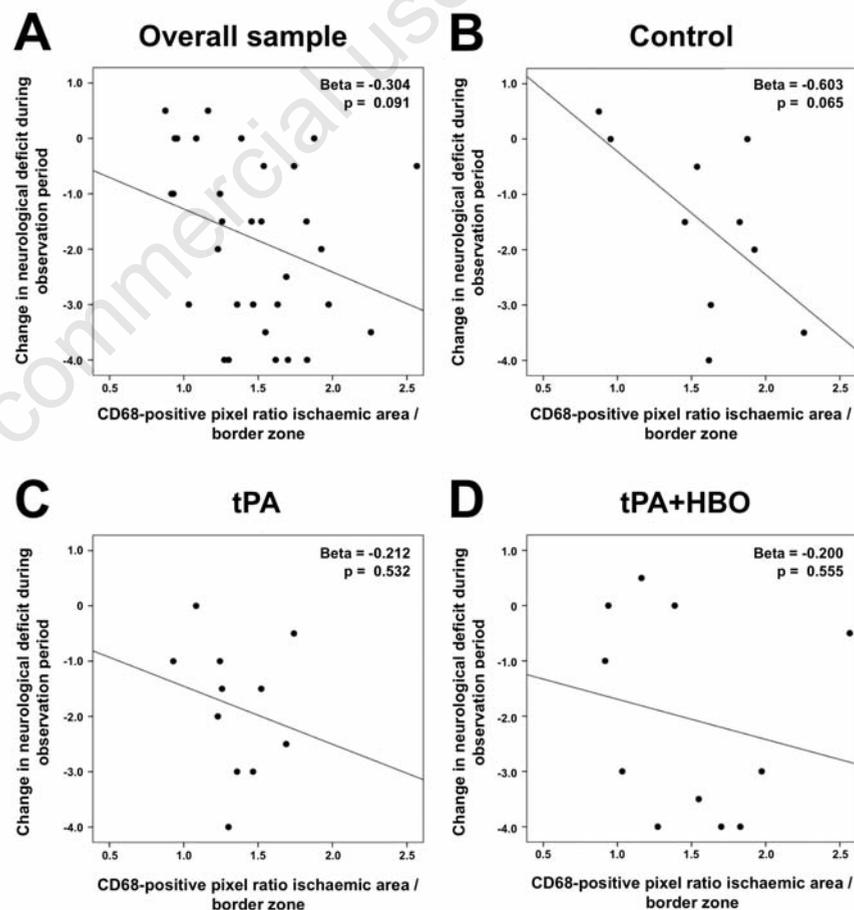


Figure 3. Scatter plots indicating the interrelation between the pixel-based ratio of CD68-immunoreactivity (as surrogate for the accumulation of macrophage-like cells) and the clinical impairment (shown as course of the neurological deficit during the overall observation period). Thereby, increasing values of the CD68-positive pixel ratio represent an increasing accumulation and tended to be associated with clinical improvement. Beta and P-value originated from linear regression analysis using the pixel ratio as independent and the change of neurological deficit as dependent factor.

identical, but activated microglia were also found in tissues devoid of CD68-staining. Additionally, considering the stroke-induced neuronal loss, Figure 6 represents the border zone from a case with an ischaemic lesion also affecting the neocortical tissue. Here, microgliosis was revealed by Iba staining (Figure 6A), whereas GFAP-immunoreactivity visualised astroglia (Figure 6B) and the neuronal loss was concomitantly detected by NeuN-immunolabelling (Figure 6C). The merged Figure 6D impressively displays the borderline of glial activation on the left side with simultaneous neuronal impairment. Finally, triple staining of Iba, GFAP and NeuN (Figure 7) exemplified the involvement of the hippocampal formation as region especially susceptible to hypoxia. Thereby, a strong microglial activation and astrocytic reaction occurred concurrently with diminished neurons in the CA1 region when focal ischaemia slightly, but not completely, affected the hippocampus.

