Ischemia-Induced Neuroinflammation Is Associated with Disrupted Development of Oligodendrocyte Progenitors in a Model of Periventricular Leukomalacia

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Abstract
Microglial activation in crossing white matter tracts is a hallmark of noncystic periventricular leukomalacia (PVL), the leading pathology underlying cerebral palsy in prematurely born infants. Recent studies indicate that neuroinflammation within an early time window can produce long-lasting defects in oligodendroglial maturation, myelination deficit, as well as disruption of transcription factors important in oligodendroglial maturation. We recently reported an ischemic mouse model of PVL, induced by unilateral neonatal carotid artery ligation, leading to selective long-lasting bilateral myelination deficits, ipsilateral thinning of the corpus callosum, ventriculomegaly, as well as evidence of axonopathy. Here, we report that permanent unilateral carotid ligation on postnatal day 5 in CD-1 mice induces an inflammatory response, as defined by microglial activation and recruitment, as well as significant changes in cytokine expression (increased IL-1β, IL-6, TGF-β1, and TNF-α) following ischemia. Transient reduction in counts of oligodendrocyte progenitor cells (OPCs) at 24 and 48 h after ischemia, a shift in OPC cell size and morphology towards the more immature form, as well as likely migration of OPCs were found. These OPC changes were topographically associated with areas showing microglial activation, and OPC counts negatively correlated with increased microglial staining. The presented data show a striking neuroinflammatory response in an ischemia-induced model of PVL, associated with oligodendroglial injury. Future studies modulating the neuroinflammatory response in this model may contribute to a better understanding of the interaction between microglia and OPCs in PVL and open opportunities for future therapies.

Introduction

Extreme prematurity, defined by a gestational age of ≤28 weeks or a birth weight of <1,500 g, affects up to 2% of all newborns in the United States [1]. A high incidence of adverse neurologic outcomes in this group calls for

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intensified research in neuroprotective options [2, 3]. Up to 20% of these infants develop cerebral palsy [4] and about 50% will develop cognitive, behavioral, attention, or socialization deficits of variable degree [5–7].

The main underlying neuropathology in this population is perinatal white matter injury (PWMI), also known as periventricular leukomalacia (PVL), which is defined as dysmyelination, neuroaxonal injury, and astrogliosis, as well as microglial activation [3, 8, 9]. The etiology of PWMI is thought to be multifactorial and encompasses perinatal infectious processes, systemic and neuroinflammation, ischemia-reperfusion, hypoxia and excitotoxicity, combined with developmental vulnerability of cellular structures, especially late oligodendrocyte progenitors and immature oligodendrocytes [5, 10–12].

Epidemiological studies strongly suggest that inflammation is associated with PWMI. Furthermore, it is postulated that active inflammatory processes may prevent regeneration and/or exacerbate brain damage in the premature infant and sensitize the brain to further injury [13–15]. While adequate glial response to injury is essential to repair [16] and regeneration, studies in radiation injury [17] and traumatic brain injury [18–21] show long-lasting microglial activation and increased expression of inflammatory cytokines for several months to years. Interestingly, a study of 7-year-old children with cerebral palsy born prematurely showed elevated basal levels of TNF-α in the plasma and in supernatants of peripheral blood mononuclear cells, supporting the notion of a prolonged inflammatory response in cerebral palsy [22]. This concept broadens the possible therapeutic window in cerebral palsy, and blocking such persistent inflammation could be of therapeutic value [23].

It has been shown that during prenatal brain development in humans, clusters of microglia are located at axonal crossroads (at crossing fibers between the internal capsule and thalamus, cingulum and corpus callosum and other regions), at sites vulnerable to PWMI in very preterm babies during time periods critical for PWMI [8, 24]. Microglial activation could be deleterious or neuroprotective, depending on the timing in relation to an initial insult, the lesion type, and the lesion location [25], and may be triggered by excitotoxic, inflammatory, hypoxic, or hypoxic-ischemic events [26–29]. Also, in normal rodents, clusters of activated microglia were detected transiently within white matter tracts (cingulum, internal and external capsules) during early postnatal development, resembling the distribution found in the normal human brain [8, 28–30].

Late oligodendrocyte progenitor cells (OPCs) exhibit selective vulnerability in PWMI [11, 31], and it has been shown that activated microglia can have deleterious effects on OPC survival and also inhibit maturation of these progenitors into myelin-producing oligodendrocytes in vitro [32–35]. Microglia can be a source of many inflammatory mediators, including cytokines such as TNF-α, chemokines, reactive oxygen/nitrogen species, as well as glutamate, which all have been demonstrated to be detrimental to oligodendroglial development in cell culture [3, 8]. Expression of the interleukins IL-1β, IL-2, and IL-6, and of TNF-α has been demonstrated in microglia in neonates with PWMI [36–38].

