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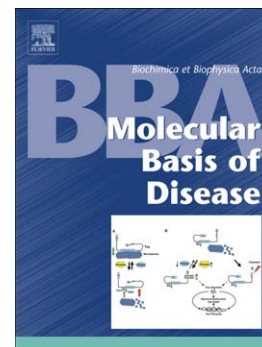
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## Mrp-8 &-14 mediate CNS injury in focal cerebral ischemia

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Running title: Role of TLR4-agonist MRP14 in experimental stroke

### Keywords

S100A8, S100A9, Toll-like receptor, MCAO, knockout mice

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**ABSTRACT**

Several reports have recently demonstrated a detrimental role of Toll-like receptors (TLR) in cerebral ischemia, while there is little information about the endogenous ligands which activate TLR-signaling. The myeloid related proteins-8 and -14 (Mrp8/S100A8; Mrp14/S100A9) have recently been characterized as endogenous TLR4-agonists, and thus may mediate TLR-activation in cerebral ischemia. Interestingly, not only TLR-mRNAs, but also Mrp8 and Mrp14 mRNA were found to be induced in mouse brain between 3 and 48h after transient 1h focal cerebral ischemia/reperfusion. Mrp-protein was expressed in the ischemic hemisphere, and co-labelled with CD11b-positive cells. To test the hypothesis that Mrp-signaling contributes to the postischemic brain damage we subjected Mrp14-deficient mice, which also lack Mrp8-protein-expression, to focal cerebral ischemia. Mrp14-deficient mice had significantly smaller lesion volumes when compared to wildtype littermates ( $130 \pm 16 \text{ mm}^3$  vs.  $105 \pm 28 \text{ mm}^3$ ) at two days after transient focal cerebral ischemia (1h), less brain swelling, and a reduced macrophage/microglia cell count in the ischemic hemisphere. We conclude that upregulation and signaling of Mrp-8 and-14 contribute to neuroinflammation and the progression of ischemic damage.

## INTRODUCTION

After focal cerebral ischemia primary brain damage results from a complex pattern of pathophysiological events including excitotoxicity, periinfarct depolarizations, inflammation, and apoptosis [1-5].

Recent expression analysis revealed that Toll-like receptors (TLR) TLR-2, -4, and -9 are induced in postischemic brain tissue [6]. Toll-like receptors (TLRs) represent a family of pattern-recognition transmembrane receptors which are able to recognize a large number of conserved structural motifs, called *pathogen-associated molecular patterns* (PAMPs). While TLRs have a crucial role in the innate immune response to invading pathogens and infection, recent findings also suggest an important role in neurodegenerative disorders: TLRs are thought to be activated by endogenous 'danger signals' released from injured or necrotic cells and contribute to the initiation of the inflammatory response as well as apoptotic cell death [3,5,7-15]. TLR2 and TLR4 were recently shown to mediate ischemic brain damage in mice, potentially by activating pro-apoptotic pathways and the release of proinflammatory cytokines [3,4,6,16-22].

Until now, it is not known which ligands activate TLR-signaling in cerebral ischemia. Molecules such as monosodium urate, hyaluronan, heat shock proteins, or high mobility group box protein 1 (Hmgb1) were shown to activate TLR-signaling and thus may potentially act as endogenous TLR-ligands in postischemic brain damage [5,18,23].

Very recently, *myeloid related protein 8* (Mrp8) and Mrp14 (also called *Calgranulin A* and *Calgranulin B* or S-100 calcium binding protein A8 [S100A8] or S100 calcium binding protein A9 [S100A9], respectively) were characterized as endogenous Toll-like receptor-4 (Tlr-4) agonists [24]. Mrp8/S100A8 and Mrp14/S100A9, as well as

S100A6 (Calcyclin) belong to the S100 protein family and are expressed in activated macrophages and microglial cells [25,26]. S100-family members are characterized by the presence of two calcium binding sites, so-called EF-hands, which modulate cell-specific functions and interactions, such as vesicle production and phagocytosis, activation and gene regulation, cell differentiation and proliferation [25]. Calprotectin, the complex of Mrp8/S100A8 and Mrp14/S100A9, is a major calcium- and zinc-binding protein of phagocytotic cells of myeloid origin and both proteins comprise about 40% of the cytosolic protein of neutrophils [27].

We hypothesized that Mrp8 and Mrp14 may contribute to postischemic brain damage by activating pro-inflammatory signaling pathways. Whereas Mrp8-deficiency causes early resorption of the mouse embryo [26], Mrp14-deficient mice, which are viable, do not express Mrp8 in the adult mice, thus allowing the functional analysis of Mrp-8 & -14 in adult mice after stroke [27,28]. Thus, we tested whether Mrp14-deficient mice were protected against cerebral ischemia and whether they have a reduced inflammatory response.

