Approximately 140,000 children are born prematurely in the United States every year. Half of all preterm infants are classified as very low birthweight (less than 1500 g), and 50% of those suffer some type of neurodevelopmental disability, including intraventricular hemorrhage (IVH) and posthemorrhagic hydrocephalus (PHH). Neonatal IVH originates from the underdeveloped germinal matrix and occurs in up to 30% of very low birthweight infants. The germinal matrix is highly vascular and vulnerable to the hemodynamic instability associated with prematurity birth. When germinal matrix blood vessels rupture, blood is released into the lateral ventricles (and into brain parenchyma in severe cases) and leads to PHH due to a combination of factors including failure of cerebrospinal fluid (CSF) absorption, CSF overproduction, and scarring of the cerebral aqueduct. The germinal matrix involutes as gestation progresses; therefore, IVH/PHH is rare in term infants.

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Current therapy for IVH focuses on treating PHH. However, the treatment of PHH alone does not address the underlying brain injury that may be a sequela of IVH. It is well established that blood released outside of the vascular system induces inflammation and oxidative stress, making immunomodulatory or antioxidant therapies promising possibilities for IVH treatment. However, the time course of inflammation after IVH is not well studied. Within the same time frame during which IVH occurs, the neonatal brain is rapidly producing the cells needed for myelina-
tion. Oligodendrocytes, the myelin-forming cells of the brain, are derived from oligodendrocyte progenitor cells (OPCs). OPCs self-renew and give rise to mature oligodendrocytes, a process that persists into adulthood. They are fragile cells that are exquisitely sensitive to excitotoxicity, inflammatory cytokines, and oxidative stress. Failure of OPC maturation has lifelong consequences including cerebral palsy and cognitive deficits.

This study was designed to examine inflammation and oxidative stress in a rat model of IVH. We hypothesized that IVH would induce inflammation and oxidative stress in white matter. Because surgical treatment for PHH typically occurs in a delayed fashion, it does not directly treat the acute inflammation induced by IVH. An understanding of the time course of IVH pathology could guide the development of acute, prolonged, or even empirical medical therapy for IVH.

Methods

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Pups from Sprague Dawley timed pregnant animals (Harlan-Envigo) were used and kept with their dams at all times, with the exception of the brief period during surgery. Animals were weaned at 21 days of age. Animals were weighed and monitored daily for failure to thrive or other adverse consequences of surgery, none of which were observed. Animals of both sexes were included in each experimental group in this study. A total of 126 rats were used in the study.

Intraventricular Injections

We modified a previously described model of neonatal IVH by using postnatal day 5 (PND5) rat pups, whose white matter development corresponds to that of preterm infants. Prior to IVH induction, pups received 0.1 mg/kg of intraperitoneal buprenorphine for pain management and were anesthetized and maintained throughout the procedure with isoflurane. Pups were secured in a warmed stereotaxic frame and nonrupture ear bars (Stoelting). The scalp was prepped with betadine, and a midline skin incision was made to expose lambda. Using a stereotaxic injector (Stoelting) equipped with a Hamilton syringe (model 802RN, 30-G, point style 4, small hub needle), the right lateral ventricle was accessed at coordinates 1.1 mm lateral, 4.6 mm anterior, and 3.3 mm deep from lambda. Injections of 150 mg/ml of hemoglobin (MP Biomedicals) prepared in saline or saline alone (controls) were delivered at a rate of 5 μl per minute, until a total of 10 μl was injected. The syringe was left in place for an additional 2 minutes to reduce retrograde flow upon removal. Wounds were closed with dermal glue, and animals were returned to their home cage. No animals died unexpectedly during the study.

Tissue Preparation

Experimental tissue was designated for biochemical or immunostaining endpoints and processed accordingly. For euthanasia, animals were deeply anesthetized with isoflurane before terminal thoracotomy and perfused with 10 ml of ice-cold phosphate-buffered saline (PBS). For tissue destined for immunostaining, an additional perfusion of 10 ml of 4% paraformaldehyde (PFA) in PBS was performed. Brains were removed and either bisected into ipsilateral and contralateral hemispheres and then flash frozen for biochemistry or were immersion fixed in 4% PFA for 18 hours at 4°C and cryoprotected in a 30% sucrose/PBS solution for immunostaining. Tissue for 37 days postinjection biochemical analysis was regionally dissected for isolation of the corpus callosum.