In terms of oligodendrocyte maturation, the development of the newborn rodent brain is equivalent to the human fetal brain at 6–7 months of gestation. Late OPCs prevail between postnatal day 2 (P2) and P7 in rodents, and at gestational weeks 23–32 in humans [39]. Based on this pattern of development, we recently reported a mouse model of PWMI [40] by performing permanent unilateral carotid artery ligation on P5 (day of birth defined as P1) in CD-1 mice and found selective vulnerability of the white matter with myelin pallor, reduced mature oligodendrocyte counts, as well as evidence of axonal injury in the corpus callosum, similar to reports from autopsy cases of PVL [41].

We then hypothesized that ischemia in our model elicits an inflammatory response, consisting of cytokines, chemokines and adhesion molecules, as well as active inflammatory cells, especially microglia. Because of the developmental migration pattern and clusters of microglia within the white matter, we further hypothesized that neuroinflammatory effects on the oligodendroglial lineage could be observed by correlating temporospatial patterns of microglia and OPCs.

The objectives of this study were (1) to quantify the temporospatial response of microglia within the white matter in our P5 ischemic model of PWMI and their spatial relationship to OPCs; (2) to quantify the cytokine response relevant to PWMI within the white matter; (3) to quantify and categorize OPC cell morphology and proliferation, and the temporospatial pattern of change following injury, and (4) to evaluate the spatial correlation between white matter areas with microglial clusters and OPC death.

Materials and Methods

Animals

This study was approved by the Johns Hopkins Animal Care and Use Committee (protocol No. MO09M422). In all, 21 CD-1 litters of 9–11 pups each were purchased from Charles River Laboratories (Wilmington, Mass., USA) at P3. The day of birth was defined as P1.
Carotid Artery Ligation

On P5, pups were placed in an incubator at 35°C for 15–30 min and were then anesthetized with isoflurane (4% induction, 1–1.2% maintenance), and the right common carotid artery was ligated (right hemisphere referred to as ipsilateral, left hemisphere as contralateral). Pups recovered at 36°C for 30–60 min and were returned to the dam. Rectal temperatures were 36 ± 0.5°C before surgery and 34.5 ± 1°C postoperatively; surgery time was standardized to 12–15 min.

Histology

A subset of animals ligated on P5 were examined at 24 h (P6, n = 10), 48 h (P7, n = 8) and 16 days (P21, n = 8) after ligation. They were compared to P6 (n = 9), P7 (n = 8) and P21 (n = 8) naïve controls. Mice were anesthetized and perfused with phosphate-buffered saline, followed by 4% paraformaldehyde in phosphate buffer for 12–18 h, and the brains were cryoprotected in sucrose and cryostat-sectioned at 40 μm. Sections were mounted onto 10 slides in parallel, each slide having sections with 400 μm displacement. All sections and images were identified using a standardized numbering system, so that antigen expression in adjacent sections of the same animal stained with different antibodies could be correlated. Slides were incubated in blocking solution, followed by primary antibody incubation at 4°C: anti-platelet-derived growth factor receptor-α antibody (PDGFα; BD Biosciences, San Jose, Calif., USA, 1:500) for detection of OPCs, anti-ionized calcium-binding adaptor molecule 1 antibody (Iba-1; Wako, Richmond, Va., USA, 1:2,000) for detection of microglia, anti-Ki67 (Abcam, Mass., USA, 1:500) for visualization of proliferation and anti-CD68 (Abcam, Mass., USA, 1:100) for detection of microglia during developmental migration. Fluorescent secondary antibodies were used to detect anti-PDGFα, anti-Ki67 and anti-CD68, and all sections were counterstained with DAPI. The Iba-1 antigen-antibody complex was visualized using an ABC ELITE kit (Vector Labs, Burlingame, Calif., USA). Adjacent sections were stained using Nissl to aid in outlining anatomic structures.

To investigate the relationship between microglia and OPCs in our injury model, microglia were evaluated using Iba-1 immunohistochemistry and their presence quantified. Since exact counting of migrating microglia within the corpus callosum proved to be difficult, we decided to quantify the fractional area stained [referred to as proportional target area (PTA)] as a means of quantitative measurement of microglial activation (described below). Corresponding sections were stained for OPC presence (PDGFα), as well as proliferation (Ki67). In the OPC population, we quantified cell count, cell size, as well as morphology, while differentiating proliferating (Ki67+) from nonproliferating OPCs.