Since Mrp-deficient mice were significantly protected in our study and have reduced macrophage/microglia cell count in a murine stroke model when compared to wild-type control mice, we conclude that Mrp8/14 contributes to postischemic tissue damage, potentially by promoting neuroinflammation via Tlr4-signaling.

## MATERIAL AND METHODS

**Animals.** Adult 9-13 weeks old male Mrp14<sup>-/-</sup> mice [28] were provided by Dr. W. Nacken (Department of Experimental Dermatology, University of Münster, Germany). Targeted deletion of Mrp14 results in a loss of both the Mrp8 and the Mrp14 proteins in mice [24,27,28]. The Mrp14<sup>-/-</sup> mice had been backcrossed to the original C57Bl/6J background for more than 10 generations. Age matched male 9-14 week old C57Bl/6J littermates were used as control in all experiments. The body weight of Mrp14<sup>+/+</sup> control mice (C57Bl/6J littermates) was  $23.7 \pm 2.7$  g compared to  $24.0 \pm 2.0$  g in Mrp14<sup>-/-</sup> mice. All animal handling and surgery were performed in accordance with the *Guidelines for the Use of Animals in Neuroscience Research* (Society for Neuroscience). All experiments were approved by the local institutional Animal Care Committee, LAGeSo No.G0382/05). Mice were housed under diurnal lighting conditions and allowed access to food and water *ad libitum*. All experiments were performed in a randomized manner, and surgery, as well as infarct volume determination were performed by investigators blinded to the groups.

**Induction of focal cerebral ischemia.** Middle cerebral artery occlusion (MCAO) was induced by inserting a silicone-coated filament (Xantopren M Mucosa and Activator NF Optosil Xantopren, Heraeus Kulzer, Wehrheim, Germany) via the internal carotid artery as described by Gertz et al. [29]. For sham control, the operation was performed without advancing the filament. Mice were anaesthetized with 2% isoflurane for induction and maintained with 1.5% isoflurane 70% N<sub>2</sub>O and 30% O<sub>2</sub> via a face mask. Anesthesia did not exceed 10 minutes. After 60 minutes the animals were re-anaesthetized for 1 min and the filament was removed to permit reperfusion. In all

animals during surgery and ischemia, body temperature was measured and maintained between 37.0 and 37.5°C with a heating pad.

**Analysis of gene expression.** Mice were deeply anaesthetized and decapitated at specified time points of reperfusion. The brains were removed rapidly from the skull. RNA derived from ipsilateral (ischemic) hemispheres, pooled from 6 wild-type C57Bl/6 mice (after MCAO, respectively sham treatment) at each time point was used for *Affymetrix GeneChip* experiments using *Affymetrix GeneChip Mouse Expression Set 430* (Affymetrix, Santa Clara, CA). For *Illumina Sentrix* arrays (Illumina, San Diego, CA), ipsi- and contralateral hemispheres of MPR14-deficient and C57Bl/6 wild-type mice were pooled from three animals each group and time point. The RNA was isolated with Trizol Reagent (Invitrogen, Karlsruhe, Germany) and was transcribed into cDNA (*SuperScript II*; Invitrogen, Karlsruhe, Germany). In order to determine the induction of specific mRNAs in the ipsilateral hemispheres of MCAO-treated animals expression values were compared to those derived from the uninjured (contralateral) hemisphere of these animals, as well as to those derived from sham-operated animals. Thus, mRNAs of the contralateral hemispheres of MCAO treated mice, as well as mRNAs from the ipsilateral brain hemispheres of sham-treated wild-type mice (C57BL/6) were used as a reference for the determination of the induction of mRNAs derived from the ipsilateral brain hemisphere of MCAO-treated mice. Commercially available Affymetrix software as well as *Beadstudio* (Illumina, San Diego, CA) was applied for data analysis of *Affymetrix GeneChips* and *Illumina Sentrix* array, respectively. Fold change (FC) was calculated using the following formula:  $FC = 2^m$ , for  $m \geq 0$ , and  $FC = (-1) \times 2^{-m}$  for  $m < 0$ , with  $m = \log_2 \text{ratio}$ . Mrp8/S100A8 expression was assessed by the probe 1419394\_at (Affymetrix-ID) and



scl22940.2.1\_25-S (Illumina-ID), Mrp14/S100A9 by probe 1448756\_at (Affymetrix-ID) and scl020202.1\_47-S (Illumina-ID), and S100A6/Calcyclin by probe 1421375\_a\_a (Affymetrix-ID) and scl22942.3.1\_64-S (Illumina-ID) respectively.