Biochemical Analysis

Brain tissue was stored at −80°C until preparation for cytokine measurements and Western blot analysis. Tissue homogenates from entire hemispheres or corpus callosum were made using an Omni Tissue Homogenizer in PBS lysis buffer with protease and phosphatase inhibitors (Halt, Thermo Fisher Scientific) and centrifuged at 12,000g for 20 minutes at 4°C. Detergent-soluble fractions were made by resuspending the tissue pellet in tissue protein extraction reagent (Thermo Fisher Scientific) with protease and phosphatase inhibitor and homogenizing prior to centrifugation to pellet cell debris. Both supernatant fractions were aliquoted for individual assays and stored at −80°C. Protein concentration for each set of homogenates was determined by BCA Protein Assay (Thermo Fisher Scientific).

A commercially available rat inflammatory cytokine detection system was used to measure IL-1β, IFNγ, IL-4, IL-5, IL-6, IL-10, IL-13, CXCL1, and TNFα protein (K15059G, Meso Scale Discovery). Following the manufacturer’s instructions with assay-specific modifications, 145 μg of protein per sample of PBS lysate was loaded onto the 96-well plate and incubated overnight at 4°C while shaking at 1000 RPM (Eppendorf MixMate). After a series of washes, Sulfo-TAG-labeled secondary antibodies were added and incubated at room temperature for 2 hours while shaking at 1000 RPM. A final series of washes was followed by the addition of read buffer immediately prior to plate reading (MSD QuickPlex SQ) and analysis (MSD Discovery Workbench v4.0).

Western blot detection was used to analyze oxidative stress and myelination in the detergent lysates from ipsilateral and contralateral hemispheres and corpus callosum of 37-day postinjection animals. Protein was normalized to 15 μg per lane in detergent lysis buffer and sample buffer (4× Protein Sample Loading Buffer, 928-40004, LiCor) and then separated by electrophoresis on precast polyacrylamide Bis-Tris gels (NuPage 4%–12% gels, WGI403, Invitrogen; and XCell4 SureLock Midi-Cell, Thermo Fisher Scientific). Antibody manufacturer instructions for reduced and/or denatured conditions for the samples were followed. Separated proteins were then transferred to nitrocellulose membranes from the gel by utilizing a dry transfer system (iBlot, IB3010, Invitrogen). After blocking the membrane to reduce nonspecific protein binding (Odyssey Blocking Buffer [PBS], 927–40000, LiCor), antibodies for 4-hydroxynonenal (4HNE; 1:2000, HNE11-S, ADI) and myelin basic protein (MBP; 1:2000, ab40390, abcam) were applied overnight at 4°C.
branes were probed with the appropriate proprietary second- 
ary antibodies specific for a near-infrared florescence imager (925-32211 and 925-68020, LiCor; Odyssey CLx Imager). Images were captured and analyzed with Image Studio (LiCor), and protein expression was normalized to GAPDH (1:10,000 rabbit anti-GAPDH, 2118s, Cell Signaling Technology).

**Immunostaining**

Fixed and cryoprotected brains were rinsed with 1× PBS before being embedded in optimal cutting temperature (OCT) compound (Tissue-Tek) and sectioned into 20-μm coronal sections and mounted onto slides. Immunostaining was performed using Sequenza immunostaining racks (Thermo Fisher Scientific). Briefly, endogenous peroxidase activity was quenched with 0.3% H₂O₂ in methanol (omitted for immunofluorescent staining) before blocking for nonspecific protein binding with 10% goat serum and 0.2% Triton X-100 in PBS. All antibodies were diluted in a PBS solution with 3% goat serum and 0.2% Triton X-100. Oxidative stress and myelination were measured by immunohistochemical staining of 15A3 (1:500 mouse anti-80HD, 12501, QED Bioscience) and MBP (1:500 rabbit anti-MBP, ab40390, abcam), respectively. Staining was amplified with an avidin-biotin kit (ABC Elite kit, PK-6100, Vector Laboratories) and developed in 3,3-diaminobenzidine tetrahydrochloride solution (DAB, VWR). Slides were incubated with a nuclear counterstain (Methyl Green, H3402, Vector Laboratories) and dehydrated before being coverslipped with Permount mounting medium (Electron Microscopy Sciences). Macrophages were labeled with immunofluorescent staining of IBA1 (1:1000, 019-19471, FUJIFILM Wako Pure Chemical Corp.) and CD68 (1:1000, MCA341R, AbD Serotec) with appropriate AlexaFluor (AF) secondary antibodies (1:200 AF647 goat anti-rabbit, A21245, Thermo Fisher Scientific; and 1:200 AF488 goat anti-mouse, A11001, Thermo Fisher Scientific). Fluorescently stained slides were coverslipped using mounting medium with DAPI counterstain (Vectorshiel HardSet, H1500, Vector Laboratories) and stored at −20°C.

