RESEARCH ARTICLE

Resident Microglia, Rather Than Blood-Derived Macrophages, Contribute to the Earlier and More Pronounced Inflammatory Reaction in the Immature Compared with the Adult Hippocampus After Hypoxia-Ischemia

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The mechanisms of neuronal injury after hypoxia–ischemia (HI) are different in the immature and the adult brain, but microglia activation has not been compared. The purpose of this study was to phenotype resident microglia and blood-derived macrophages in the hippocampus after HI in neonatal (postnatal day 9, P9) or adult (3 months of age, 3mo) mice. Unilateral brain injury after HI was induced in Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ male mice on P9 ($n = 34$) or at 3mo ($n = 53$) using the Vannucci model. Resident microglia (Cx3cr1-GFP+) proliferated and were activated earlier after HI in the P9 (1–3 days) than that in the 3mo hippocampus, but remained longer in the adult brain (3–7 days). Blood-derived macrophages (Ccr2-RFP+) peaked 3 days after HI in both immature (P9) and adult (3mo) hippocampi but were twice as frequent in adult brains, 41% vs. 21% of all microglia/macrophages. CCL2 expression was three times higher in the P9 hippocampi, indicating that the proinflammatory response was more pronounced in the immature brain after HI. This corresponded well with the higher numbers of galectin-3-positive resident microglia in the P9 hippocampi, but did not correlate with CD16/32- or CD206-positive resident microglia or blood-derived macrophages. In conclusion, resident microglia, rather than infiltrating blood-derived macrophages, proliferate and are activated earlier in the immature than in the adult brain, but remain increased longer in the adult brain. The inflammatory response is more pronounced in the immature brain, and this correlate well with galectin-3 expression in resident microglia.

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Key words: hypoxic-ischemic encephalopathy, CCL2, neuroinflammation, galectin-3

Introduction

There is increasing evidence showing that inflammation accounts for the progression of hypoxic–ischemic encephalopathy in neonates as well as stroke in adults (Vexler and Yenari, 2009; Wang et al., 2007). The inflammatory response after ischemia and reperfusion starts at the vascular level, driven by nontranscriptional events triggered by hypoxia, shear stress, and reactive oxygen species (ROS) production (Iadecola and Anrather, 2011). Resident microglia are among the first cells to respond to ischemic brain injury. Microglia exert neurotoxic functions through the production of ROS via NADPH oxidase, proinflammatory cytokines, and matrix metalloproteinase-9. These events precede leukocyte infiltration into the brain and may play a pivotal role in mediating the initial increase in blood-brain barrier (BBB) permeability and the early infiltration of circulating leukocytes into the brain (Jin et al., 2010).

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Monocytes can be divided into two subsets based on expression of Ly6C in mice. The Ly6C^{hi} subsets express chemokine receptor 2 (Ccr2) and low levels of CX3C-chemokine receptor 1 (Cx3cr1). Conversely, the Ly6C^{low} subsets lack Ccr2 but express high levels of Cx3cr1. The expression of Ccr2 and the capacity to migrate toward the Ccr2 ligand CC-chemokine ligand 2 (CCL2) is consistent with the role of this chemokine and its receptor in the recruitment of monocytes to inflammatory lesions, so the subset of monocytes that expresses Ccr2 is known as the 'inflammatory' subset (Gordon and Taylor, 2005). Within inflamed tissues, Ccr2^{+}Cx3cr1^{low} Ly6C^{+} monocytes differentiate into macrophages (blood-derived macrophages). In the absence of inflammation, Ccr2^{-}Cx3cr1^{hi-} Ly6C^{-} monocytes are postulated to enter the tissues and replenish the 'tissue-resident' macrophage, known as resident microglia in the brain (Gordon and Taylor, 2005). To distinguish resident microglia from blood-derived macrophages in the brain after injury, Saederup et al. generated red fluorescent protein (RFP)-Ccr2 knock-in mice (Ccr2$^{RFP/RFP}$) and crossed them with green fluorescent protein (GFP)-Cx3cr1 mice (Cx3cr1$^{GFP/GFP}$) (Saederup et al., 2010). This enabled them to discriminate between resident microglia and blood-derived macrophages in a model of experimental autoimmune encephalitis using Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ double transgenic mice.

Although inflammatory responses after ischemic stroke have been reported to be different in neonates compared with adults (Fernández-López et al., 2014; Vexler and Yenari, 2009), the relative contributions of resident microglia and blood-derived macrophages to the inflammatory response in the immature and mature hippocampus remain unclear. Our group previously demonstrated that apoptotic mechanisms of injury were activated to a greater extent in the cornu ammonis (CA) 1 and the dentate gyrus (DG) of the immature hippocampus, whereas excitotoxic-necrotic mechanisms were activated more in the adult hippocampal region, particularly the CA region (Zhu et al., 2005). In the present study, we investigated inflammatory responses to brain injury after HI in the mouse hippocampus on postnatal day 9 (P9) or at 3 months of age (3mo), focusing on the characterization of resident microglia and blood-derived macrophages using the same brain injury paradigm in Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ double transgenic mice.

Abbreviations

BBB	blood-brain barrier
Ccr2	chemokine receptor 2
CA	cornu ammonis
Cx3cr1	CX3C-chemokine receptor 1
CCL2	Ccr2 ligand CC-chemokine ligand 2
DG	dentate gyrus
GFP	green fluorescent protein
HI	hypoxia–ischemia
Iba1	ionized calcium binding adaptor molecule 1
RFP	red fluorescent protein
ROS	reactive oxygen species

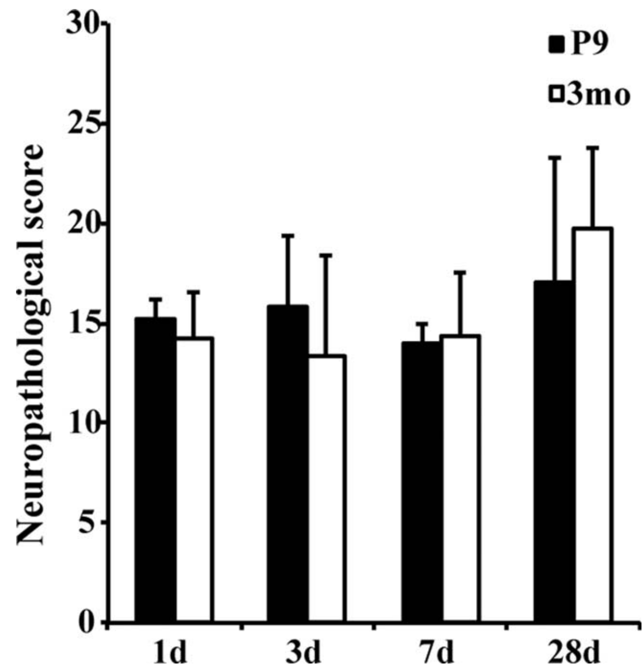


FIGURE 1: Brain injury after HI. The total neuropathological scores of Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ mice on P9 or at 3mo 1 day (1d: P9 pups, $n=4$; 3mo mice, $n=5$), 3 days (3d: P9 pups, $n=4$; 3mo mice, $n=3$), 7 days (7d: P9 pups, $n=3$; 3mo mice, $n=3$), and 28 days (28d: P9 pups, $n=3$; 3mo mice, $n=3$) after HI.