**Assessment of infarct volume.** At 48 h after the induction of ischemia for 60 min, mice were deeply anaesthetized and sacrificed; the brains were removed rapidly from the skull and snap frozen in 2-methylbutane on dry ice. Brains were sectioned (12  $\mu$ m) on a microtome, dried overnight, and stained with hematoxylin (Merck, Darmstadt, Germany). The sections were digitized, the area of infarction was quantified on a PC using Sigma Scan Pro Software (Jandel Scientific, San Rafael, CA), and infarct volumes were calculated. The 'indirect' infarct volume was calculated as the volume of the contralateral hemisphere minus the non-infarcted volume of the ipsilateral hemisphere. The difference between 'direct' and 'indirect' infarct volumes represents brain swelling [30]. There was no need to exclude animals due to insufficient occlusion of the middle cerebral artery, because there were no animals without an infarct in both groups (MRP14<sup>-/-</sup> and MRP 14<sup>+/+</sup>).

**Quantification of inflammatory cells in the ischemic hemisphere.** The number of cd11b-positive cells was determined by counting all cd11b-positive cells at interaural position No. III (distance to bregma 3.9 mm) in whole ischemic hemisphere of mice at 48 h after induction of cerebral ischemia (MCAO) for 60 min.

**Statistical analysis.** Student's *t*-test for independent samples was applied to determine statistical significance between the mean values of two study groups if not stated otherwise. P-values below 0.05 were considered statistically significant. If groups were

not normal distributed, Mann-Whitney-U-test was used (comparison of infarct volumes and cd11b-positive cells). As not stated otherwise, values are given as mean value  $\pm$  standard deviation (SD).

**Immunohistochemistry.** Staining was performed on fresh frozen tissue harvested at 48 h of reperfusion. From the fresh frozen tissue, 12  $\mu$ m coronar cryosections at interaural positions 6.6, 5.3, 3.9, 1.9, and 0 mm were thaw-mounted onto glass slides. Slides were air-dried for 30 min and fixed for 5 min in  $-20^{\circ}\text{C}$  methanol and acetone (1:1), dried for 15 min and washed in PBS for 5 min. Next, the sections were incubated in blocking solution containing 3 % normal goat serum and 0.3 % Triton X-100 (Sigma) in PBS for 60 min. The slides were incubated over night at  $4^{\circ}\text{C}$  with a polyclonal rabbit anti-Mrp14/S100A9 antibody (gift from W. Nacken, Münster, Germany) [24] at a dilution of 1:300. For the detection of primary antibody, slides were incubated with Alexa488 conjugated goat anti-rabbit IgG (Invitrogen) at 1:200 for 1 hr at room temperature. To determine the lineage of Mrp14-positive cells, additional staining of endothelial cells was performed using rat anti-CD31 (550274; BD Pharmingen, San Jose, CA, USA), while rat anti-CD11b (Santa Cruz Biotechnology inc., CA) was used at 1:200 to stain macrophages/microglia. Secondary antibodies were obtained commercially from *Jackson ImmunoResearch* (West Grove, PA).

## RESULTS

### *Mrp8 and Mrp14 mRNA are induced after focal cerebral ischemia in the mouse.*

Expression values for S100A family members were determined using *Affymetrix GeneChip arrays* (Set 430) in combination with transcripts pooled from six ipsilateral mouse brain hemispheres at the time points 1, 3, 6, 12, and 24 h of reperfusion after 1 h of middle cerebral artery occlusion and compared to sham treated mice. Nearly 2000 genes out of more than 39.000 mRNA transcripts were up-regulated significantly at least twofold with a p-value below 0.05 (data not shown). Several induced genes are related to inflammation or apoptosis (KEGG Pathway Database; BioCarta Charting Pathways of Life), including Mrp8/S100A8 and Mrp14/S100A9 (table 1). The *Affymetrix GeneChip* results revealed a significant up-regulation of Mrp8 as well as Mrp14 mRNA during the first day of reperfusion, reaching the maximal induction at 12 h of reperfusion after induction of cerebral ischemia when compared to sham treated mice. Moreover, other members of the S100A-family were also found to be induced in postischemic brain tissue. After a minor downregulation at very early time points, induction of most S100A-family members started around 6 to 12 h after the start of reperfusion (table 1).

Apart from *Affymetrix GeneChip* analysis, which was performed to investigate early mRNA expression patterns 'over time', gene expression was also compared between ipsilateral and contralateral hemispheres (comparison 'over space'). Gene expression profiles were determined from ipsilateral (as well as contralateral) brain hemispheres (n = 3) at 48 h of reperfusion after 1 h MCAO (respectively at 72 h after 30 min MCAO) to compare gene-specific expression in the ischemic hemisphere not only with the sham-operated mice as reference (as done for the initial *Affymetrix* screening experiment), but also to allow comparison between ipsi- and contralateral hemispheres.

Up-regulation of Mrp8 mRNA (Target ID: scl22940.2.1\_25-S; *Illumina Sentix arrays*), as well as Mrp14 mRNA (Target ID: scl020202.1\_47-S; *Illumina Sentix arrays*) was confirmed by *Illumina sentix* arrays in the ipsilateral hemisphere at 48 h, but already declines at 72 h after the induction of the ischemia (data not shown).