## Discussion

The present study addressed inflammatory reactions following focal cerebral ischaemia as a stroke research area with high priority,<sup>3</sup> taking into account tPA as the only treatment currently promising in a clinical setting<sup>23</sup> and furthermore investigating HBO as potential neuroprotective co-medication to attenuate deleterious side effects of tPA.<sup>4,24,48,49</sup> Special emphasis was given to a translational relevant experimental setting by precluding preconditioning attempts prior to stroke, using an embolic stroke model to mimic human conditions, and choosing a relatively long-term observation period of 4 weeks.<sup>1,4,62</sup> Especially the latter feature of the present study achieves great relevance due to the known peak of inflammatory processes in later phases of stroke,<sup>7</sup> which was neglected in experimental studies with shorter observation periods. Moreover, as conclusion from a study by Li *et al.* who demonstrated regionally different patterns of macrophages and neutrophils after MCAO,<sup>63</sup> more than one ischaemia-related area was investigated.

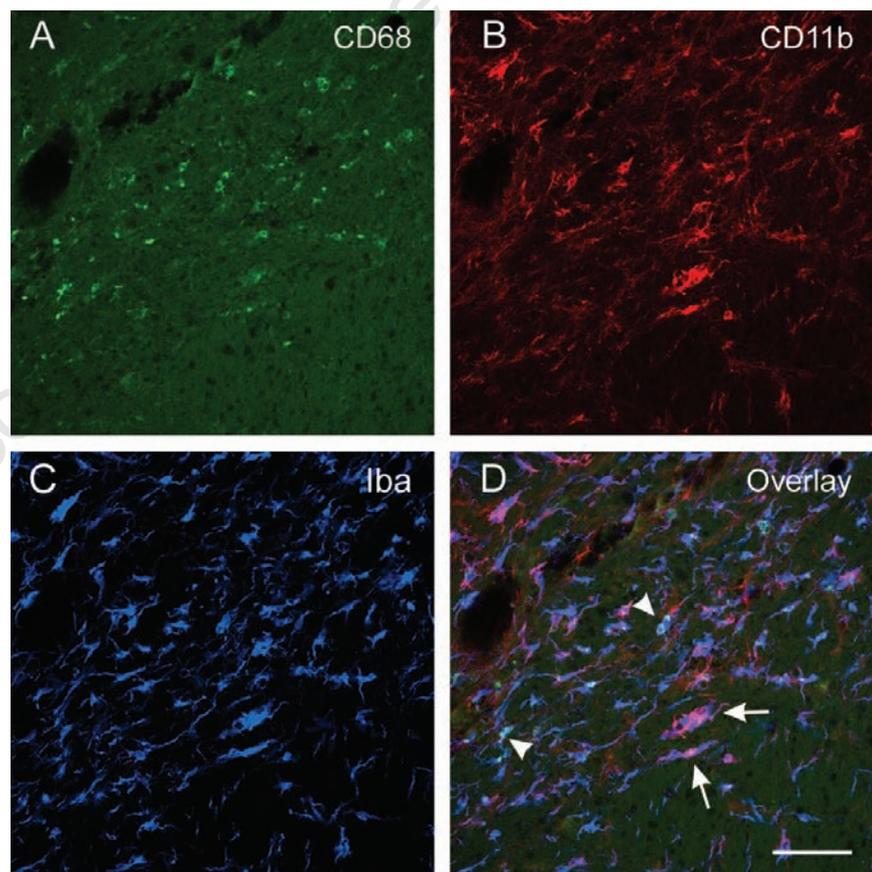
### Course and impact of macrophage-like cell accumulation

In its first part, the present study reveals the spatio-temporal course of CD68-immunoreactivity as marker for the accumulation of macrophage-like cells, which has often been used in previous experimental stroke studies (e.g., Benjelloun *et al.*,<sup>58</sup> and Qiao *et al.*<sup>64</sup>).