OPC Cell Count and Morphology Quantification

Fluorescent images were acquired using the Zeiss Axioskop M5 microscope with ApoTome functionality, using structured illumination to optimize resolution. Using this technique, the total exposure of the specimen is slightly greater because the grid projection is typically not completely opaque. However, the resolution is comparable to that achieved by either confocal or deconvolution techniques. To reduce bias due to bleaching of the fluorescent antibody, all images used for analysis were acquired the first time the slides were illuminated under the microscope. Mosaic settings, Z stack settings, and anatomical regions were defined and set in the microscope software prior to acquisition to reduce illumination time and thereby bleaching, using the robotic microscope stage.

Fig. 1. Microglial distribution and morphology in developing white matter in controls and after P5 carotid artery ligation. Iba-1 immunohistochemical staining of microglia is shown at the level of the anterior commissure (a–f) and at the level of the rostral hippocampus (g–n). Twenty-four to 48 h after ligation (P6–P7), there is a notable increase in microglial staining in several regions: Mid-CC, clusters within the SVWM, the ASVZ-r, and the EC/PSVZ-r. CD68+ cells were found in controls (o, p) and ligated mice (not shown) in clusters within the SVWM at anterior levels (o) and in the EC/PSVZ-r at posterior levels (p) on P6–P7 but not on P21, indicating that these regions are migrational zones for invading monophagocytic cells in the developing brain [8, 30]. Dashed lines indicate the areas examined for quantitative analysis. The asterisk indicates the location of higher magnification inset. HIPP = Hippocampus. Scale bar = 50 μm for a–o, 10 μm for p.
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Control

Ligated at P5

P6

P7

P21

P7

Con

Lig at P5

CD68

Con P7

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Fluorescent channels switched automatically and were acquired sequentially for each field of view, in the same order for each sample. Regions to be acquired were selected based on the DAPI staining. All images and channels were postprocessed in the Zeiss Axiovision software following standardized protocols for optimal quantification. For verification of correct regional identification, overview mosaic images of the DAPI channel of each analyzed brain section were acquired using a ×10 microscope objective.

To understand the pattern of OPC cell damage in ligated animals, we outlined the PDGFRα+ cells and measured the cell length/height ratio as well as the cell area. For quantification of OPCs and their proliferation, images were taken under a ×40 objective in anatomically defined regions for anti-PDGFRα/anti-Ki67+/DAPI+ triple-stained sections, with 5 Z stack layers at 3-μm intervals. Within the selected regions, PDGFRα+ cells and PDGFRα+/Ki67+ cells were manually counted and their density (cell count per area) used for statistical analysis. All PDGFRα+ cell counts were normalized to an area of 10,000 μm². The following anatomic regions were selected: at the rostrocaudal level of the anterior commissure, we selected Mid-CC limited to mid-440 μm, ASVZ-r excluding one...
ependymal cell layer, and SVWM, at the level of the rostral hippocampus, we selected the PSVZ-r and EC as two separate regions identified by DAPI staining (fig. 2).

To quantify morphological changes in OPCs 24 and 48 h after ligation, PDGFRα+ cells were outlined manually in images of Mid-CC using the Zeiss Axiovision software. Cell area and aspect ratio, defined as cell width (along the corpus callosum) divided by cell height, were measured.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

For quantitative reverse transcriptase polymerase chain reaction (PCR), P5 ligated pups and age-matched controls were examined at 6 h (n = 10 controls, 11 ligated), 12 h (n = 11 controls, 11 ligated), 24 h (n = 16 controls, 18 ligated), 48 h (n = 17 controls, 17 ligated), and 16 days (P21) after ligation (n = 8 controls, 7 ligated). To understand whether cytokines may be involved in the pathology leading to OPC death and/or affecting oligodendrocytic maturation, we measured the RNA expression levels of IL-1β, IL-6, TNF-α and TGF-β1 at 6, 12, 24, and 48 h, and 16 days after ligation, as well as the expression levels of IL-2 and INF-γ 48 h after ligation. Total RNA was extracted from a 1-mm-thick mid-sagittal section of fresh-frozen ligated and mouse brains (excluding the cerebellum) using the RNAsesy Lipid Tissue Mini Kit (Qiagen, Mississauga, Ont., Canada) according to the manufacturer’s instructions. RNA quantity was measured using the Nanodrop ND-1000 UV Spectrometer (Nanodrop Technologies, Wilmington, Del., USA) and 0.5–1 μg of DNase-treated total RNA was used to generate complementary DNA using Superscript III™ reverse transcriptase (Invitrogen, Carlsbad, Calif, USA) following the manufacturer’s instructions. Predesigned Taqman primers were used in conjunction with TagMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif, USA) to assay the expression of IL-1β (Mm001336189_m1), IL-2 (Mm00434256_m1), IL-6 (Mm00446190_m1), INF-γ (Mm99999071_m1), TNF-α (Mm00443258_m1), TGF-β1 (Mm01227699_m1) and eukaryotic 18S rRNA (4310893E). The reactions were run and analyzed using an ABI PRISM 7900 real-time PCR system (Applied Biosystems). Samples were run in triplicate and the results averaged. Relative levels were normalized against 18S rRNA. All analyses were performed blinded to study conditions.