**Image Analysis and Quantification**

All slides were imaged using Axio Scan.Z1 (Zeiss) at 20× magnification, and digital pathology was quantified with Histo Image software (Indica Labs). This software allows for automated quantification of staining on the basis of user-set thresholds to optimized algorithms and user-defined anatomical areas. For brightfield images, the area Quantification v1.0 algorithm was used. For fluorescent images, the Object Colocalization FL v1.0 algorithm was used. Analysis was performed in a blinded manner. White matter of the corpus callosum and external capsule was traced on the basis of anatomical landmarks and extended laterally along the edge of the ventricle and was restricted to tissue clearly identified as white matter. Cerebral cortex directly dorsal to the traced white matter extending to the cortical surface was analyzed. Areas of tissue irregularity or processing artifact were excluded. The different immunohistochemical analyses at each time point were performed on tissue from the same animals, representative of the brain from 1.0 mm through −3.0 mm from bregma. Ventricular width measurements were quantified by the ratio of ventricular width divided by total brain width taken in the coronal plane at the anterior commissure in single sections stained for MBP.

**Myeloid Cell Enrichment and Gene Expression**

Animals were euthanized and exsanguinated as described above. Brains were processed for myeloid cell enrichment using discontinuous Percoll centrifugation, as previously described. The resultant myeloid fraction at the 30/70 Percoll interface was harvested, resuspended with Hanks balanced salt solution (HBSS; 14175-079, Thermo Fisher Scientific) to wash Percoll from cells and subsequently pelleted by centrifugation at 3000g for 5 minutes at 4°C. HBSS was aspirated, and the pellet was lysed for RNA extraction using RLT Plus lysis buffer with 0.1% beta mercaptoethanol for subsequent processing with RNAasy Plus Micro kit (74034, Qiagen). RNA quantity and quality were analyzed using NanoDrop 2000 (Thermo Fisher Scientific). Approximately 50 ng of total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (4368814, Applied Biosystems). Resultant cDNA was quantified for gene expression changes using TaqMan low density array (TLDAs, TaqMan Gene Expression Array card, Thermo Fisher Scientific). Gene expression changes were calculated using the 2^ΔΔCt method, with saline-injected pups as the reference group.

**Statistical Analysis**

Statistical analysis for immunohistochemistry experiments was performed by our institution’s Department of Statistics. Statistics for weight gain, cytokine induction, 4HNE Western blot, and gene expression were assessed via one-way ANOVA (time x treatment [saline vs IVH]). Significant main effects of treatment were analyzed using Holm-Šídák post hoc comparisons; where interactions between time and treatment group were detected, a Student t-test was used to test for significance at each time point. Gene array data were analyzed via one-way ANOVA and Dunnett’s multiple comparisons test. Where only two experimental groups were present (anatomical measurements, MBP Western blot), a Student t-test was used. Data analyses were conducted using general linear repeated mixed models for IBA1, CD68, and 15A3 as response variables with a compound symmetry covariance structure. The models were run using the log-transformed values. The covariates other than treatment were time point, side, region, and sex. Covariates in the final models, including interactions, were chosen using backward selection and a significance level of 0.05. To generate p values between control and IVH groups at each time point, values were log transformed and a t-test was performed.

**Results**

IVH Induces a Reduction in the Rate of Weight Gain

There was no significant difference in weight between littermates assigned to control and IVH groups prior to
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had gained 34.0% ± 8.1% of their preinjection body weight and IVH ani-

mals had gained 8.0% ± 5.6% (p = 0.004, n = 47–59 per group). Forty-eight hours after IVH induction, control animals

had gained 23.6% ± 8.8% (p = 0.03, n = 20–25 per group). Seven days after IVH induction (postnatal day 12), IVH

animals had no significant difference in weight gain compared with control animals.