Focussing on the control group that represents the regular course of invading

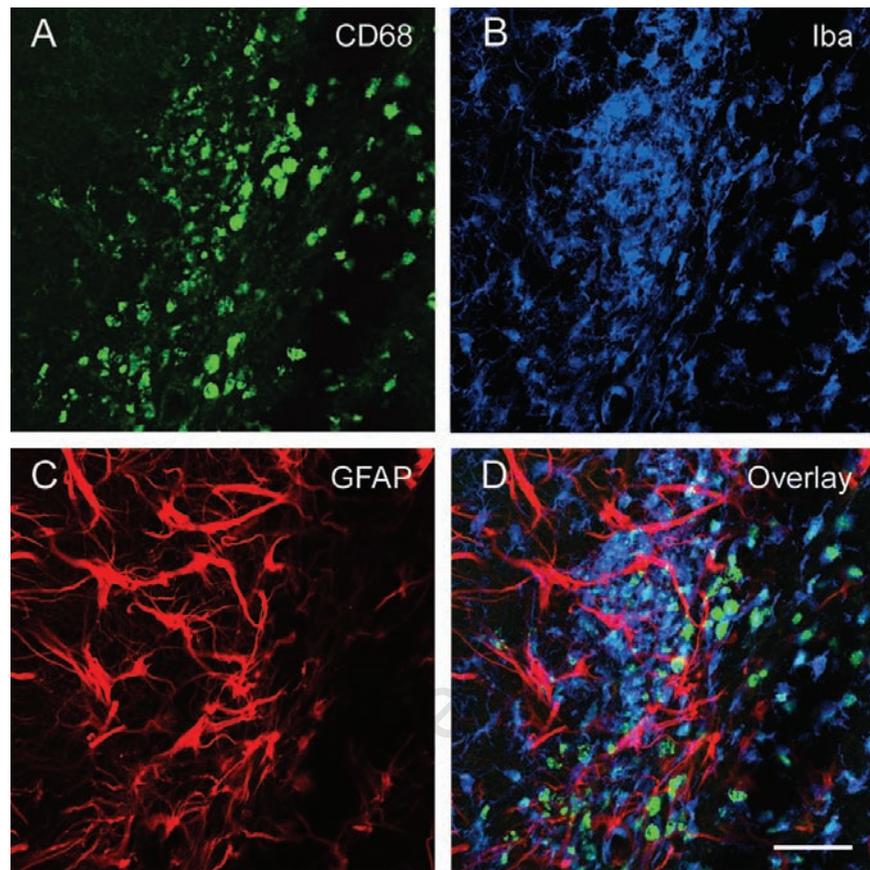
macrophage-like cells following focal cerebral ischaemia, a considerable increase was seen from 24 h to 7 days with long-lasting high levels up to day 28. In general, time-dependent changes have been summarised in several previous reports (e.g., Danton and Dietrich,<sup>9</sup> Jin *et al.*,<sup>32</sup> Stoll *et al.*<sup>13</sup> and Tomita and Fukuuchi<sup>14</sup>), wherein, considering different influences of permanent vs transient ischaemia on post-stroke inflammation, an infiltration of leukocytes during the first hours and days has been described as ensured. Going into detail: in a notably comprehensive study, Clark *et al.* provided information about the development of infarct size and inflammatory mechanisms after permanent MCAO in rats and presented a model of 4 stages (*neuronal death, inflammation, organization and resolution*) within the first 30 days after onset of ischaemia.<sup>65</sup> Additionally, stage II (*inflammation*) was sub-

divided into an *initial/acute phase* and a *delayed/chronic phase*, starting within the first 12 h, ending at day 5 and representing the infiltration of neutrophils; but abundant macrophages were also observed in stage III (*organization*), which ended at day 15.<sup>65</sup> Comparable data of leukocyte invasion were provided, amongst others, by Kato *et al.* after transient MCAO in rats,<sup>66</sup> Jander *et al.* and Kleinschnitz *et al.* after photochemically induced cortical ischaemia in rats,<sup>19,67</sup> from Weston *et al.* after endothelin-caused MCAO in rats,<sup>68</sup> and from Mena *et al.* who studied 137 post-mortem cases of human cerebral infarction.<sup>69</sup> Typically, leukocyte invasion starts with a hypoxia-related production of inflammatory mediators by brain cells, which causes adhesion molecules being expressed on endothelial surfaces [e.g., the intercellular adhesion molecule 1 (ICAM-1)] thus enabling leukocyte