Statistical Analysis

Data are expressed as means ± standard deviation for each group of mice. Two-way ANOVAs were performed to determine any effects of ligation status and of time after ligation on the measured variables in each analyzed region and to evaluate any interaction between ligation status and the time after ligation. The 6- to 48-hour time points were used as acute outcome measures, while the 16-day postligation (P21) time point was used as a late outcome measure and therefore, separate ANOVA tests were performed for this latter time point. In addition, unpaired t tests were performed to evaluate any significant differences between the means of measured variables in control and ligated groups at each time point after ligation. Correlations between OPCs and microglia were evaluated using Pearson’s correlation coefficient. For all figures, *p < 0.05, **p < 0.01 and ***p < 0.001 were used. Graphs were plotted and statistics assessed using the program GraphPad Prism 5.0 (GraphPad Software) and IBM SPSS 19.
staining in control or ligated animals, suggesting that the systemic migration of monophagocytic cells is no longer present at this later stage of development.

**Microglial Response within the Corpus Callosum after Unilateral Ligation**

Activation of microglia was quantified by measuring the Iba-1+ PTA within Mid-CC (a), the SVWM (b), the ASVZ-r (c), and the EC/PSVZ-r (d) at P6/24 h after ligation (n = 5 controls, 6 ligated), P7/48 h after ligation (n = 7 controls, 7 ligated), and P21/16 days after ligation (n = 5 controls, 4 ligated). In ligated animals, there was a significant increase of Iba-1 in Mid-CC (ANOVA, p < 0.001), right SVWM (ANOVA, p < 0.05), bilateral ASVZ-r (ANOVA, left p < 0.05; right p < 0.05), and right EC/PSVZ-r (ANOVA, p < 0.01) across all time points while a marginally significant interaction was found between ligation status and time after ligation only in ASVZ-r (ANOVA, p = 0.05). Post hoc t tests showed significant increases in Iba-1 in ligated animals compared to controls in Mid-CC at 24 h and 48 h after ligation (a), but only at 24 h after ligation in the right SVWM (b) and bilateral ASVZ-r (c). In the EC/PSVZ-r (d), Iba-1 PTA was significantly increased on the ligated side at 48 h after ligation, and in this area only, remained significantly increased through P21. Error bars represent 1 standard deviation.

**Fig. 3.** Microglial activation after P5 right carotid artery ligation. Activation of microglia was quantified by measuring the Iba-1+ PTA within Mid-CC (a), the SVWM (b), the ASVZ-r (c), and the EC/PSVZ-r (d) at P6/24 h after ligation (n = 5 controls, 6 ligated), P7/48 h after ligation (n = 7 controls, 7 ligated), and P21/16 days after ligation (n = 5 controls, 4 ligated). In ligated animals, there was a significant increase of Iba-1 in Mid-CC (ANOVA, p < 0.001), right SVWM (ANOVA, p < 0.05), bilateral ASVZ-r (ANOVA, left p < 0.05; right p < 0.05), and right EC/PSVZ-r (ANOVA, p < 0.01) across all time points while a marginally significant interaction was found between ligation status and time after ligation only in ASVZ-r (ANOVA, p = 0.05). Post hoc t tests showed significant increases in Iba-1 in ligated animals compared to controls in Mid-CC at 24 h and 48 h after ligation (a), but only at 24 h after ligation in the right SVWM (b) and bilateral ASVZ-r (c). In the EC/PSVZ-r (d), Iba-1 PTA was significantly increased on the ligated side at 48 h after ligation, and in this area only, remained significantly increased through P21. Error bars represent 1 standard deviation.

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Since we observed transient increases in microglial PTA in regions such as the Mid-CC after ligation, we hypothesized that ligation could also be accelerating microglial migration from clusters in the SVWM into other regions of the white matter. Therefore, we calculated an Iba-1 PTA Mid-CC/SVWM ratio (for the left and right hemisphere separately), assuming that with increased microglial migration this ratio would be increased, as more microglia would leave the clusters and move into other regions of the corpus callosum. Iba-1 Mid-CC/SVWM ratios increased bilaterally in ligated animals, but this effect was transient (fig. 4a; ANOVA p < 0.01, ligation × time after ligation p < 0.01). There was a highly significant bilateral increase in this ratio in ligated animals at 24 h after ligation, but 48 h after ligation this ratio was comparable in controls and ligated mice, suggesting accelerated microglial migration from SVWM towards the Mid-CC in injured mice.