Materials and Methods

All animal experiments were approved by the local Animal Ethics Committee at Karolinska Institutet (Ethical approval no.N249/13).

Animals

To generate Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ double transgenic mice, Cx3cr1$^{GFP/GFP}$ and Ccr2$^{RFP/RFP}$ mice purchased from the Jackson Laboratory were crossed and first generation littermates were used. Cx3cr1$^{GFP/GFP}$ and Ccr2$^{RFP/RFP}$ mice are on a C57BL/6 genetic background. The presence of the transgenes in the offspring was confirmed by performing PCR. All mice were kept in a humidity-controlled room with a 12h light–dark cycle (lights on at 0700 h). Food and water were available *ad libitum*.

Induction of HI

Unilateral HI was induced in Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ male mice on P9 ($n=34$, mean body weight 5.0 ± 1.0 g) or at 3mo ($n=53$, mean body weight 27.0 ± 2.4 g) according to the Vannucci model, with some modification (Rice et al., 1981; Zhu et al., 2005). Eleven mice at 3mo were excluded from the study because of the absence of detectable brain injury. Animals were anesthetized with isoflurane and the right common carotid artery was ligated with a 6-0 silk suture (Ethicon Inc.). The animals were returned to the dam for 2 h (P9) or the home cage for 1h (3mo) and then placed in a chamber perfused with a humidified gas mixture (10% oxygen in nitrogen) for 50 min (P9) or 75 min (3mo) at 36°C to produce a similar extent of brain injury. Control P9 male pups ($n=3$) and 3mo male mice ($n=3$) were neither subjected to ligation nor hypoxia.

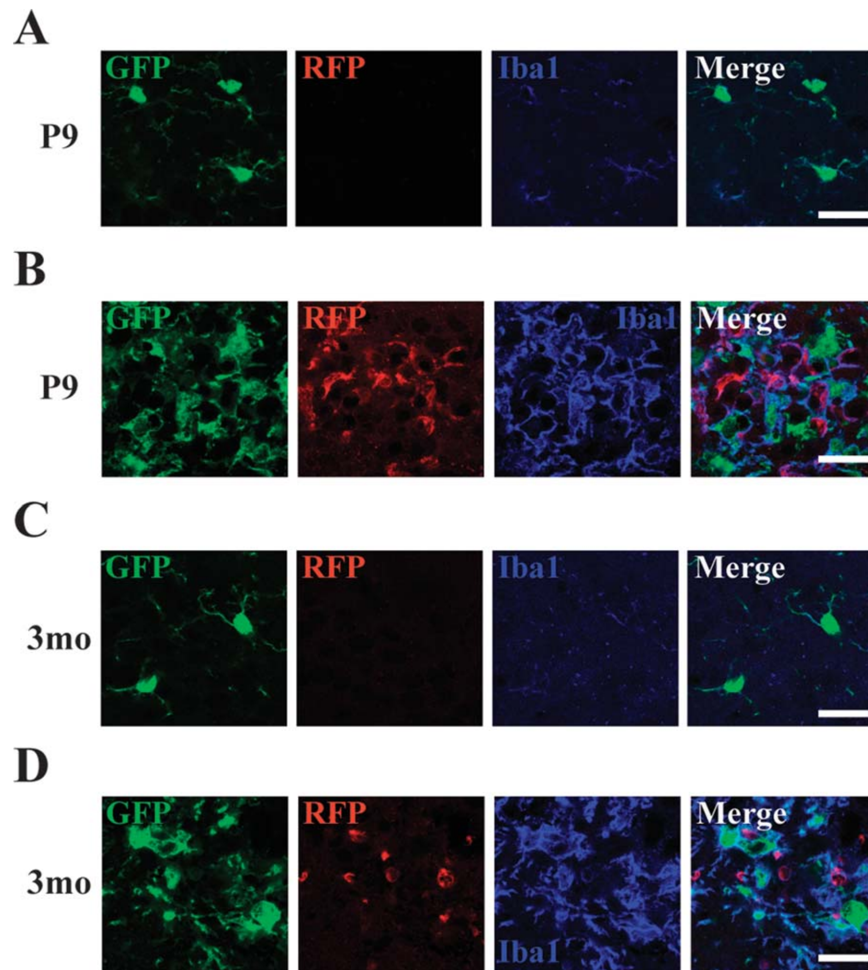


FIGURE 2: Iba1 expression in resident microglia and blood-derived macrophages. Representative images of GFP (green), RFP (red), and Iba1 (blue) staining in the hippocampus of the control (A and C) and 3 days after HI (B and D) in the P9 pups and the 3mo mice. Scale bars: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Tissue Preparation and Cutting

Animals were killed 1 day (1d: P9, n=4; 3mo, n=5), 3 days (3d: P9, n=4; 3mo, n=3), 7 days (7d: P9, n=3; 3mo, n=3), or 28 days (28d: P9, n=3; 3mo, n=3) after HI for histological analysis. Animals were anesthetized with 50 mg/kg sodium pentobarbital (APL, Stockholm) and perfusion-fixed with a 6% formaldehyde solution (Histofix, Histolab products AB, Gothenburg). The brains were immersion-fixed in the same fixative for 24 h at 4°C after perfusion and then soaked overnight in graded concentrations of sucrose solution (10%, 20%, and 30%). The right hemisphere was cut into 40 μm sagittal sections in a series of 10 using a sliding microtome (Leica SM2010R). The sections were stored in a cryoprotection solution at −20°C until staining.

Evaluation of Brain Injury

Brain injury in different regions was evaluated in cresyl violet-stained sections using a semiquantitative neuropathological scoring system as described earlier (Hagberg et al., 2004). The cortical injury was graded from 0 to 4, 0 being no observable injury and 4 confluent infarction encompassing most of the cerebral cortex. The damage in the hippocampus, striatum, and thalamus was assessed both with respect to hypotrophy (shrinkage) (0–3) and observable cell injury/infraction (0–3) resulting in a neuropathological score for each brain region (0–6). The total score (0–22) was the sum of the scores for all four regions.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Osman et al., 2014). To characterize resident microglia and blood-derived macrophages phenotype, sections were incubated at 4°C for 24 h with primary antibody rabbit anti-DsRed polyclonal antibody (1:1000; Clontech) in combination with either goat anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:500; Abcam), rat anti-galectin-3 (1:500; eBioscience), rat anti-CD16/32 (1:100; BD Biosciences), or goat anti-CD206 (1:100; R&D Systems), followed by an appropriate secondary antibody for 2 h at room temperature. Microglia proliferation was assessed by the expression of Ki67. Sections were incubated at 4°C for 24 h with primary antibody rabbit anti-Ki67 polyclonal antibody (1:500; Abcam), followed by an appropriate secondary antibody for 1 h at room temperature.