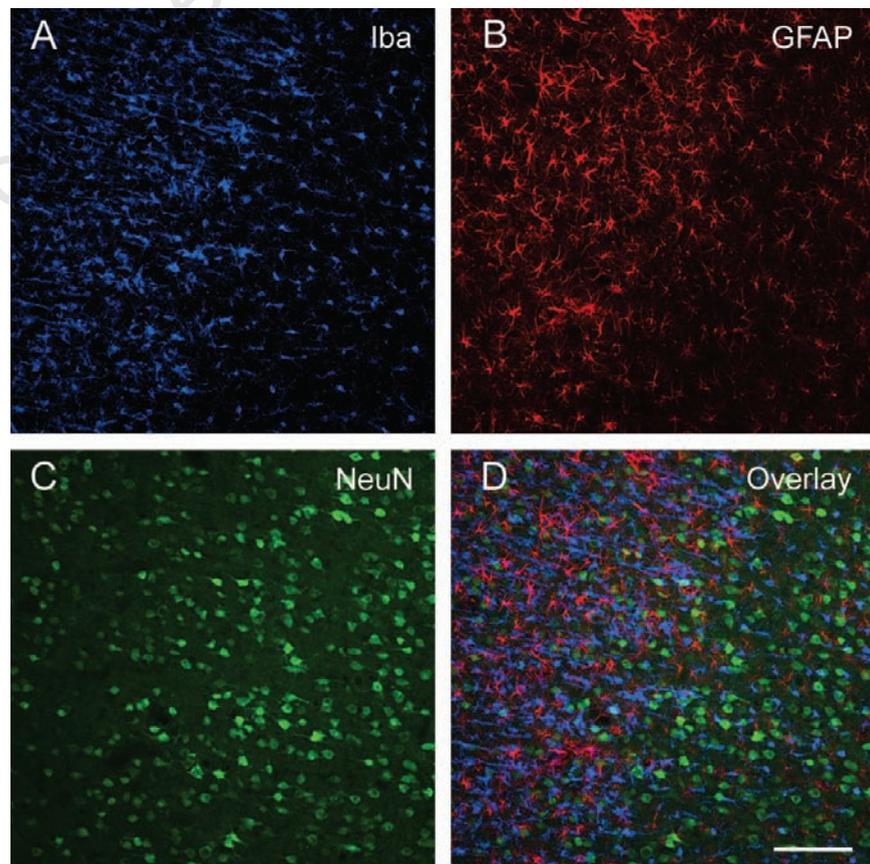


**Figure 4.** Triple fluorescence labelling of markers for macrophages and microglia, 4 weeks after embolic stroke in the right middle cerebral artery territory. (A) The staining of CD68 with FITC-conjugated monoclonal mouse antibodies was enhanced by Cy2-tagged anti-FITC, and combined with (B) the immunodetection of CD11b based on biotinylated mouse antibodies visualized by Cy3-streptavidin, and (C) Cy5-immunolabelling of ionized calcium binding adaptor molecule 1 (Iba) in microglial cells. The merge of the three staining patterns reveals a microglial population co-expressing CD11b and Iba (purple, arrows), but in the centre also cells exist, displaying all three markers (white, arrowheads). Scale bar: 50 µm.

**Figure 5.** Concomitant immunofluorescence staining of CD68 in macrophage-like cells, ionized calcium binding adaptor molecule 1 (Iba) in microglial cells and glial fibrillary acidic protein (GFAP) in astroglia 4 weeks after embolic stroke in the border zone of the infarct. (A) CD68-immunoreactivity addressed with biotinylated monoclonal mouse antibodies and visualised by Cy2-conjugated streptavidin, and (B) Iba revealed by Cy5-immunolabelling were counterstained in (C) with Cy3-coupled mouse-anti-GFAP. By merging of staining patterns in (D) activated micro- and astroglia were also elucidated around the centre of the infarct with infiltrated CD68-immunopositive macrophages. Scale bar: 50  $\mu$ m.