**OPC Qualitative Assessment**

To evaluate OPC morphology and proliferation, sections immunostained for PDGFRα and Ki67 were examined. In control mice on P6, many OPCs (PDGFRα+) in the Mid-CC showed elongated cell bodies, branched processes, a small nucleus and only a few were proliferating (PDGFRα+/Ki67+; fig. 2a). Clusters of OPCs were seen in SVWM and EC with a high number of proliferating OPCs (fig. 2c, g). Interestingly, these two regions correspond to areas where dense microglial clusters were noted at the same age (fig. 1). The proliferating OPCs appeared rounder, with fewer branched processes, and had a larger nucleus when compared to nonproliferating OPCs in the same areas, reflecting a more immature stage. In ligated animals, there was a notable decrease in the number of OPCs in the Mid-CC (fig. 2b), SVWM (fig. 2d), and PSVZ-r (fig. 2h). Furthermore, within the Mid-CC, the surviving OPCs appeared rounder with smaller nucleus and cytoplasm and fewer branched processes and were not Ki67+, likely representing a less mature OPC population that is not proliferating.

**OPC Response to Unilateral Ligation**

Quantitative analysis of OPCs was carried out on P6 and P7, ages at which OPCs are still the predominant developmental stage of the oligodendroglial lineage.

**Cell Counts and Proliferation.** In Mid-CC, there was no significant overall effect of ligation on OPC counts; however, there was a significant interaction between ligation status and time after ligation, and post hoc t tests showed a significant reduction in ligated mice at 24 h after ligation (fig. 5a). There was no significant effect of ligation on OPC proliferation in this area (fig. 5b). Ligation significantly decreased OPC counts across all times.

**Fig. 4.** Shift in regional ratios suggests migration of microglia and OPCs. The ratio, Iba-1 PTA in Mid-CC/Iba-1 PTA SVWM, was examined at 24, 48 h, and 16 days (P21) after ligation as a measure of migration (n = 6 per group at each age) (a). At 24 h after ligation, there was a highly significant bilateral increase in this ratio in ligated animals, but 48 h after ligation this ratio was comparable in controls and ligated mice, suggesting accelerated microglial migration from SVWM towards the Mid-CC in injured mice. A similar ratio was calculated using OPC count Mid-CC/SVWM (n = 6 per group at each age) (b), which showed a significant bilateral increase only at 48 h after ligation. Since the OPC count in Mid-CC was reduced at 24 h after ligation but had normalized at 48 h after ligation (fig. 4), this increased ratio is likely reflecting migration of OPCs from the SVWM to Mid-CC. Error bars represent 1 standard deviation.
Fig. 5. OPC response after P5 right carotid artery ligation. The total number of OPCs (PDGFRα+ cells) and proliferating OPCs (PDGFRα+/Ki67+ cells) per 10,000 μm² were determined in the Mid-CC (a, b), the SVWM (e, f) and the PSVZ-r (g, h) at 24 h after ligation/P6 (n = 8 controls, 7 ligated) and 48 h after ligation/P7 (n = 6 controls, 7 ligated). In the Mid-CC, ANOVA showed no significant overall effect of ligation on OPC counts; however, there was a significant interaction between ligation status and time after ligation (ANOVA, p < 0.01), and post hoc t tests showed a significant reduction in OPC counts on the ligated side at 24 h after ligation (fig. 5 a, b), but not in any other assessed region (ANOVA, p < 0.01), and post hoc t tests showed significantly lower proliferating OPC counts in right PSVZ-r at 24 h after ligation and in right PSVZ-r (ANOVA, p < 0.01); post hoc t tests showed significantly reduced OPC counts across all time points except in bilateral SVWM (ANOVA, left, p < 0.05; right, p < 0.05), and in right PSVZ-r (ANOVA, p < 0.01); post hoc t tests showed significantly reduced OPC counts in right PSVZ-r at 24 h after ligation (g) and in right SVWM at 48 h after ligation (e). Ligation significantly reduced proliferating OPC counts in right PSVZ-r (h), but not in any other assessed region (ANOVA, p < 0.01), and post hoc t tests showed significantly lower proliferating OPC counts in PSVZ-r on the ligated side at 24 h after ligation and on the contralateral side at 48 h after ligation. Since the OPCs in Mid-CC appeared to have a different morphology in ligated animals compared to controls (fig. 3), OPCs were manually outlined and average cell size (c) and length/width ratio (d) were calculated in this region at 24 and 48 h after ligation, as well as in age-matched controls. Small, mid, and large cells were defined as 20–50, 50–70 and 70+ μm², respectively. Interestingly, this analysis showed significantly smaller OPCs with a smaller length/height ratio (reflecting rounder appearance) in Mid-CC of ligated animals compared to age-matched controls at 24 h after ligation. Error bars represent 1 standard deviation.