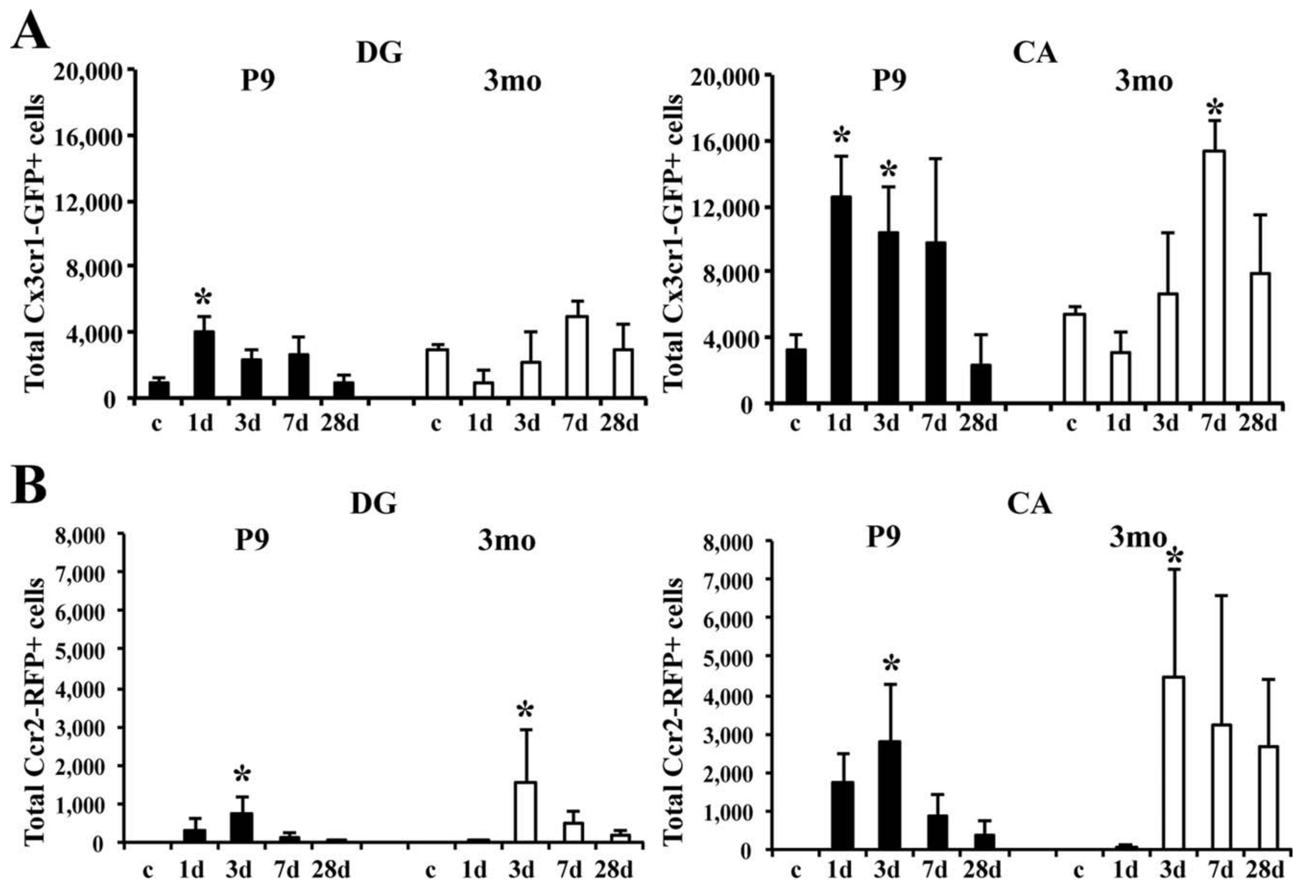


FIGURE 3: Quantification of resident microglia and blood-derived macrophages in the hippocampus after HI. The total number of Cx3cr1-GFP+ cells (A) and Ccr2-RFP+ cells (B) in the DG and the CA regions 1 day (1d: P9 pups, $n = 4$; 3mo mice, $n = 5$), 3 days (3 d: P9 pups, $n = 4$; 3mo mice, $n = 3$), 7 days (7d: P9 pups, $n = 3$; 3mo mice, $n = 3$), and 28 days (28d: P9 pups, $n = 3$; 3mo mice, $n = 3$) after HI. *$P < 0.05$: significant differences compared with the corresponding control group (c: $n = 3$ in each age group).

Cell Counting

The analysis of resident microglia and blood-derived macrophages was performed by quantifying Cx3cr1-GFP-positive (Cx3cr1-GFP+) and Ccr2-RFP-positive (Ccr2-RFP+) cells in the entire dorsal DG region of the hippocampus (the granule cell layer including the subgranular zone) and the entire dorsal CA region of the hippocampus (the pyramidal cell layer from CA1-3) (Fanselow and Dong, 2010; Qiu et al., 2007). For quantification of immunopositive cells in the DG and the CA regions, ten consecutive 0.5μm *z*-stack images of the sections were acquired using Axio Imager M2 microscope with Apotome attachment (Carl Zeiss) at 20× magnification and compressed in a single image (Kadam et al., 2015). All immunopositive cells were counted in three to four sections per animal spaced 400 μm apart. The total number of cells was obtained by multiplying the number of cells by the sampling fractions. To evaluate the proliferation of microglia, 100 Cx3cr1-GFP+ cells were quantified and examined for coexpression of Ki67.

Protein Extraction and CCL2 Assay

Animals were killed 1 day (P9, $n = 9$; 3mo, $n = 8$) after HI for CCL2 assay. Animals were deeply anesthetized with isoflurane and perfused with cold phosphate-buffered saline (pH 7.4) (Life technologies). Brains were removed and hemispheres were separated. Hippocampi were dissected from the right hemisphere and placed in a precooled 2.0 mL Eppendorf tube in dry ice. Ice-cold extraction buffer consisting of 50 mM Tris HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA (all from Sigma-Aldrich), and protease inhibitor cocktail (Completemini; Roche) was added, and the tissue was homogenized by sonication. Homogenates were centrifuged at 10,000*g* at 4°C for 10 min and the supernatants were collected. Protein concentration was determined using the BCA protein assay kit (Life technologies), and samples were aliquoted and stored at −20°C. CCL2 concentrations were determined using Quantikine Elisa kit (R&D systems). Samples were assayed in duplicates, and the assay was run according to the manufacturer instructions. Results were expressed as picogram/milligram protein (pg/mg protein).