IVH Induces an Acute Wave of Cytokine Production and Oxidative Stress

Analysis of cytokine production from whole-brain lysate (without differentiating white matter from cortex) revealed an acute increase of proinflammatory cytokines that rapidly subsided after IVH (Fig. 2). TNFα protein levels were significantly elevated 3 hours after IVH (p < 0.0001), CXCL1 was significantly elevated 3 and 6 hours after IVH (p < 0.0001 for both), IL-1β was also significantly elevated 3 and 6 hours after IVH (p = 0.0006 and p = 0.001, respectively), IL-6 was significantly elevated 3 and 6 hours after IVH (p = 0.0014 and p < 0.0001, respectively). Injury-induced responses were similar for the contralateral hemisphere (see Supplemental Fig. 1 for contralateral hemisphere results). The remaining cytokines assayed were not significantly changed after IVH.

Using a Western blot for 4HNE at the same time points as the cytokine analyses, we observed that oxidative stress was elevated 24 hours after IVH (Fig. 3; p < 0.0001). This effect was present in the contralateral hemisphere from injection as well (see Supplemental Fig. 2).

IVH Induces Acute Inflammation in Both Cerebral Cortex and White Matter

To better determine the regional inflammatory response to IVH, we performed histological analysis of IBA1, a marker for macrophages and microglia,21 and CD68, a marker of macrophages with increased lysosomal activity, regardless of microglial or blood monocyte origin.22 We analyzed tissue 3 hours, 24 hours, and 7 days after IVH in cortex and white matter both ipsilateral and contralateral to the injection site. These time points were selected on the basis of our cytokine measurements to capture the time of maximal proinflammatory cytokine response (3 hours), the time at which most proinflammatory cytokine production had subsided (24 hours), and a subacute/chronic time point (7 days). Data presented here are from the hemisphere ipsilateral to hemoglobin injection. Contralateral hemisphere data showed similar effects and are presented as Supplemental Figs. 3 and 4.

There was a significant effect of IVH and region (i.e., cortex vs white matter) on IBA1-positive cells, as well as an interaction between time point and region. IBA1-positive cells were significantly increased in white matter at 3 and 24 hours after IVH induction (p = 0.002 and 0.03, respectively). This effect was not present in cortex, demonstrating a site-specific inflammatory response within areas of developing white matter. Seven days after IVH induction, there was no longer a significant effect of IVH on IBA1 labeling in white matter or cortex (Fig. 4).

CD68-positive cells were significantly increased within both white matter and cortex at 3 and 24 hours after IVH induction (p = 0.002 and p = 0.02, respectively, for white matter; p = 0.004 and p = 0.01 for cortex). Seven days after IVH, there was an increase in CD68 labeling in cortex (p = 0.02) but not in white matter (Fig. 5). On the contralateral side of injection, both IBA1 and CD68 labeling followed the same patterns as the ipsilateral side, although statistical significance was not present at as many time points (see Supplemental Fig. 4). An overview of IBA1 and CD68 labeling 24 hours after injection (where both were elevated in the IVH group) is shown in Supplemental Fig. 5.

Post-IVH Induction of CCL2/CCR2 Pathway in Myeloid Cell Fraction

Various reports have linked brain injury to the recruitment of peripheral immune cells, notably monocytes, which is driven by the chemokine CCL2 and its putative receptor CCR2.23 To understand whether our IVH model drives similar signaling pathways, we purified the myeloid fraction of cells from whole brain and analyzed expression profiles of CCL2 and CCR2. Our model of IVH induced a significant increase in CCL2 at 3 hours after IVH (p < 0.001) and a significant decrease in CCL2 72 hours after IVH (p = 0.01). CCR2 was increased at 3, 24, and 72 hours after IVH (p < 0.0001, p < 0.0001, and p = 0.0006, respectively), visually peaking 24 hours after IVH (Fig. 6).

Oxidative Stress in White Matter Is Elevated 24 Hours After IVH Induction

Inflammation has been linked to oxidative tissue injury, and OPCs are particularly vulnerable to oxidative stress.9 We used 15A3, an antibody that labels oxidized nucleic acids,24 to delineate the regional spread of oxidative stress over time. As with IBA1 and CD68, there was a significant elevation of oxidative stress within white matter 24 hours after

FIG. 1. Weight gain after IVH induction. Twenty-four hours after IVH induction, control animals had gained 17.8% ± 6.3% of their preinjection body weight and IVH animals had gained 8.0% ± 5.6% (p = 0.004, n = 47–59 per group). Forty-eight hours after IVH induction, control animals had gained 34.0% ± 8.1% of their preinjection body weight and IVH animals had gained 23.6% ± 8.8% (p = 0.03, n = 20–25 per group). Seven days (7D) after injection, there was no significant difference in weight gain from the day of injection in control and IVH animals (n = 12–16 per group). *p < 0.05.