***Mrp14 protein is expressed in cd11b-positive cells in the ischemic mouse brain.*** The induction of Mrp14 protein in postischemic brain tissue was confirmed by immunohistochemistry at 48 h of reperfusion after 1 h MCAO. Mrp14 protein expression was not detected in the non-ischemic, contralateral hemisphere. By contrast, a strong upregulation was found in the ipsilateral hemisphere with highest expression in the peri-infarct region (figure 1) and lower signal density in the infarct core. Double labeling (figure 2) identified the Mrp14-immunopositive cells as microglia and infiltrating myeloid cells/macrophages. In contrast, Mrp14 positive signal did not colocalized with endothelial cells or neurons of the ischemic hemisphere, but were often found in the immediate vicinity of small vessels (figure 2).

***Mrp14<sup>-/-</sup> mice have a smaller direct infarcts and reduced brain swelling when compared to Mrp14<sup>+/+</sup> mice.*** To test whether the Mrp14 protein is of functional relevance in cerebral ischemia, infarct volumes of male Mrp14-deficient mice and littermates after 1 h MCAO and 2 d of reperfusion were compared: infarct volumes of Mrp14<sup>-/-</sup> mice were significantly smaller than infarct volumes of Mrp14<sup>+/+</sup> mice (figure 3). The mean direct infarct volume of littermates (Mrp14<sup>+/+</sup>; n = 11) was 24 percent larger than that observed in Mrp14-deficient mice (n = 11) (mean value  $\pm$  standard deviation: 130 mm<sup>3</sup>  $\pm$  16 vs. 105 mm<sup>3</sup>  $\pm$  28; p = 0.016) (figure 3A). There was only a tendency without significance towards larger indirect infarct volumes in

Mrp14<sup>+/+</sup> mice when compared to Mrp14-deficient mice (mean  $\pm$  SD: 74 mm<sup>3</sup>  $\pm$  20 vs. 83 mm<sup>3</sup>  $\pm$  8;  $p = 0.18$ ) (figure 3B), whereas the calculated brain swelling was significantly larger in Mrp14<sup>+/+</sup> mice when compared to Mrp14-knockout mice (mean  $\pm$  SD: 47 mm<sup>3</sup>  $\pm$  13 vs. 31 mm<sup>3</sup>  $\pm$  13;  $p = 0.005$ ) (figure 3C).

***Mrp-8 and -14 contribute to the inflammatory response in postischemic brain tissue.***

There were significantly less Cd11b-positive cells (macrophages/activated microglia) found in the ischemic hemisphere at 48 h of reperfusion in Mrp14-deficient mice (727  $\pm$  124;  $p_{t-test} = 0.003$ ), when compared to Mrp14<sup>+/+</sup> wild-type littermates (1107  $\pm$  136) (figure 4A). Moreover, visualization of inflammatory cell numbers and infarct volumes revealed that Mrp14<sup>+/+</sup> mice have an increased inflammatory cell count when compared to Mrp14-deficient mice, an observation which is also true for animals with similar infarct sizes (figure 4B).

## DISCUSSION

This study has the following major findings: 1. S100A genes (including Mrp8 and Mrp14) are significantly induced in cerebral ischemia 2. Mrp14-deficiency decreases brain swelling and the direct infarct volume at 48 h of reperfusion 3. Mrp14-deficient mice have a reduced inflammatory cell count in postischemic brain tissue.

MRP8- and MRP14-positive cells were detected in various brain pathologies, including Alzheimer's plaques, multiple sclerosis lesions, experimental allergic encephalomyelitis, cerebral malaria, and traumatic brain lesions [25,31]. Ischemic human brains showed ramified microglial cells expressing both Mrp8 and Mrp14 in the first three days after insult in the periinfarctional area [32,33]. Furthermore, the Mrp8 transcript was upregulated by oxidative stress such as ultraviolet A irradiation and H<sub>2</sub>O<sub>2</sub> [34]. Thus, our current data, which demonstrates an induction of Mrp8 and Mrp14 as well as of other S100A-family members in cerebral ischemia, is in good agreement with previous reports.

Some members of the S100-protein family, such as Mrp8 and Mrp14, are secreted from cells upon stimulation, exerting cytokine- and chemokine-like extracellular activities [35]. Mrp8 and -14 were shown to impair endothelial integrity, cause endothelial damage, and Mrp8 is one of the most potent chemotactic factors described [36,37]. Accordingly, release of the heterodimer Mrp8/14 (S100A8/A9) substantially amplifies neutrophil recruitment in gouty inflammation [38,39], and high concentrations of calprotectin (complex of Mrp8 and Mrp14) in humans result in a syndrome with recurrent infections, inflammation, and hyperzincaemia [40]. Thus, our observation of a reduced infarct volume in Mrp14-deficient mice could be explained by a reduced inflammation, which was demonstrated by a reduced brain swelling (figure 3C) and a reduced inflammatory cell number in the ischemic hemisphere

(figure 4). The observation that leukocyte infiltration between Mrp14-deficient and wild-type littermates significantly differs also in those mice that do not have significant differences in lesion size argues against a simple secondary effect due to different infarct volumes.