Statistical Analysis

All values were presented as mean ± SD. All statistical tests were carried out using GraphPad Prism6 (GraphPad Inc.). Unpaired Student's *t* test and chi-square test were used when comparing two groups. One-way ANOVA followed by Dunnett's *post-hoc* test was used when comparing different time points after HI with the corresponding control value. Two-way ANOVA followed by Bonferroni's *post-hoc* test was used to analyze the neuropathological score. *P*-values < 0.05 were considered statistically significant.

Results

Brain Injury After HI

There were no significant differences in the mortality rates after induction of HI (P9, 32.4%; 3mo, 47.6%; $P = 0.18$) or the duration of anesthesia (P9, $n = 23$, 467 ± 93 sec; 3mo, $n = 22$, 426 ± 85 sec; $P = 0.14$) between the P9 pups and the 3mo mice. The total neuropathological scores were not different between the P9 pups and the 3mo mice for any time point after HI after titrating the duration of hypoxic exposure (Fig. 1).

Quantification of Resident Microglia and Blood-Derived Macrophages After HI in the Hippocampus

We first confirmed that Cx3cr1-GFP+ and Ccr2-RFP+ cells overlapped with Iba1 (Fig. 2). In the control group of both the P9 pups and the 3mo mice, Cx3cr1-GFP+ cells were evenly distributed throughout the hippocampus (Supp. Info. Fig. 1). The Cx3cr1-GFP+ cells increased 1 day after HI in the DG and the CA regions of the P9 pups, whereas they increased 7 days after HI in the CA region of the 3mo mice (Fig. 3A). The ratio of Cx3cr1-GFP+/Ki67+ cells increased 1 day after HI in the DG region of the P9 pups, whereas there were no significant changes in the ratios in the DG region of the 3mo mice (Fig. 4). In the CA region, the ratio of Cx3cr1-GFP+/Ki67+ cells peaked transiently 3 days after HI in the P9 pups, but they remained increased for at least 7 days in the 3mo mice (Fig. 4).

Ccr2-RFP+ cells were neither detected in the DG nor the CA regions in any of the controls (Supp. Info. Fig. 1). One day after HI, Ccr2-RFP+ cells appeared in the hippocampus of the P9 pups, whereas they were very few in the 3mo mice, even though degenerating cells were detected in cresyl violet-stained sections, particularly in the CA region (Figs. 3B and 5). After that, the Ccr2-RFP+ cells increased in the DG and the CA regions and peaked 3 days after HI in both the P9 pups and the 3mo mice, and then decreased gradually (Fig. 3B). The ratio of infiltrating Ccr2-RFP+ cells in the CA region was $21.0\% \pm 6.2\%$ in the P9 pups and $41.3\% \pm 27.9\%$ in the 3mo mice.

Quantification of Activated Resident Microglia and Blood-Derived Macrophages After HI in the Hippocampus

Cx3cr1-GFP and galectin-3 double-positive (Cx3cr1-GFP+/galectin-3+) cells were very few in the control hippocampus of both the P9 pups and the 3mo mice (Fig. 6B,C). Cx3cr1-GFP+/galectin-3+ cells were increased 1 day and 3 days after HI in the P9 pups, whereas they reached a peak later in the 3mo mice (Fig. 6B). Importantly, the number of Cx3cr1-GFP+/galectin-3+ double positive cells was much higher in the immature than in the adult hippocampi, more than nine-fold higher in the immature brains ($3{,}193.4 \pm 827.5$ vs. 341.1 ± 464.6, respectively), constituting $25.2\% \pm 2.7\%$ of all Cx3cr1-GFP+ cells in the immature, but only $8.8\% \pm 8.9\%$ in the adult brains 1 day after HI in the CA region (Fig. 6B).

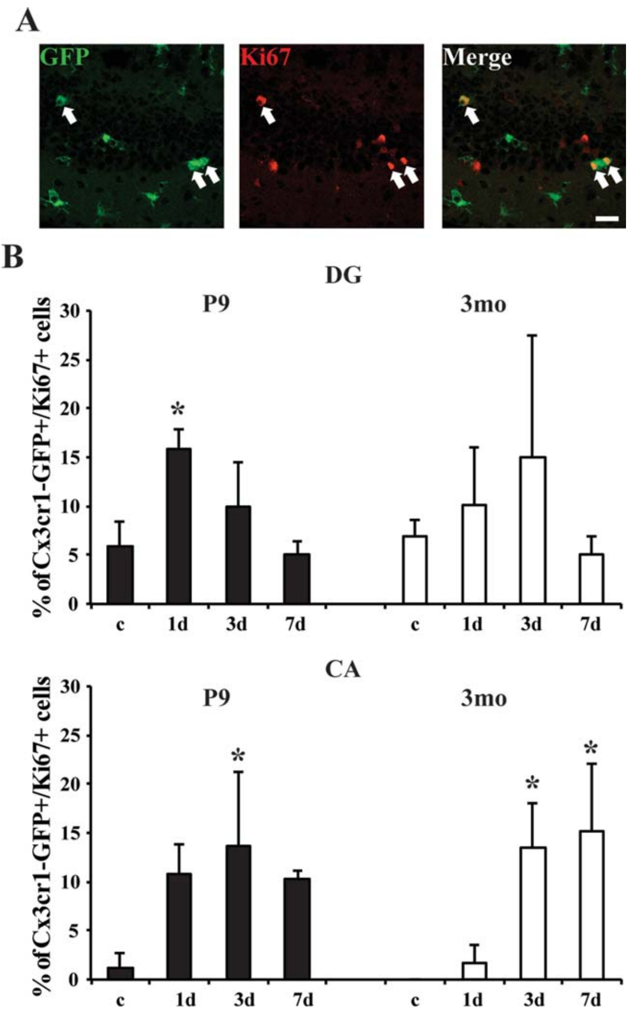


FIGURE 4: Ki67 expression in resident microglia after HI. Representative images of Cx3cr1-GFP+/Ki67+ cells (white arrows) in the hippocampus (A). Scale bar: 20 µm. The percentage of Cx3cr1-GFP+/Ki67+ cells in the DG and the CA regions 1 day (1d: P9 pups, $n = 4$; 3mo mice, $n = 5$), 3 days (3d: P9 pups, $n = 4$; 3mo mice, $n = 3$), and 7 days (7d: P9 pups, $n = 3$; 3mo mice, $n = 3$) after HI (B). *$P < 0.05$: significant differences compared with the corresponding control group (c: $n = 3$ in each age group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Ccr2-RFP and galectin-3 double-positive (Ccr2-RFP+/galectin-3+) cells in the DG and the CA regions increased transiently 3 days after HI in both the P9 pups and the 3mo mice (Fig. 6C). In the adult mice, the increase was more pronounced, but the variation was considerable (Fig. 6C).