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after IVH induction (p = 0.01). This was sustained 7 days after IVH induction (p = 0.002). There was no increase in oxidative stress in the cortex in response to IVH (Fig. 7).

**IVH Leads to Reduced Myelin in the Corpus Callosum**

To determine the long-term effect of IVH on white matter pathology, we quantified MBP density with both immunohistochemistry on fixed tissue and Western blot of isolated corpus callosum from 42-day-old animals, 37 days postinjection. Animals with IVH had a trend toward less myelin density when analyzed via immunohistochemistry (p = 0.12), but Western blot revealed a significant reduction in MBP signal 37 days after IVH (Fig. 8; p = 0.01).

**Ventriculomegaly Develops Gradually After IVH**

Ventricular measurements were made in tissue slices stained for MBP in animals 37 days after IVH induction. The ratio of ventricular width to total brain width was significantly increased in animals with IVH versus control saline injection (Fig. 9; p = 0.003).

**Discussion**

Here we demonstrate that our IVH model initiates a rapid wave of inflammation followed by oxidative stress. While others have shown that inflammation occurs as a consequence of IVH, our results are the first to show the temporal relationship between cytokine and cellular inflammatory responses, and oxidative stress after IVH. This is similar to the spread of inflammation and oxidative stress after neurotrauma, where focal injury can have widespread effects.

Our biochemical data (Figs. 2 and 3) indicate that IVH incites a rapid wave of cytokine production even contralateral to the side of injection, but that this effect subsides relatively quickly. This is followed by elevated oxidative stress. Since IVH induces the same pathological mecha-
isms as other forms of neonatal brain injury, such as hypoxia, ischemia, and infection, IVH and those processes could have a cumulative effect on white matter injury. While nutritional deficits have been linked to white matter injury, the effect of brain injury on weight gain we observed merits further investigation. Interestingly, the effect of IVH on weight gain resolved over the same time frame that white matter inflammation resolved, indicating that the proinflammatory state in the brain induced by IVH may decrease appetite or ability to gain weight.

We used IBA1 and CD68 to track immune reactivity throughout the brain after IVH. In contrast to IBA1, CD68 was elevated in both the white matter and cortex 3 hours after IVH. This effect was not as robust in contralateral white matter; however, it followed the same trend as ipsilateral white matter and both the contralateral and ipsilateral cortex. As with IBA1 reactivity, this demonstrates that the inflammatory effects of IVH are widespread throughout the brain and not isolated to the area adjacent to IVH origin. While microglia and macrophages can co-express IBA1 and CD68, recent data have shown that a CD68-positive/IBA1-negative microglial subset is associated with white matter injury in human autopsy subjects. Our analysis was not designed to measure the colocalization, or

FIG. 3. Oxidative stress after IVH in the hemisphere ipsilateral to saline or hemoglobin injection. There was a significant increase in 4HNE 24 hours after IVH induction (0.76 ± 0.3 normalized signal vs 0.2 ± 0.06 normalized signal in controls at 3 hours, p < 0.0001, n = 5–6 per group except the 7D control group in which n = 1). *p < 0.05.

FIG. 4. Microglia and macrophages increase after IVH. Quantitative analysis of IBA1 immunohistochemistry in the cerebral cortex (A) showed no effect of IVH on IBA1 labeling (B). Quantitative analysis of IBA1 labeling in white matter (C) showed a significant effect of IVH at 3 and 24 hours after IVH (91.3 ± 45.2 vs 17.5 ± 8.4 cells/mm² in controls at 3 hours, p = 0.002, n = 3–5 per group; 172.3 ± 65.4 vs 75.2 ± 30.6 cells/mm² in controls at 24 hours, p = 0.03, n = 3–5 per group; D). Data from hemisphere ipsilateral to saline or hemoglobin injection. *p < 0.05.
lack thereof, of IBA1 and CD68. However, it demonstrates that both of these markers of innate immune cells are elevated after IVH. Our evidence that IVH causes rapid and sustained inflammation throughout the brain suggests that modulating this inflammation may be a therapeutic target for IVH. Another observation from our histological studies is that IBA1-positive cells steadily increased in the cortex regardless of experimental group. We hypothesize that this is part of the normal process of microglia-mediated synaptic pruning that occurs during development. The finding that the CCL2/CCR2 pathway is activated in response to IVH suggests that the increase in innate immune cells after IVH are attributable at least in part to recruitment of blood-borne macrophages into the brain.