Vogl et al. [24] recently demonstrate that Mrp8 and Mrp14 are endogenous activators of Toll-like receptor-4 signaling, which promotes lethal, endotoxin-induced shock. Thus, our data, which demonstrates that Mrp8/Mrp14 significantly contribute to ischemic brain injury and inflammation, is in good agreement with recent data, which demonstrate that TLR4 contributes to neuroinflammation and to ischemic brain injury [9,18,19], whereas mild stimulation of TLR-signaling is able to induce preconditioning effects [41,42]. However, because Mrp14 not only activates TLR4, but also the receptor for advanced glycation end products (RAGE) [35], it can not be excluded that further receptors besides TLR4 may be involved in Mrp8/14-mediated postischemic damage.

Whether the deleterious effect of Mrp14 observed in our study is due to a systemic effect (e.g. by invading leukocytes) or due to a central nervous system effect of Mrp14 signaling remains yet to be investigated. This could be achieved by the use of cell- or tissue-specific (chimeric) Mrp14 knockout mice. Moreover, the contribution of other S100A genes (table 1) in postischemic brain injury deserves further studies, as well as the question if the difference of infarct volumes at 48 h of reperfusion also persists at later time points (at which edema will be largely resolved).

The Toll-like receptor 4 (TLR4) is expressed mainly by microglia and is thought to mediate microglial activation and expression of pro-inflammatory mediators in a response to a variety of ligands [4]. Accordingly, the exacerbation of damage after stroke caused by prior immobilization stress (which triggers systemic and brain

inflammation) was attenuated in TLR-4 mutant mice [4,16]. TLR4 signaling results in NF- $\kappa$ b formation and therefore in transcription of proinflammatory signals, such as TNF-alpha and Il-1 $\beta$ , that activate the innate immune system [3,7,11,43,44].

Besides Mrp8/14, other endogenous TLR4-agonists such as hyaluronan [23] or Hmgb1 (high mobility group box protein 1) [45], heat shock proteins and monosodium urat represent further endogenous 'danger signals' [14], which are released by necrotic cells, and which lead to the initiation of the inflammatory immune response [8,22,38,45,46]. However, since our study do not prove a causal relationship between the neuroprotective effect of MRP14-deficiency and the hypothesized MRP8/14-mediated TLR4-signaling, further studies need to be performed, e.g. by the use of MRP14/TLR4-double knockout mice.

To summarize, we herewith demonstrate an up regulation of S100A-family members, including Mrp8 and Mrp14 after ischemic brain injury. Furthermore, we observed a significant exacerbation of brain damage and inflammatory cell infiltration in wild-type littermates mice when compared to Mrp-knockout mice, suggesting a detrimental and pro-inflammatory effect of Mrp8/14 mediated signaling in murine focal experimental stroke.

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## TABLES

gene symbol	Accession number	FC 1h	FC 3h	FC 6h	FC 12h	FC 24h
<b>S100A5</b>	1449927_at	n. d.	-1.71	n. d.	-1.11	2.22
<b>S100A6</b>	1421375_a_a	1.41	1.47	1.15	2.81	3.46
<b>S100A8</b>	1419394_s_a	n. d.	-1.51	1.19	4.32	3.81
<b>S100A9</b>	1448756_at	n. d.	-1.46	-1.20	1.99	1.97
<b>S100A10</b>	1416762_at	-1.23	-1.25	1.02	1.70	1.79
<b>S100A13</b>	1418704_at	-1.43	1.21	1.54	1.80	1.72

**Table 1. Expression of S100-family genes in ischemic mouse brain.** mRNA expression level was measured after extracting RNA from whole ischemic hemispheres (n = 6), at various time points of reperfusion after 1 hr MCAO by using *Affymetrix GeneChip Mouse Expression Set 430* (Affymetrix, Santa Clara, CA). Expression ratio was calculated by the use of the ratio of signal intensity in ipsilateral/ischemic hemisphere and the signal of the non-ischemic hemisphere of sham-operated animals of the same wild-type strain (C57Bl6). The regulation of different S100-family genes is displayed as fold change (FC), downregulation is indicated by ‘-’.