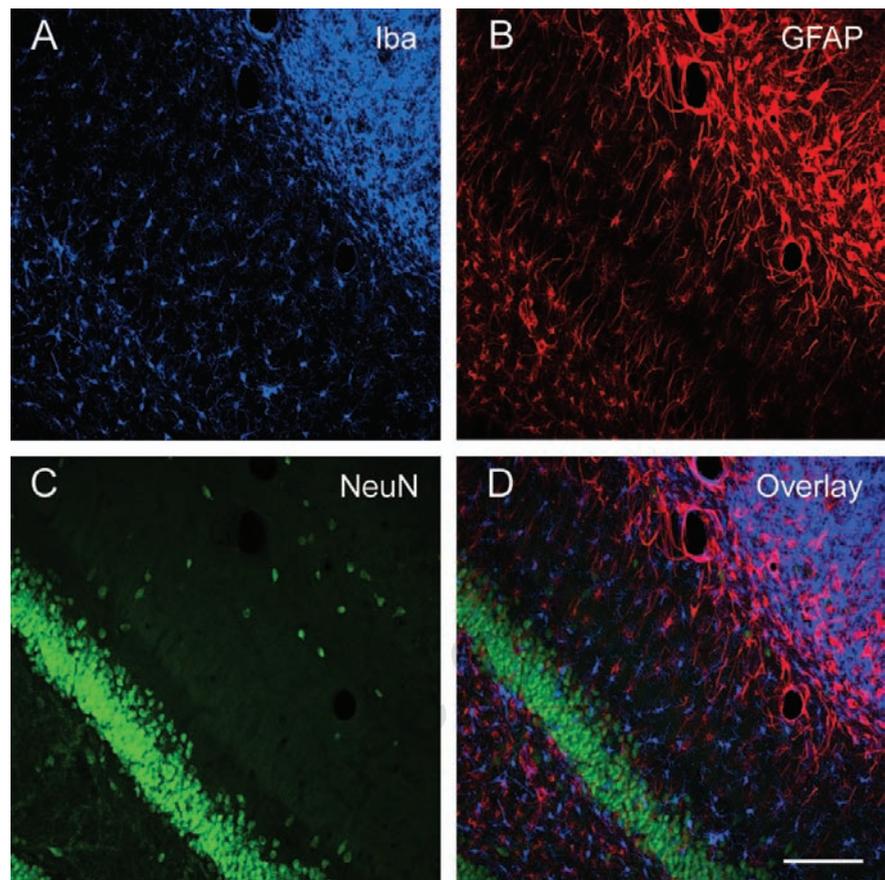
**Figure 6.** Stroke-induced glial activation corresponding to neuronal loss in the neo-cortex. Four weeks after embolic stroke, the outer rim of the infarct zone in the left part of the pictures displays in (A) activated microglia revealed by Cy5-immunolabelling of ionized calcium binding adaptor molecule 1 (Iba) and in (B) a strong, infarct-induced astrogliosis shown by Cy3-staining of glial fibrillary acidic protein (GFAP). In (C), the Cy2-immunolabelling of the pan-neuronal marker NeuN reveals the loss of neuronal structures in the area of infarction. The overlay of staining patterns in (D) visualises the border between the stroke-affected region (displaying gliosis and neuronal loss) and the apparently healthy tissue (without apparent glial activation and non-affected neuronal staining). Scale bar: 100  $\mu$ m.



infiltration.<sup>7,15,32,70,71</sup> Several studies focussed on the roles of such invading cells: Gidday *et al.*,<sup>36</sup> Justicia *et al.*,<sup>37</sup> Romanic *et al.*,<sup>72</sup> and Wang *et al.*<sup>38</sup> demonstrated that leukocytes are a major source of MMP-9 following experimental stroke, and therefore contribute to the post-ischaemic blood-brain barrier disruption.<sup>26</sup> These findings are in line with Rosell *et al.*,<sup>73</sup> who confirmed the suggested association of neutrophil infiltration with local MMP-9 increase in patients after fatal cases of stroke. However, other reports indicate that MMPs might also be expressed by neurons.<sup>74</sup> Predominantly deleterious effects were reported for MMPs in the acute phase following stroke, but recent studies also suggested regenerative properties of these enzymes in later phases.<sup>29,75</sup> Concerning the maintenance of the inflammatory response after focal cerebral ischaemia, a recent study from Qiao *et al.* demonstrated that in contrast to mild ischaemia with only a transient inflammation, a long-lasting response up to weeks exists following a substantial infarction.<sup>64</sup> This was supported by data from Karki *et al.*,<sup>76</sup> who found a long-lasting presence of macrophages (up to 1 year) in the lesion border after transient MCAO in rats.