As seen via Western blot and immunohistochemical analysis, oxidative stress follows the acute inflammatory response. The regional analysis afforded by immunohistochemistry demonstrates that oxidative stress is concentrated in white matter. There were no statistically significant effects or notable trends in oxidative stress in the cerebral cortex. In white matter, oxidative stress was elevated 24 hours and sustained 7 days after IVH induction. Further work is needed to determine how sustained inflammation post-IVH leads to subsequent and persistent oxidative stress and tissue injury within white matter.

The anatomical changes seen in this injury model are similar to those described in similar injury paradigms. It should be noted that our injury was induced at an earlier age than prior hemoglobin injection studies in rats, in order to capture the developmental stage that most closely mimics the preterm human neonate. As we observed white matter loss in our Western blot analysis but not histological measurements, we consider our model to represent a mild or moderate grade of clinical IVH. We do not know to what degree our ventricular changes are from white matter loss or elevated intracranial pressure. Future studies utilizing intracranial pressure monitoring and serial in vivo imaging in individual animals will help to establish the relationship between increased CSF pressure and volume loss, both of which could simultaneously change ventricular size. This unclear relationship among volume loss, increased CSF, and other variables such as head circumference complicates the care of patients with IVH and results in a lack of standardized criteria for surgical intervention.

**FIG. 5.** Macrophage reactivity increases after IVH. Quantitative analysis of CD68 immunohistochemistry in the cerebral cortex (A) showed a significant effect of IVH 3 hours, 24 hours, and 7 days (7D) after induction (88.5 ± 35.6 vs 10.9 ± 9.2 cells/mm² in controls at 3 hours, p = 0.004, n = 3–5 per group; 19.7 ± 8.5 vs 6.4 ± 1.2 cells/mm² in controls at 24 hours, p = 0.01, n = 3–5 per group; 0.8 ± 0.5 vs 0.2 ± 0.1 cells/mm² in controls at 7D, p = 0.02, n = 5–6 per group; B). Quantitative analysis of CD68 labeling in white matter (C) showed a significant effect of IVH 3 and 24 hours after induction (187.5 ± 60.3 vs 42.0 ± 19.7 cells/mm² in controls at 3 hours, p = 0.002, n = 3–5 per group; 205.8 ± 60.4 vs 101.6 ± 23.4 cells/mm² in controls at 24 hours, p = 0.02, n = 3–5 per group; D). Data from hemisphere ipsilateral to saline or hemoglobin injection. *p < 0.05.
FIG. 6. CCL2/CCR2 is induced in myeloid cells after IVH. A: rtPCR of the myeloid fraction of whole brain revealed a significant increase in CCL2 induction 3 hours after IVH (4.2 log₂ fold change from saline controls at 3 hours, p < 0.001, n = 4 per group). There was a significant decrease in CCL2 induction 72 hours after IVH (−1.9 log₂ fold change from controls at 3 hours, p = 0.01, n = 4 per group). B: CCR2 was increased at 3, 24, and 72 hours after IVH (2.2 log₂ fold change, 3.4 log₂ fold change, and 1.8 log₂ fold change from saline control at 3 hours, p < 0.0001, p < 0.0001, and p = 0.0006, respectively). **p < 0.05.