**FIGURE LEGENDS**

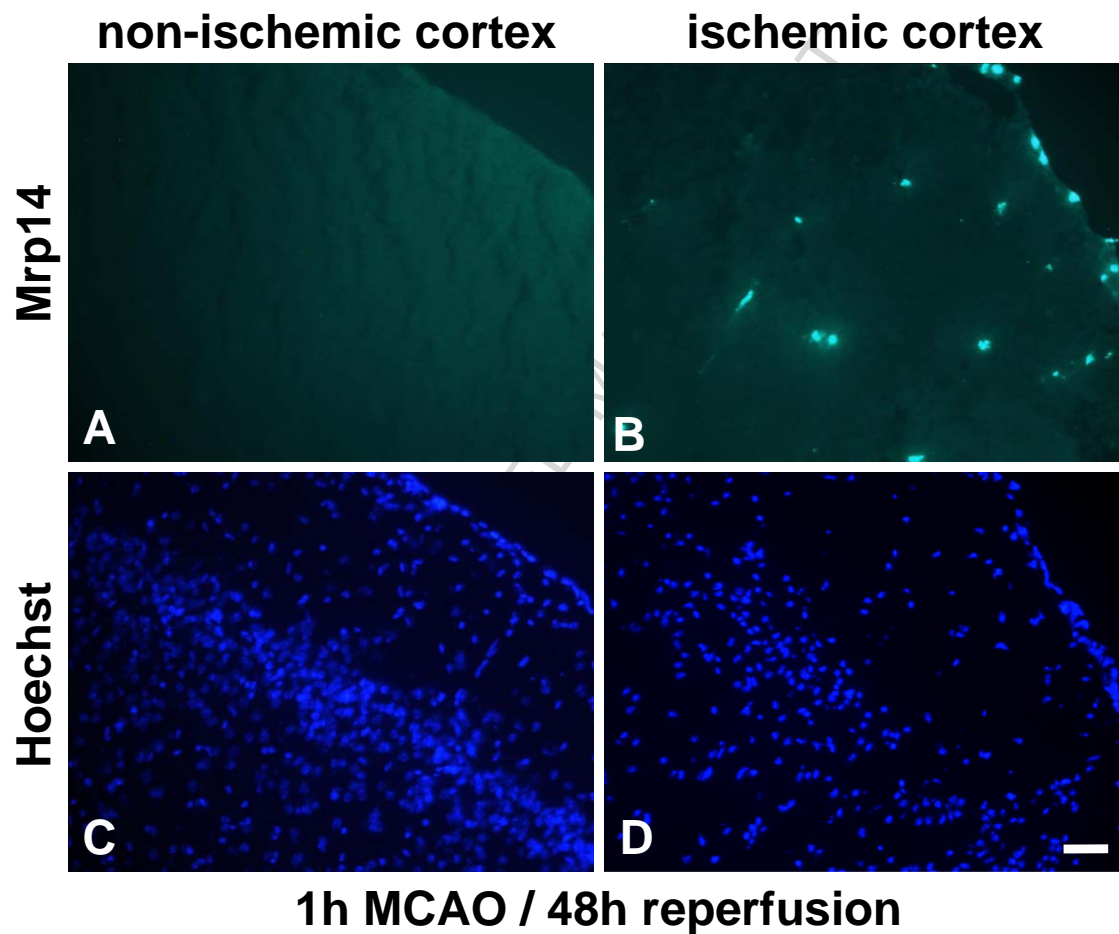
**Figure 1. Mrp14 protein is induced in in the ischemic mouse brain.** Mouse brain tissue 48 hr after 1 hr MCAO was stained with polyclonal rabbit anti-Mrp14 antibody (Vogl et al. 2007). Mrp14-positive cells are almost exclusively observed in the ipsilateral (ischemic) hemisphere. They are scattered around the peri-infarctial border zone (**B**) and partially also in the core of the ischemic tissue, but are not found in the contralateral (non-ischemic) hemisphere (**A**). Image **A** is derived from a region of the contralateral hemisphere which corresponds to the peri-infarct region (**B**) of the ischemic hemisphere. Figure **C** and **D**: Hoechst 33258 staining of the cell nuclei in the regions shown in A, respectively B. Scale bar, 80  $\mu\text{m}$ .