As noted above, in ligated animals, we found a transient reduction in total OPC count in Mid-CC 24 h after ligation, away from the proliferative zones. At 24 h after ligation, we found significantly reduced OPC size distribution and OPC morphology compared to age-matched control animals. In ligated mice, there were significantly more small cells (20–50 μm²) than in controls, but significantly fewer large cells >70 μm² (fig. 5c). At 24 h after ligation, the OPC length/width ratio was significantly less in ligated animals than in age-matched controls (fig. 5d). However, at 48 h after ligation, there was no significant difference in the size or shape of OPCs in between ligated and control mice.

OPC Size and Shape Analysis. OPC size and morphology (which reflects maturation) were examined in Mid-CC, away from the proliferative zones. At 24 h after ligation, we found significantly altered OPC size distribution and OPC morphology compared to age-matched control animals. In ligated mice, there were significantly more small cells (20–50 μm²) than in controls, but significantly fewer large cells >70 μm² (fig. 5c). At 24 h after ligation, the OPC length/width ratio was significantly less in ligated animals than in age-matched controls (fig. 5d). However, at 48 h after ligation, there was no significant difference in the size or shape of OPCs in between ligated and control mice.

As noted above, in ligated animals, we found a transient reduction in total OPC count in Mid-CC 24 h after ligation.
ligation, but at 48 h after ligation, this number had fully recovered. No significant changes in cell proliferation were seen in Mid-CC, but there was a significant reduction of total OPC counts in the SVWM of ligated animals compared to controls at 48 h after ligation. We therefore postulated that OPCs may migrate from SVWM to the Mid-CC, replenishing that population of OPCs. As a measure of migration, the ratio between OPC counts in Mid-CC/SVWM was calculated. In ligated animals, there was a significant bilateral increase in this ratio at 48 h after ligation (fig. 4b), implying a higher rate of OPC migration from the SVWM clusters towards Mid-CC. It is of interest to note that the change in this index of migration for microglia (fig. 4a) precedes the change in this index for OPCs.

**Relationship between OPCs and Microglia after Unilateral Ligation**

In ligated animals, most areas with increased microglial activation or migration showed fewer OPCs or proliferating OPCs; we, therefore, used Pearson’s correlation to examine associations between Iba-1 PTA values and total OPC or proliferating OPC counts at 24 and 48 h after ligation. At 48 h after ligation, there was a significant negative correlation between ASVZ-r OPC counts and the adjacent SVWM Iba-1 on the ligated side (r = −0.88, p < 0.05, n = 5) (a). Such correlation was not significant in control animals (n = 4). At 24 and 48 h after ligation, there was a significant negative correlation between SVWM proliferating OPC counts (ligated side) and Mid-CC Iba-1 in ligated animals (r = −0.58, p < 0.05, n = 12) but not in controls (n = 12).

**Proinflammatory Cytokine Upregulation**

Using quantitative reverse transcriptase PCR, we measured mRNA expression levels for the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, and for the growth factor TGF-β1 normalized to 18S rRNA in ligated and control mice 6, 12, 24 and 48 h after ligation, as well as on P21. IL-2 and IFN-γ mRNA levels were measured 48 h after ligation, but there was no expression detected in ligated or control brains. In ligated mice, there was a significant increase in TNF-α gene expression across 6–48 h after ligation (ANOVA, p < 0.001). Although there was no significant interaction of ligation and time after ligation, t tests showed a significant increase at 6 and 24 h (fig. 7a). IL-1β and IL-6 showed a
trend for increased gene expression in ligated animals across 6–48 h (ANOVA: IL-1β, p = 0.06; IL-6, p = 0.09); t tests showed an initial significant increase of IL-1β and IL-6 at 6 h, followed by a significant decrease at 48 h (fig. 7b, c). In ligated animals, there was an increase in TGF-β1 gene expression across 6–48 h (ANOVA, p < 0.05), but there was no interaction of ligation and time after ligation, and unpaired t tests did not reveal significant differences at any individual time point (d). Error bars represent 1 standard deviation.