FIG. 7. IVH selectively increases oxidative stress in the white matter of brain ipsilateral to injury. Quantitative immunohistochemistry for 15A3 in the cerebral cortex (A) revealed no effect of IVH (B). Quantitative immunohistochemistry for 15A3 in white matter (C) revealed an increase in oxidative stress 24 hours (31.3% ± 7.8% positive area vs 7.7% ± 4.3% positive area in controls, p = 0.01, n = 3–4 per group) and 7 days (7D) after IVH (10.2% ± 4.5% positive area vs 5.5% ± 0.4% positive area in controls, p = 0.002, n = 5–6 per group; D). *p < 0.05.
Although we chose PND5 as the time point for IVH induction on the basis of white matter development, there are also immunological changes in the developing brain that could affect response to IVH. There have been several recent advances in the understanding of macrophage developmental biology, from establishing the true origin of resident microglia to the important physiological functions of resident microglia in synaptic pruning and OPC survival.\textsuperscript{36} Much of this work has been done in mouse models, so it is difficult to link a particular stage of brain immunological development in humans to a corresponding age in rat pups. As knowledge of immunological development in humans and animals improves, it will help to fine-tune animal models to better recapitulate the human disease phenotype. Although our experiments were designed to define the effects IVH on macrophages, there are clearly changes in macrophages over time in the absence of IVH (Figs. 4 and 5). Therefore, just as developmental stage affects OPC response to injury,\textsuperscript{18} immunological response could also vary by developmental stage. Future studies could empirically answer questions regarding the timing of IVH on acute neuroinflammation and final degree of white matter loss and would provide insight about how the timing of IVH affects outcome. A more complex analysis of inflammation after IVH, such as macrophage phenotyping,\textsuperscript{37} could help determine if inflammation provides a reparative role as well, as in other diseases.\textsuperscript{38,39}

It is important to note that the cytokine response sub-

**FIG. 8.** IVH leads to a reduction in white matter. Immunohistochemistry for MBP (A) at 37 days (37D) postinjection showed a trend toward less MBP labeling in animals that had IVH (B; 49.0% ± 21.4% positive area after IVH vs 68.6% ± 10.9% positive area in controls, p = 0.12, n = 4–9 per group; C). Western blot of MBP performed on isolated corpus callosum revealed a significant reduction in MBP 37D after IVH (D; 0.46 ± 0.07 normalized MBP signal after IVH vs 0.63 ± 0.11 normalized signal in controls, p = 0.01, n = 5–6 per group). All Western blot data are shown. \(^*\)p < 0.05.
also been observed in a genetic model of hydrocephalus.45 Since developing white matter is composed of OPCs—was confined to white matter in our histological evaluation. Since developing white matter is composed of OPCs, there are especially vulnerable to injury. Microvascular pathology may play a role as well, as extravascular hemoglobin induces vasoconstriction.43 This is supported by earlier work showing hippocampal inflammation in a similar rat IVH model32 and in humans.44 A robust white matter inflammatory response has also been observed in a genetic model of hydrocephalus.45 Understanding the time course of inflammation and oxidative tissue injury after IVH in humans can help guide the timing of surgical therapies that reduce intraventricular blood products and therefore inflammation and oxidative stress. Our findings of an acute peak of inflammation after IVH suggest that anti-inflammatory treatment would need to be applied early after, or even before, IVH.

**Conclusions**

These findings show that IVH induces a rapid inflammatory response, preceding a peak of oxidative stress that was confined to white matter in our histological evaluation. Since developing white matter is composed of OPCs that are especially vulnerable to injury, the inflammatory and oxidative burden from IVH may contribute to brain parenchymal injury in addition to causing hydrocephalus. This is supported by earlier work showing hippocampal inflammation in a similar rat IVH model32 and in humans.44 A robust white matter inflammatory response has also been observed in a genetic model of hydrocephalus.45 Understanding the time course of inflammation and oxidative tissue injury after IVH in humans can help guide the timing of surgical therapies that reduce intraventricular blood products and therefore inflammation and oxidative stress. Our findings of an acute peak of inflammation after IVH suggest that anti-inflammatory treatment would need to be applied early after, or even before, IVH.

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**References**


**Disclosures**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**

Conception and design: Miller, Gensel, Morganti. Acquisition of data: Miller, Goulding, Vogel, Morganti. Analysis and interpretation of data: all authors. Drafting the article: Miller, Goulding. Critically revising the article: Miller, Goulding, Vogel, Gensel, Morganti. Reviewed submitted version of manuscript: Miller, Goulding, Vogel, Gensel, Morganti. Approved the final version of the manuscript on behalf of all authors: Miller. Statistical analysis: Gensel, Stromberg. Administrative/technical/material support: Goulding. Study supervision: Miller.

**Supplemental Information**

Online-Only Content

Supplemental material is available with the online version of the article.

**Supplemental Figs. 1–5.** https://thejns.org/doi/suppl/10.3171/ 2020.5.PEDS20124.

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