The CCL2 Concentration in the Hippocampus One Day After HI

The concentration of the proinflammatory chemokine CCL2 was approximately three times higher in the ipsilateral

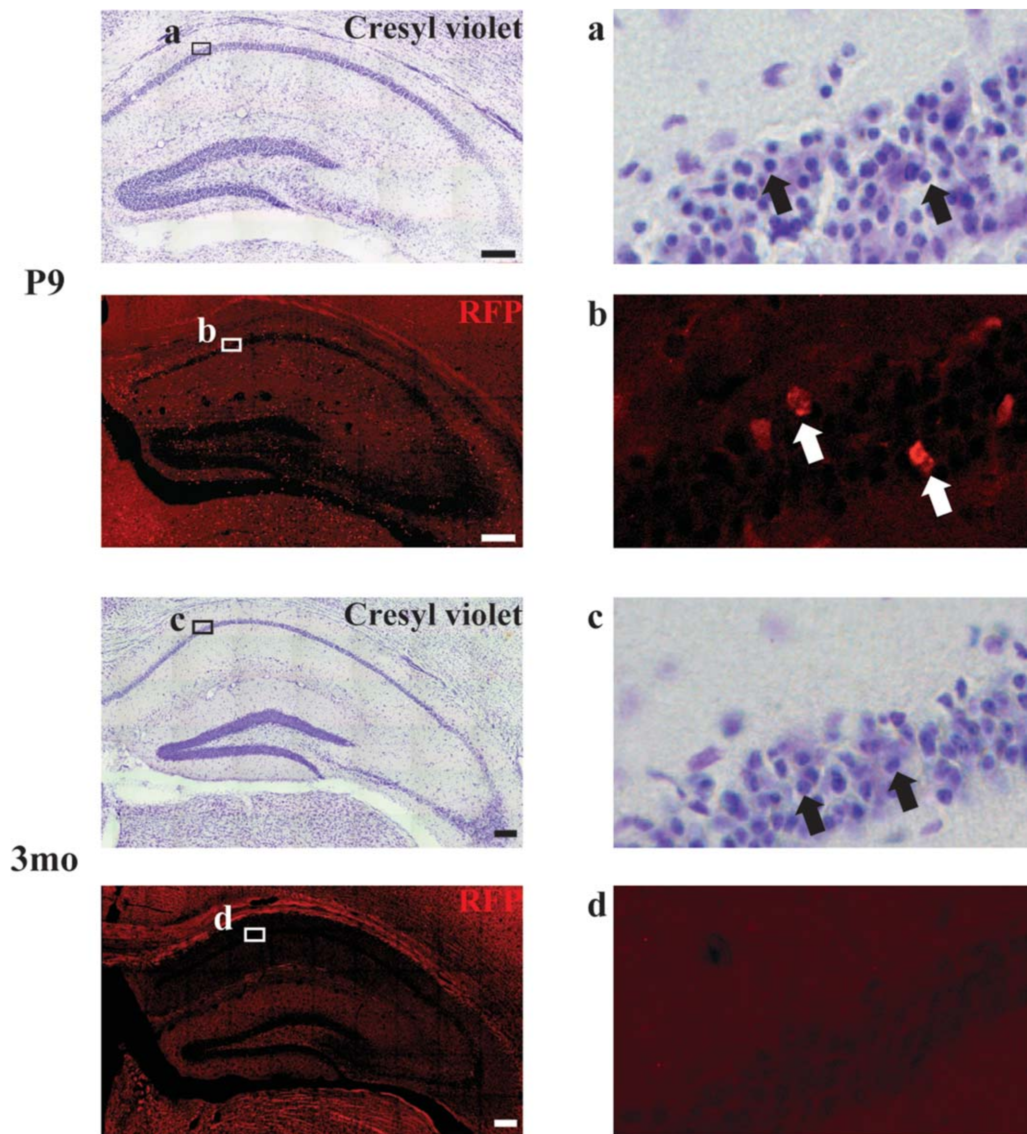


FIGURE 5: Blood-derived macrophages infiltration in the hippocampus 1 day after HI. Representative images of cresyl violet and RFP (red) staining in the ipsilateral hippocampus 1 day after HI in the P9 pups and the 3mo mice. Scale bars: 200 µm. The areas in the small rectangles are shown at higher magnification in the right column (a–d). Black arrows and white arrows indicate degenerating cells and Ccr2-RFP+ cells, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hippocampus of the P9 pups compared with the 3mo mice 1 day after HI (377.6 ± 231.9 and 140.9 ± 193.2 pg/mg protein, respectively) (Fig. 7).

Expression of Cd16/32 and Cd206 in Resident Microglia and Blood-Derived Macrophages After HI

In the P9 pups, Cx3cr1-GFP and CD16/32 double-positive (Cx3cr1-GFP+/CD16/32+) cells briefly increased 3 days after HI. In contrast, in the 3mo mice, the Cx3cr1-GFP+/CD16/32+ cells increased 3 days and remained at the same level at least up to 7 days after HI (Figs. 8 and 9A). Cx3cr1-GFP and CD206 double-positive (Cx3cr1-GFP+/CD206+) cells increased transiently 3 days after HI in the DG region of both the P9 pups and the 3mo mice (Figs. 8 and 9B). Also in the CA region the Cx3cr1-GFP+/CD206+ cells peaked transiently 3 days after HI in the P9 pups, but they remained increased for at least 7 days in the 3mo mice (Figs. 8 and 9B).

Ccr2-RFP and CD16/32 double positive (Ccr2-RFP+/CD16/32+) cells in the DG region increased 3 days after HI in both the P9 pups and the 3mo mice (Figs. 7 and 8C). Ccr2-RFP+/CD16/32+ cells were detected in the CA region of the P9 pups, but there were no significant changes (Figs. 8 and 9C). They increased in the CA region of the 3mo mice 3 days after HI (Figs. 8 and 9C). Ccr2-RFP and CD206 double positive (Ccr2-RFP+/CD206+) cells in the DG region transiently increased 3 days after HI in both the P9 pups and the 3mo mice (Figs. 8 and 9D). There were no