**Discussion**

Microglial activation at axonal crossroads is a hallmark of noncystic periventricular white matter injury in very preterm infants [25, 43, 44]. Recent studies indicate that neuroinflammation can produce long-lasting defects in oligodendroglial maturation, myelination deficits, an increase in oligodendroglial progenitors, as well as a disruption of the expression of transcription factors important in oligodendroglial maturation [3, 45]. The concept of ongoing neuroinflammation predisposing a patient to further injury, or preventing repair or regeneration after an initial insult may open possibilities for new therapies.
within a broader time window [23] and calls for adapted animal models to test experimental therapies. We recently reported an ischemic model of PVL with long-lasting bilateral myelination defects as assessed at 2 months of age [40]. We used this model to study whether ischemia would result in neuroinflammation and whether changes in this inflammatory response would be associated with OPC injury. Here we report that ischemia on P5 in CD-1 mice induces a region-specific neuroinflammatory response, as defined by microglial activation and recruitment/migration along the white matter, as well as changes in mRNA levels of a subset of proinflammatory cytokines. We find that microglial response is altered at least up to 16 days after the initial ischemic insult in the EC of the ischemic hemisphere, where the most significant long-term myelination deficits are seen at 2 months of age [40]. Along with these topographic changes in microglial activation, we found a transient reduction in OPC counts and proliferating OPC counts. Interestingly, in addition to a reduction in total number of OPCs, as defined by PDGFRα staining, quantitative morphologic analysis suggested that most OPCs seen in the postischemic brain were less differentiated cells (smaller, rounder cells with fewer processes) when compared to controls. Finally, we show that the striking microglial response following ischemia was significantly associated with the reduction of OPCs.

Noninfectious insults to the CNS like hypoxia-ischemia, stroke and exposure to excitotoxicity have been shown to induce inflammatory reactions in the immature brain involving inflammatory cells, mainly microglia/macrophages. While the mechanisms for microglial activation in infectious settings have been well studied, the initial signals after sterile tissue damage are less well characterized [43, 46, 47]. Microglia play important roles during brain development that regulate apoptosis, vascularization, axonal development and myelination [5, 48]. In humans, they become increased in the forebrain at 16–22 weeks of gestation, and reach peak prominence in cerebral white matter in the third trimester [44, 49, 50]. On P6 and P7, we could detect CD68+ (presumed to be recently blood-derived) macrophage/microglial clusters within the supraventricular corpus callosum and the EC (fig. 1), as has been shown during early postnatal development in rodents and the normal human brain [28–30]. Interestingly, these macrophage/microglial clusters were in near proximity to the site of OPC proliferation (fig. 1, 2). One should note that late OPCs prevail between P2 and P7 in rodents, and at gestational weeks 23–32 in human fetuses [39].

Microgliosis is a prominent feature of PWMI in humans and expression of IL-1β, IL-2, IL-6 and TNF-α was demonstrated on microglia/macrophages in autopsy samples from neonates with PWMI [8, 36–38]. In the presented model, we found that the microglial response 24 h after ligation, and in some regions also 48 h after ligation, was not restricted to the ligated hemisphere, but was bilateral. We further observed a temporary significant shift of microglial staining between the supraventricular clusters and the Mid-CC, which was stronger on the nonligated side, suggesting recruitment/migration of microglia from microglial clusters to other white matter regions (fig. 4). This finding is in line with our prior report [40] of white matter abnormalities and axonopathy in the bilateral corpus callosum at 2 months following unilateral neonatal ischemia. On P21, however, highly significant microgliosis among the regions of interest was only detected in the EC of the ipsilateral hemisphere in ligated animals (fig. 1, 3), where ligation of the internal carotid artery caused the most severe long-term myelination deficits in our model [40].

We detected a transient significant increase in gene expression of inflammatory cytokines IL-1β, IL-6 and TNFα in the brain from 6 h after ligation. The question, whether and how the neuroinflammatory response has deleterious effects on OPC proliferation, survival and development, has been suggested in several studies, but is not yet completely addressed [34, 35, 51]. Studies have shown that activated microglia can release or upregulate various cytokines, chemokines, and enzymes including IL-1β, TNF-α, IL-6, TGF-β1, macrophage colony-stimulating factors, and induced nitric oxide synthase [52]. A recent study showed that systemic IL-1β injection between P1 and P5 leads to long-term myelination deficits in Swiss mice while an increase in PDGFRα+ OPCs was seen on P5 [45]. Apart from cytokine-induced toxicity [3] and possible disruption of oligodendroglial maturation process [45], high levels of nitric oxide generated by induced nitric oxide synthase can damage neighboring neurons/axons by inhibiting mitochondrial respiration and increasing glutamate release from neurons and glia. Nitric oxide is also oxidized to reactive oxygen species, to which developing oligodendroglia show great vulnerability [28, 35, 53].