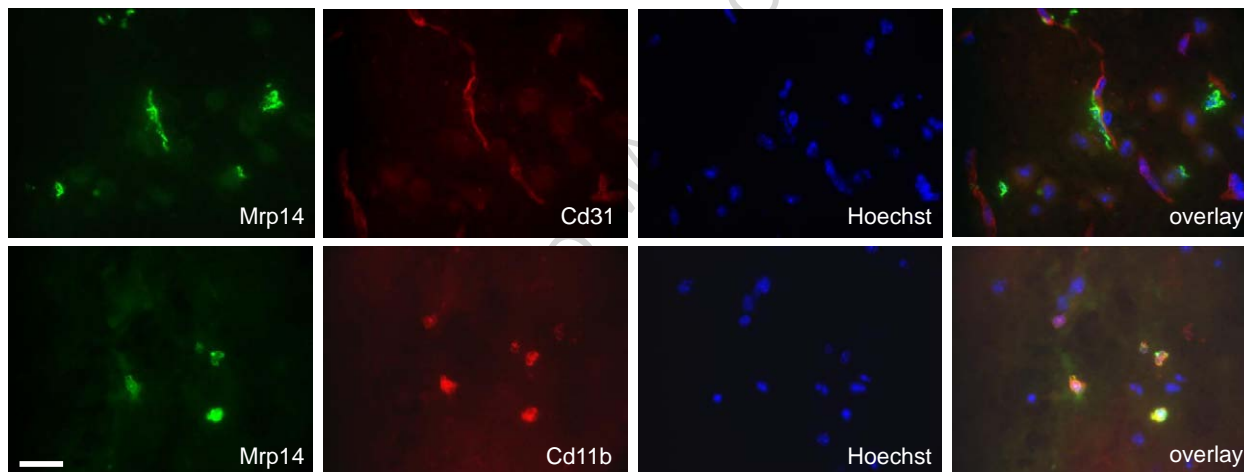
**Figure 2. Identification of Mrp14 expressing cells in the ischemic brain tissue.** Double staining of ischemic mouse brain tissue 48 h after 1 h MCAO in the ischemic hemisphere. Immunohistochemical staining of **Mrp14** (first column), and cell specific marker (second column) for endothelial cells (**Cd31**), respectively invading macrophages/microglial cells (**Cd11b**). Cell nuclei were stained using Hoechst 33258 stain, which stains DNA. The merge of the three different stainings is displayed in the forth column (**overlay**). Mrp14 positive cells were identified as invading macrophages / activated microglia, and are found mainly in the peri-infarct region of the ischemic hemisphere. Cells localized in the ischemic tissue were also found around vessels as exemplified in the right upper image (Mrp14/Hoechst/Cd31-overlay). Scale bar, 50 $\mu\text{m}$ .

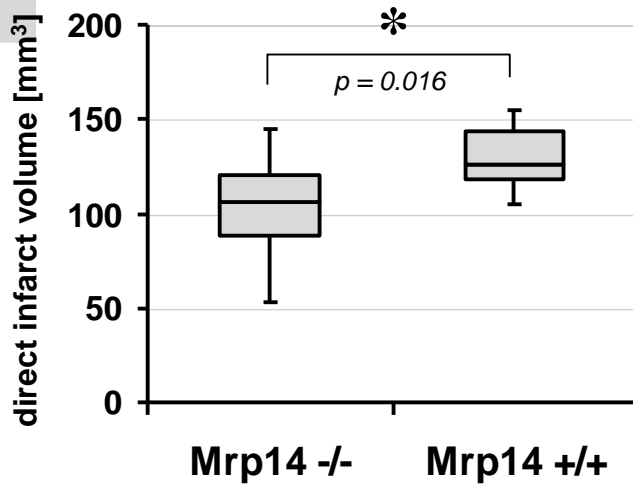
**Figure 3. Comparison of infarct volumes of Mrp14<sup>-/-</sup> and wildtype littermates (Mrp14<sup>+/+</sup>).** Direct infarct volume after 1h induction of cerebral ischemia at two days of reperfusion is significantly ( $p = 0.016$ ) smaller in Mrp14-knockout mice (**Mrp14<sup>-/-</sup>**) ( $n = 11$ ) when compared to wild-type littermates (**Mrp14<sup>+/+</sup>**) ( $n = 11$ ) (figure 3A). Infarct volume is visualized as box-and-whisker plots. Statistical analysis was performed using the *Mann-Whitney-U*-test. In contrast to the significant differences of the direct infarct volumes (figure 3A) and the calculated edema ( $p = 0.005$ ) (figure 3C), there was only a tendency without statistical significance of the indirect infarct volume (figure 3B). Indirect infarct volumes were calculated as the volume of the contralateral hemispheres minus the non-infarcted volumes of the ipsilateral hemispheres. Brain swelling was calculated as the direct minus the indirect infarct volumes. In all box plots, the top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the line in the middle represents the 50th percentile. The whiskers (the lines that extend out the top and bottom of the box) represent the highest and lowest values that are not outliers or extreme values. Outliers (values that are between 1.5 and 3 times the interquartile range) and extreme values (values that are more than 3 times the interquartile range) are represented by points.

**Figure 4. Comparison of inflammatory cell count in the ischemic hemisphere of Mrp14<sup>-/-</sup> and wildtype littermates (Mrp14<sup>+/+</sup>).** The number of cd11b-positive cells was determined by counting all cd11b-positive cells at interaural position No. III (distance to bregma 3.9 mm) in whole ischemic hemisphere of Mrp14-deficient, as well as Mrp14<sup>+/+</sup> control mice at 48 h after induction of cerebral ischemia (MCAO) for 60 min. There are significant ( $p = 0.003$ ) less infiltrating macrophages/microglia in the ischemic hemisphere of Mrp14-deficient mice when compared to the ischemic hemisphere of wild-type littermates (**Mrp14<sup>+/+</sup>**) ( $n = 4$ , respectively  $n = 5$ ) (Figure 4A). Whole cell counts in the ipsilateral hemispheres are visualized as box-and-whisker plots. Figure 4B: correlation between inflammatory cell counts (number of cd11b-positive cells in the ischemic hemisphere in Mrp14-deficient and Mrp14<sup>+/+</sup> mice / wild-type littermates) and indirect infarct sizes.