tPA was investigated in the present study due to its widespread use as a recanalisation drug with generally positive effects concerning the neurological outcome in acute stroke patients.<sup>23</sup> Based on the knowledge that tPA activates also MMPs,<sup>25,27</sup> which is even contributing to BBB impairment and increases the potential risk of haemorrhagic transformation,<sup>24,26</sup> HBO was chosen to inhibit these side effects. This rationale was supported by reports from Liu *et al.* and Veltkamp *et al.*,<sup>77-79</sup> who demonstrated reduced MMP-9 levels due to normobaric and hyperbaric hyperoxia after experimental stroke in rats. Furthermore, HBO was found to decrease the accumulation of neutrophils,<sup>45,46</sup> possibly based on a HBO-related reduction of ICAM-1.<sup>80,81</sup> In the present study, the accumulation of macrophage-like cells in the ischaemic area increases in a nearly identical way from 24 h to 7 days irrespective of treatment (see Figure 2A), which means that a combined application of HBO at 2.4 ata and tPA does not encompass a decreased inflammatory response during the first 7 days following experimental stroke. Similarly, Hjelde *et al.* reported neutrophil accumulation after permanent MCAO in rats following treatment with HBO at 2.0 ata or room air under ambient pressure.<sup>47</sup> Focussing on the time course following day 7, a decreasing tendency of macrophage-like accumulation was observed in the tPA+HBO group.

Taking these results together with our previous work that showed a trend to worsened long-term clinical outcome (as defined by a com-



**Figure 7.** Simultaneous detection of glia and neurons 4 weeks after embolic stroke in the hippocampal border zone. Activated microglia are shown in (A) by ionized calcium binding adaptor molecule 1 (Iba)-immunostaining (Cy5), whereas (B) demonstrates the astrogliosis revealed by Cy3-staining of glial fibrillary acidic protein (GFAP) and (C) displays a slightly diminished Cy2-staining of neuronal nuclei (NeuN) in the area of and surrounding the pyramidal layer of the CA1 region. By merging the staining patterns in (D) the stroke-induced gliosis becomes even clearer (in the upper right corner of the picture). Scale bar: 100  $\mu$ m.

bined endpoint of neurological deficit and premature death) in tPA+HBO-treated subjects *vs* the control and tPA group,<sup>50</sup> the inhibition of macrophage-like accumulation in the ischaemic area has to be discussed in a critical manner. To further explore the general clinical impact of the obtained cellular changes, a functional perspective was added, addressing the relationship between the amount of macrophage-like cell accumulation and the reduction of neurological deficit during the overall observation period. Interestingly, these variables tended to be associated negatively, leading to the conclusion that an increased accumulation of macrophage-like cells might cause clinical improvement. Although it is important to note that the found association failed statistical significance, our data are in line with findings from a number of recent studies: Inácio *et al.* and Justicia *et al.* aimed to reduce the local inflammatory response after cerebral ischaemia via antibodies recognising

the vascular cell adhesion molecule-1 or via an inhibition of macrophage migration while using factor-depleted mice.<sup>82,83</sup> Furthermore, Shechter *et al.* controlled macrophage invasion after spinal cord injury.<sup>84</sup> Taken together, these attempts did not lead to beneficial effects. In parallel, Madinier *et al.* reported that the drug-induced reduction of the post-ischaemic inflammatory response, especially the microglia activation, causes a long-term impairment of neuronal plasticity.<sup>85</sup> Moreover, Smirkin *et al.* and Matsumoto *et al.* identified populations of invading cells in the ischaemic area after transient MCAO in rats playing regenerative roles, e.g., by producing neuroprotective factors.<sup>86,87</sup> Finally, Manoonkitiwongsa *et al.* found a strong association between the microvessel density and the macrophage count in relation to impaired brain tissue after transient MCAO in rats and concluded that these microvessels enable the infiltration of macrophages, compatible to the clean-up hypothesis.<sup>88</sup>