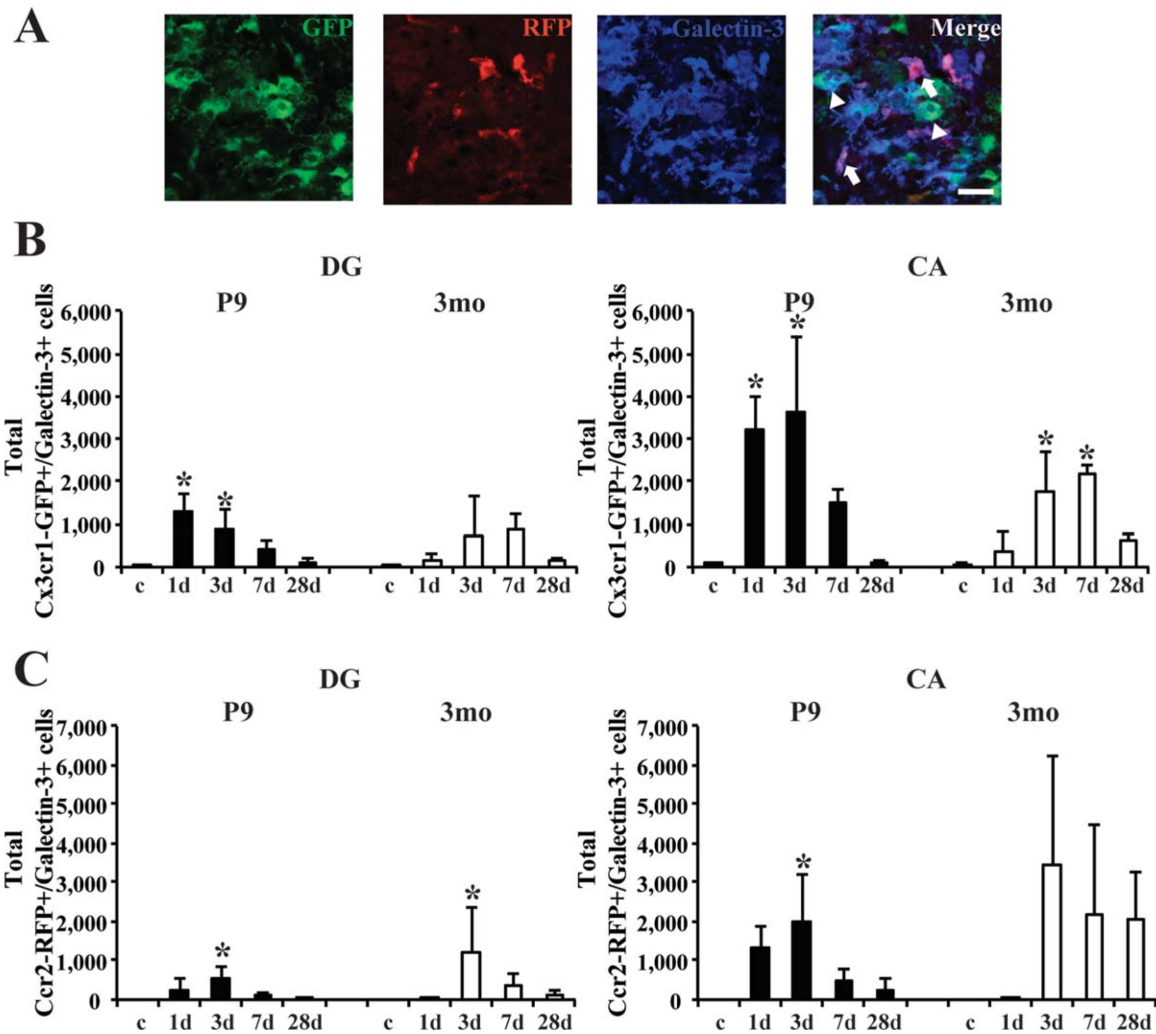


FIGURE 6: Galectin-3 expression in resident microglia and blood-derived macrophages after HI. Representative images of GFP (green), RFP (red), and galectin-3 (blue) staining in the hippocampus, where double-positive cells are indicated by white arrow heads (Cx3cr1-GFP+/galectin-3+ cells) and white arrows (Ccr2-RFP+/galectin-3+) cells) (**A**). Scale bars: 20 µm. The total number of Cx3cr1-GFP+/galectin-3+ cells (**B**) and Ccr2-RFP+/galectin-3+ cells (**C**) in the DG and the CA regions 1 day (1d: P9 pups, $n = 4$; 3mo mice, $n = 5$), 3 days (3d: P9 pups, $n = 4$; 3mo mice, $n = 3$), 7 days (7d: P9 pups, $n = 3$; 3mo mice, $n = 3$), and 28 days (28d: P9 pups, $n = 3$; 3mo mice, $n = 3$) after HI. *$P < 0.05$: significant differences compared with the corresponding control group (c: $n = 3$ in each age group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significant changes in Ccr2-RFP+/CD206+ cells in the CA region of the P9 pups, whereas those cells in the same region of the 3mo mice increased 3 days after HI (Figs. 8 and 9D).

Discussion

This is the first report, to our knowledge, demonstrating age-dependent differences in the activation of resident microglia and infiltrating blood-derived macrophages in the hippocampus after hypoxic–ischemic brain injury. To distinguish resident microglia and blood-derived macrophages in ischemic brain injury, several studies have been performed using transplanted bone marrow cells from a mouse ubiquitously expressing GFP under the control of a beta-actin promoter after depleting the intrinsic monocyte population by irradiation (Schilling et al., 2003; Tanaka et al., 2003). A disadvantage with this approach is the possibility that the irradiation used to deplete the endogenous bone marrow may affect the BBB and augment cell infiltration (Hanisch and Kettenmann, 2007). To circumvent this, a recent report employed cranium-shielded irradiation in bone marrow chimera experiments when addressing the role of blood-derived macrophages in ischemic stroke, concluding that blood-derived macrophages recruited via Ccr2 are essential for maintaining integrity of the neurovascular unit following brain ischemia (Gliem et al., 2012), at least in adult mice. However, Hanisch et al. mentioned that myeloid precursors may be artificially mobilized into circulation by transplantation of bone marrow, which is not physiological (Hanisch

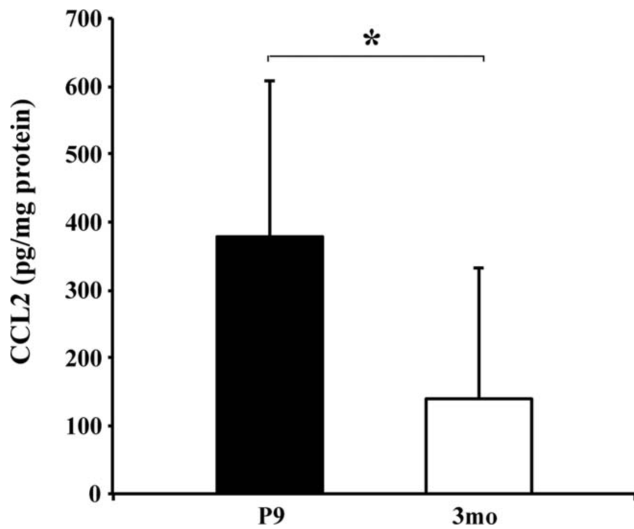
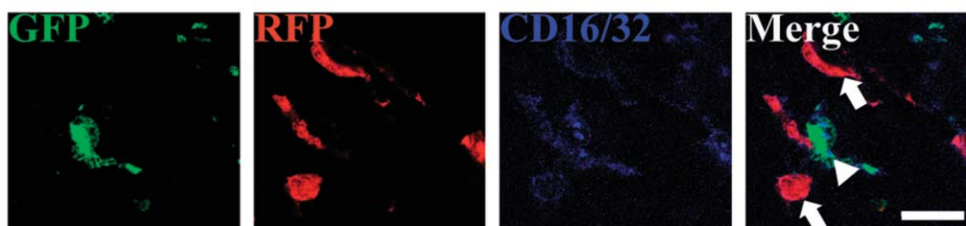