The developmental vulnerability of oligodendroglial progenitors due to impaired antioxidant defenses, increased expression of calcium-permeable glutamate receptors (AMPA receptors mainly on cell body, NMDA receptors mainly on cell processes) and glutamate transporters, as well as susceptibility to cytokine-triggered in-
jury has been established [54–56]. While we show that after a transient decrease, there is recovery in OPC counts at 48 h after ligation in the Mid-CC, morphologic analysis of the same region revealed a significant shift in cell size towards smaller, less mature cells (fig. 5). We conclude that ischemia leads to a shift toward less mature OPCs, a finding that is in line with suggested maturational arrest of oligodendrocytes in PVL. Regional OPC cell count ratios between sites of OPC proliferation (SVWM) towards areas with more mature OPCs (Mid-CC) revealed a significant shift at 48 h after ligation, showing relatively more cells in the Mid-CC than in age-matched controls (fig. 4). This may indicate OPC migration from sites of proliferation to sites of injury in ligated animals. In the current study, we did not assess the intercellular matrix and the role of hyaluronan [57], chondroitin sulfates and other proteoglycans, which may be blocking the differentiation of OPCs, as shown in multiple sclerosis models and autopsy samples, [58, 59] but we plan to investigate these in future studies.

Considering the strong proximity of supraventricular areas of OPC proliferation (fig. 2) to microglial clusters (fig. 1) within the white matter, as well as the slower recovery of OPC cell count on P7 in these regions, in contrast to the Mid-CC (fig. 5), it is also plausible that the proximity to activated microglia may contribute to decreased OPC counts after ligation. Following this notion, we found that microglial Iba-1 PTA increase in the dense SVWM cluster was associated with decreased OPC counts in the adjacent subventricular zone. Furthermore, decreased OPC proliferation in the white matter on the ligated side was associated with increased Iba-1 PTA in Mid-CC, which appears to be a global measure of microglial response after ligation. These correlations were not seen in control brains. While we cannot ascertain a causal relationship, it is conceivable that the activated microglia may release cytokines, inflammatory products or have phagocytic activity that could result in reduced OPC cell counts. Many inflammatory mediators and products have been demonstrated in microglia in neonates with PWMI [36–38] and have been shown to be detrimental for oligodendroglial development in cell culture [3, 8].

The interaction between microglia and OPCs in PWMI is complex and requires further investigation. Cell culture studies with endotoxin-activated microglia and OPCs have demonstrated that microglia may secrete factors which, depending on the experimental setting and activator, increase or reduce OPC survival [32, 58, 60]. However, activated microglia increase survival and reduce apoptosis of mature oligodendrocytes, independent of an activating agent [51]. Microglia have been shown to secrete many growth/trophic factors such as brain-derived neurotrophic factor, bFGF, NGF, IGF-1, TGF-β, and ciliary neurotrophic factor [61–63], most of which also support oligodendroglial survival and differentiation. They can also generate free radicals, secrete injurious proinflammatory cytokines, and contribute to excitotoxicity [5, 8]. Thus, microglia play a dual role in the maintenance of tissue homeostasis and repair. A recent study using the mouse cuprizone model, where remyelination occurs spontaneously after toxin-induced primary demyelination, examined genome-wide gene expression analysis of microglia from the corpus callosum during demyelination and remyelination and characterized a microglial phenotype that supports remyelination that was present at the onset of demyelination and persisted throughout remyelination. These microglia expressed cytokines and chemokines that activate and recruit endogenous OPCs to the lesion site and deliver trophic support during remyelination [64].

While we show increased microglial activation along with increased expression of proinflammatory cytokines, one limitation in our study is that we did not further characterize whether the activated microglia are pro- or anti-inflammatory. It is possible that the microglial response seen in our study during the late phase (P21) involves different mechanisms than the initial microglial activation, and is perhaps more a reparative or trophic response rather than destructive. Along with this notion is the fact that none of our proinflammatory cytokines were still up-regulated at this late stage. Future studies with this model will need to address the role of neuroinflammatory priming and the potential for tertiary injury [23] in older animals. Another limitation of this study is that astrocytes could be an important part of the neuroinflammatory process in this model [56, 65], but while we showed ipsilateral astrogliosis in this model at P60 [40], at early postnatal ages our assessment using the GFAP marker proved insensitive, as it also stained progenitor cells and radial glial cells (data not shown). We are planning to utilize newer, more specific astrocyte markers in our future studies to further characterize the role of astrocytes following acute ischemia.

In summary, we report an ischemia-induced neonatal mouse model of PVL in which we find significant neuroinflammation and selective vulnerability of the white matter. Temporospatial study of the interaction of microglia and OPCs after injury, as well as the cytokine profile indicate that neuroinflammation plays an important role.
role in ischemia-induced oligodendrogial injury. Future studies utilizing immunomodulatory agents in this ischemic model can aid in a better understanding of the diverse roles of neuroinflammation within the vulnerable developmental period of PWMI and open possibilities for new protective therapies.

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References


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