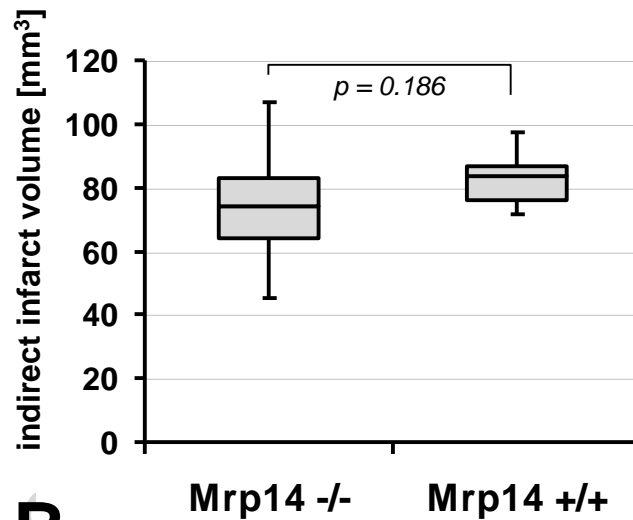






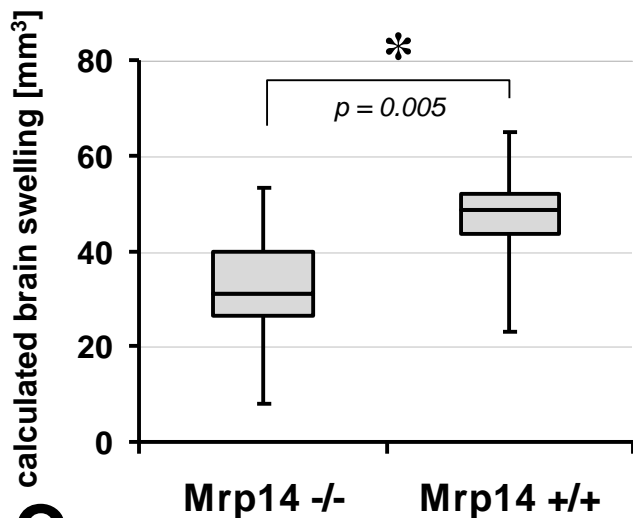
**A**

60 min MCAO / 48 h reperfusion



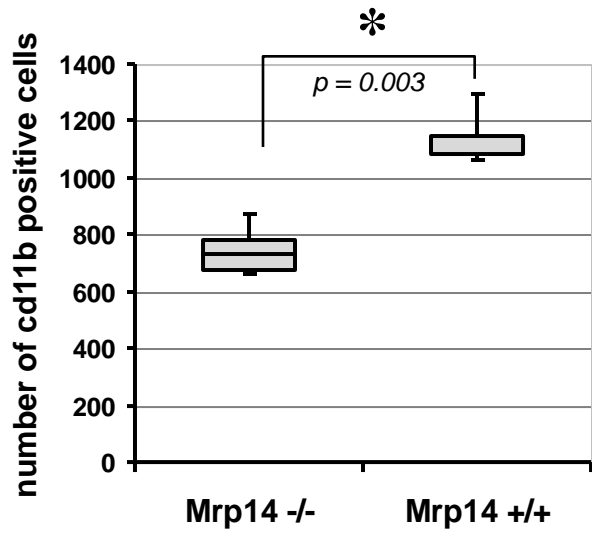
**B**

60 min MCAO / 48 h reperfusion

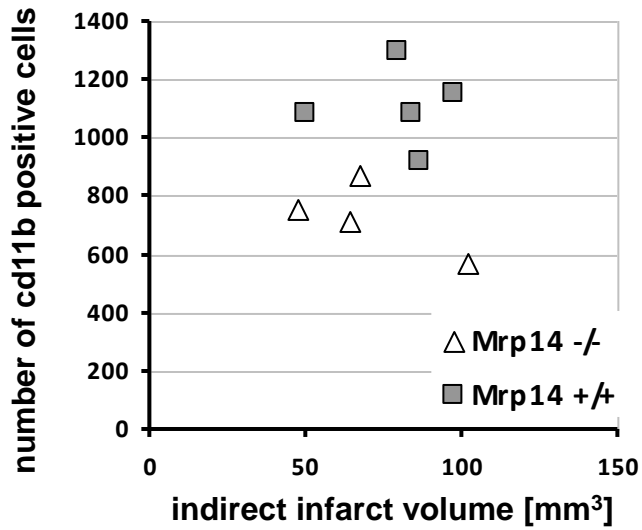


**C**

60 min MCAO / 48 h reperfusion



**A** 60 min MCAO / 48 h reperfusion



**B** 60 min MCAO / 48 h reperfusion