## Long-term stroke-induced cellular changes

The second part of the present study was focussed on the attempt to differentiate delayed components of the inflammatory response, known to be highly complex.<sup>8,9,13,14,89,90</sup> Additionally, neurons were addressed due to their embedding in the neurovascular unit concept.<sup>5,6,10</sup> Microglial and astroglial activation was described by numerous reports following cerebral ischaemia indicating their role as key elements.<sup>13,28,32,55,65,66,91-93</sup> In this regard, recent studies elucidated microglia as a source of pro-inflammatory mediators such as tumor necrosis factor in early stages of ischaemia.<sup>32,33,94-96</sup> However, neuroprotective properties of microglia have also been discussed.<sup>12,28,32,97-100</sup>

Summarising data from the present study, a strong microglial activation was still found at 4 weeks following focal cerebral ischaemia, indicating that microglia play not only a role in early phases following stroke. This activation was mainly co-localised with macrophage-like cells as indicated by CD68. Interestingly, at this time point the general microglia activation resulting in altered cell morphology appears as dominant effect when compared with the occurrence of CD68-positive cells, even if some Iba/CD68-co-expressing cells exist. Our findings are in line with a previous report by Gelderblom *et al.* who investigated the time course of post-stroke inflammation up to 7 days after MCAO in mice and found a larger proportion of microglia vs macrophages in this interval.<sup>101</sup> Furthermore, the present data are in accordance with the reported microglial activation observed even 4 weeks after MCAO.<sup>102</sup> The activated microglia were thereby primarily associated to the area of ischaemia-related impairment as shown by neuronal loss, which was described previously in a comparable manner but with markedly shorter observation periods.<sup>71,93,103</sup> Focussing on astrocytes, at 4 weeks a strong circular astrogliosis was found in the rim around CD68-positive cells (see Figures 5 and 6) representing the border zone of ischaemia enwrapping the infarct core. Such an organization has been described previously (e.g., by Verkhatsky and Butte<sup>104</sup>), whereas Clark *et al.* investigated this issue in a long-term course and found that this process starts to form up at 24 h after MCAO simultaneously to neutrophil infiltration and lasts up to day 30, enabling the demarcation of the necrotic core.<sup>65</sup>

## Methodological considerations

The relatively small number of subjects limits the validity of the study. However, the performed semiquantitative evaluation allows a tendency of spatio-temporal alterations for

macrophage-like cells after experimental embolic stroke, which is of high relevance regarding translational issues. The found long-term course towards a decreased macrophage-like cell accumulation in the combined treatment group (tPA+HBO) is presumably related to the co-application of HBO, but due to the lack of a separate HBO group the definitive proof remains open. Caused by the overlapping target structures of available antibodies in this field, it represents an ongoing challenge to address neural subpopulations specifically, e.g., for the clarification on proportional invading macrophages, differentiated from activated resident microglia, as addressed by previous reviews (e.g., Dijkstra *et al.*,<sup>105</sup> Matsumoto *et al.*,<sup>18</sup> and Stoll *et al.*<sup>13</sup>). Especially for microglia, it remains uncertain if the obtained circular organization originates from proliferation of resident microglia or invading processes. In the past, several lectins (e.g., from *Griffonia simplicifolia*<sup>106</sup>) were useful markers for microglia but co-stained vessels,<sup>57,107</sup> which was not desired in the present study.

## Conclusions

This study reveals a spatio-temporal pattern of accumulating macrophage-like cells after experimental ischaemic stroke with a significant increase from 24 h to 7 days post ischaemia, which remained up to 4 weeks, indicating a long-term inflammatory response. Taking into account tPA as a widely used recanalisation drug and HBO as a potential neuroprotective approach, a tendency in the combined treatment group to a decreased accumulation of macrophage-like cells starting at day 14 needs to be discussed in a critical manner, since recent research has ascribed long-term inflammatory responses as important for repair and neuronal plasticity. Following experimental stroke, a long-lasting glial activation was found simultaneously with neuronal loss. Thereby, microglia were mainly observed within the ischaemic area whereas astrogliosis occurred primarily in the ischaemic border zone.

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