FIGURE 7: The inflammatory response in the hippocampus 1 day after HI. The CCL2 concentrations in the ipsilateral hippocampus 1 day after HI (P9 pups, $n = 9$; 3mo mice, $n = 8$). *$P < 0.05$: significant differences compared with the 3mo mice.

and Kettenmann, 2007). Dénes et al. demonstrated that there were no significant changes in the ischemic brain damage between Cx3cr1$^{GFP/+}$ and wild type mice, and Saederup et al. showed that monocyte/macrophage recruitment after thioglycollate-induced peritonitis was not significantly changed between Cx3cr1$^{GFP/+}$Ccr2$^{RFP/+}$ and wild type mice (Dénes et al., 2008; Saederup et al., 2010). Moreover, we showed that Cx3cr1-GFP+ and Ccr2-RFP+ cells overlapped completely with Iba1, a marker expressed in both resident microglia and blood-derived macrophages, as reported previously (Cardona et al., 2006). Thus, we believe that this strain of mice can be used to investigate the contribution of resident microglia and blood-derived macrophages after HI without influence of the transgene expression.

Ivacko et al. showed that B4-isolectin-positive cells, a population constituting resident microglia as well as blood-derived macrophages, infiltrated the DG region and the pyramidal cell layer 24 hours after HI in P7 rats, and suggested that HI-induced macrophage accumulation in the immature brain proceeded more rapidly than that in the mature brain (Ivacko et al., 1996). This is consistent with our findings, but we could further identify the resident microglia as primarily responsible for this difference between the immature and the adult brain. Galectin-3 has been reported to be produced by activated microglia/macrophages after HI and to be required for resident microglia activation response to HI (Burguillos et al., 2015; Lalancette-Hébert et al., 2012; Yan et al., 2009). We have previously shown that galectin-3-positive microglia is detectable only in areas with overt brain injury (Li et al., 2011). In the present study, we showed that CCL2 expression in the ipsilateral hippocampus of the P9 pups was higher than that of the 3mo mice after HI, indicating that the inflammatory response was more pronounced in the immature brain. Importantly, we demonstrate that galectin-3 expression was higher in the immature brain and that this expression correlated well with the more pronounced proinflammatory response in young animals, consistent with our finding that galectin-3 released from microglia cells can activate other surrounding immune cells (Burguillos et al., 2015). Our data reveal that resident microglia in the P9 pups



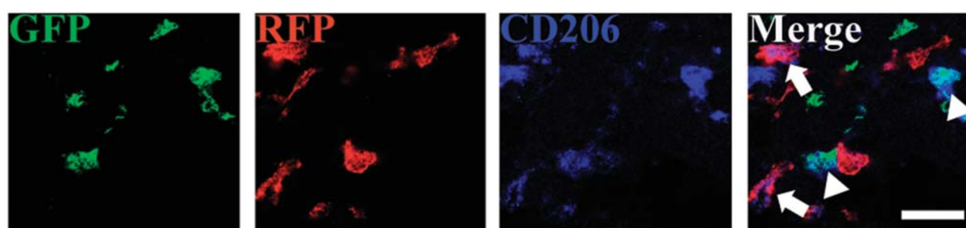


FIGURE 8: CD16/32 and CD206 expression in resident microglia and blood-derived macrophages after HI. Representative images of GFP (green), RFP (red), and CD16/32 (A)/CD206 (B) (blue) staining in the hippocampus, where double-positive cells are indicated by white arrow heads [Cx3cr1-GFP+/CD16/32+ cells (A) and Cx3cr1-GFP+/CD206+ cells (B)] and white arrow [Ccr2-RFP+/CD16/32+ cells (A), and Ccr2-RFP+/CD206+ cells (B)]. Scale bars: 20 μm. [Color figure can be viewed in the online issue, which is available at wiley onlinelibrary.com.]

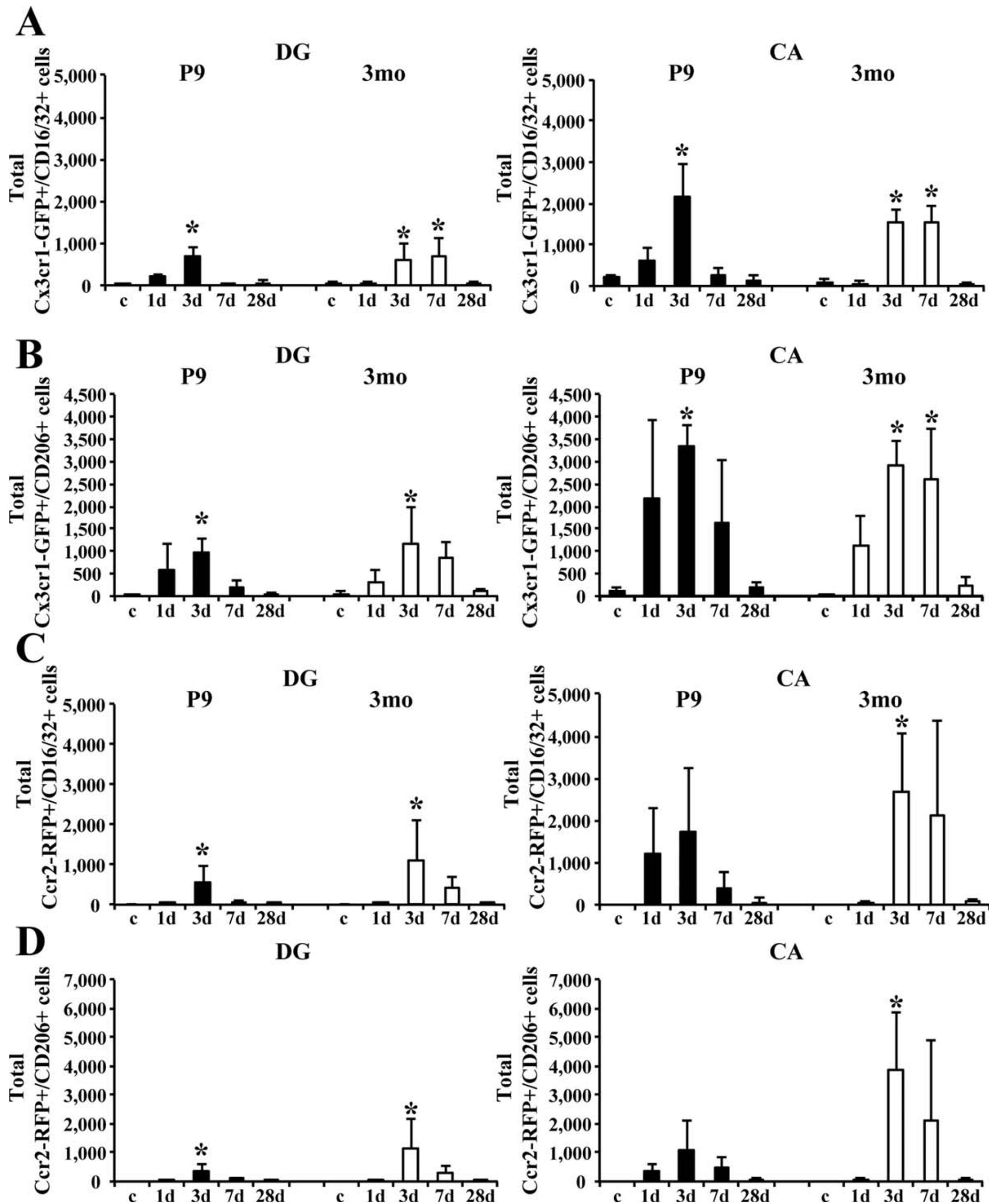


FIGURE 9: Quantification of CD16/32 and CD206 expression in resident microglia and blood-derived macrophages after HI. The total number of Cx3cr1-GFP+/CD16/32+ cells (**A**), Cx3cr1-GFP+/CD206+ cells (**B**), Ccr2-RFP+/CD16/32+ cells (**C**), and Ccr2-RFP+/CD206+ cells (**D**) in the DG and the CA regions 1 day (1d: P9 pups, $n=4$; 3mo mice, $n=5$), 3 days (3d: P9 pups, $n=4$; 3mo mice, $n=3$), 7 days (7d: P9 pups, $n=3$; 3mo mice, $n=3$), and 28 days (28d: P9 pups, $n=3$; 3mo mice, $n=3$) after HI. $^*P<0.05$: significant differences compared with the corresponding control group (c: $n=3$ in each age group).

proliferated and were activated earlier in the DG and the CA regions after HI earlier than that in the 3mo mice, and that activated microglia in the 3mo hippocampus increased progressively after the early stage of HI brain injury, consistent with the report by Perego et al (Perego et al., 2011). We previously showed that microglia/macrophage proliferation from 1 day to 7 days after HI was higher after HI in the juvenile (P21) than in the immature (P9) hippocampus (Qiu et al., 2007). When taking into account the results of the ratios of Cx3cr1-GFP+/Ki67+ cells, we can safely presume

that the increases in resident microglia proliferation account for the early accumulation of resident microglia in the DG region of the P9 pups, and that proliferation of resident microglia play a part also in the accumulation of these cells in the CA region of the P9 pups 3 days after HI. In the 3mo mice, our data are consistent with the report that resident microglia, but not Ccr2-positive infiltrating cells, frequently proliferated between 3 and 7 days after stroke in adult mice (Li et al., 2013). Moreover, we showed that blood-derived macrophages began infiltrating the hippocampus in the P9 pups 1 day after HI, but few blood-derived macrophages infiltrated the hippocampus of the 3mo mice at the same time point. Denker et al. reported that the population of blood-derived macrophages ($CD45^{high}$ macrophages) in the injured hemisphere 24 hours after HI of P7 rats was $8.8 \pm 6.7\%$ and the majority of macrophages were resident microglia ($CD45^{low}$ and $CD45^{med}$ macrophages) as judged by flow cytometry (Denker et al., 2007), which is consistent with our results. CCL2 has been reported to increase BBB permeability *in vivo* (Stamatovic et al., 2005). Muramatsu et al. suggested that the BBB in P7 rats is more vulnerable to HI than that in P21 rats (Muramatsu et al., 1997). This possibly explains, at least partly, our findings that HI induces accumulation and activation of resident microglia as well as infiltration of blood-derived macrophages earlier in the immature hippocampus than that in the mature hippocampus. However, there is a conflicting report demonstrating that the immature BBB is more resistant to HI brain damage than its adult counterpart (Fernández-López et al., 2012), so further studies are needed to resolve this issue.

We characterized the phenotype of resident microglia and blood-derived macrophages after HI using CD16/32 and CD206 as proposed, representative M1 (proinflammatory), and M2 (anti-inflammatory) macrophage markers (Biber et al., 2014). In the 3mo hippocampus, resident microglia expressed M1 or M2 macrophage markers 3 days and 7 days after HI, whereas blood-derived macrophages transiently expressed these markers only 3 days after HI. Hu et al. examined the polarization of microglia/macrophages after HI using adult rats (Hu et al., 2012). They showed that expression of the M2 macrophage marker CD206 increased transiently at the early stage of brain injury after HI, but expression of the M1 macrophage marker CD16/32 increased over time in the cortex and striatum of adult rats. Taken together, this indicates that both resident microglia and blood-derived macrophages contribute to the expression of M1 and M2 phenotypes at the early stage of brain injury after HI, but that mainly resident microglia contribute to the expression of M1 and M2 phenotypes later during recovery after HI in the 3mo hippocampus, which was different from the pattern observed in the P9 hippocampus. Based on this study and previous reports, we found that the observed expression of galectin-3, rather than the differences in the time-course of the two M1 and M2 phenotype macrophage markers after HI between the immature and the mature hippocampus, correlated with the age-dependent effects of HI (Fernández-López et al., 2014). However, a single M1 or M2 phenotype marker does not classify individual microglia as pro- or anti-inflammatory, rather, the combined expression of multiple markers produce a continuous spectrum of functional heterogeneity (Biber et al., 2014), and more comprehensive approaches are needed to more accurately phenotype individual microglia.

In summary, there were obvious differences in the responses of resident microglia and blood-derived macrophages to brain injury after HI between the immature and the mature hippocampus. Our results indicate that resident microglia, rather than infiltrating blood-derived macrophages, proliferate and are activated earlier in the immature than in the adult brain, but remain increased longer in the adult brain and contribute to the more pronounced inflammation in the immature brain. These age-dependent differences correlate better with galectin-3 expression than with the M1 (CD16/32) and M2 (CD206) phenotype macrophage markers in the hippocampus after HI.

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