White Matter Protection in Congenital Heart Surgery

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Background—Neurodevelopmental delays in motor skills and white matter (WM) injury have been documented in congenital heart disease and after pediatric cardiac surgery. The lack of a suitable animal model has hampered our understanding of the cellular mechanisms underlying WM injury in these patients. Our aim is to identify an optimal surgical strategy for WM protection to reduce neurological injury in congenital heart disease patients.

Methods and Results—We developed a porcine cardiopulmonary bypass model that displays area-dependent WM maturation. In this model, WM injury was identified after cardiopulmonary bypass–induced ischemia-reperfusion injury. The degree of injury was inversely correlated with the maturation stage, which indicates maturation-dependent vulnerability of WM. Within different oligodendrocyte developmental stages, we show selective vulnerability of $O4^+$ preoligodendrocytes, whereas oligodendrocyte progenitor cells were resistant to insults. This indicates that immature WM is vulnerable to cardiopulmonary bypass–induced injury but has an intrinsic potential for recovery mediated by endogenous oligodendrocyte progenitor cells. Oligodendrocyte progenitor cell number decreased with age, which suggests that earlier repair allows successful WM development. Oligodendrocyte progenitor cell proliferation was observed within a few days after cardiopulmonary bypass–induced ischemia-reperfusion injury; however, by 4 weeks, arrested oligodendrocyte maturation and delayed myelination were detected. Logistic model confirmed that maintenance of higher oxygenation and reduction of inflammation were effective in minimizing the risk of injury at immature stages of WM development.

Conclusions—Primary repair in neonates and young infants potentially provides successful WM development in congenital heart disease patients. Cardiac surgery during this susceptible period should avoid ischemia-reperfusion injury and minimize inflammation to prevent long-term WM-related neurological impairment. **(*Circulation*. 2012; 125:859-871.)**

Key Words: cardiopulmonary bypass ■ congenital heart disease ■ surgery ■ brain ■ white matter injury

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Congenital heart disease (CHD) is the leading birth defect, affecting almost 1 in every 100 infants born each year.[1] Significant advances have been made over the last 3 decades in reducing the mortality risk for patients with CHD,[2,3] so that by the end of the next decade, almost 1 in 150 young adults will have some form of CHD.[3] One of the most important concerns in this growing population is impaired neurological development.[4] There is accumulating evidence that CHD patients requiring surgical correction with cardiopulmonary bypass (CPB) early in life are at significant risk of neurological deficits.[5–7] The personal and societal costs of both gross and subtle neurological morbidity are inestimable; however, there has been surprisingly little cellular and molecular investigation of the impact of CPB on brain development.

Clinical Perspective on p 871

The most common neurological deficits seen in children after cardiac surgery are fine and gross motor deficits.[8–10] These symptoms are consistent with diffuse white matter (WM) injury.[11,12] Recent clinical magnetic resonance imaging studies have demonstrated a high incidence (25% to 55%) of newly developed WM injury in infants after cardiac surgery.[13–15] Causes of neurological morbidity associated with CPB include an exaggerated systemic inflammatory response syndrome (SIRS), as well as risk of ischemia-reperfusion and reoxygenation injury (I/R injury).[16,17] Specific susceptibility of developing WM to inflammation and ischemia has been identified both in clinical and laboratory studies.[12,18] Understanding the cellular and molecular events that result in such WM injury and alter WM development is

Received June 7, 2011; accepted January 4, 2012.

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Guest Editor for this article was Costantino Iadecola, MD.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.048215/-/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.111.048215**

of crucial importance to develop targeted therapies that will minimize the risk of neurological deficits in CHD patients.[19]

The cellular and molecular processes underlying WM development, including oligodendrocyte maturation and myelination of axons, have been explored extensively.[20,21] Significant advances have been made in understanding the mechanisms of endogenous myelin repair by oligodendrocyte progenitor cells (OPCs) using rodent models[22,23]; however, a large-animal model, anatomically and physiologically closer to humans, is needed to elucidate the cellular and molecular mechanisms of CPB-related developing WM injury[24] that occurs in humans.

The porcine model has been widely used for the investigation of CPB.[17,25] Furthermore, the porcine brain has similar anatomic structure to the human brain[26] and shares more physiological and metabolic similarities to humans than other large mammals.[27] Therefore, we first examined the adequacy of a porcine model for the investigation of WM development by establishing methods for detection and analysis of oligodendrocyte lineage cells.[12,28] We then assessed the effects of standard CPB on oligodendrocyte lineage cell development and on myelination. Finally, we studied the risk of WM injury caused by CPB-related SIRS and I/R injury at various maturational stages of WM development.

Methods

Animals

A total of 48 female Yorkshire piglets were involved in the present study. Normal WM development was evaluated at 1, 3, and 7 weeks of age by use of an immunohistochemical and anatomic approach (each n=5). The number of OPCs was assessed at the age of 12 weeks (n=3). To investigate the effects of CPB on WM development, 30 animals at 3 weeks of age were randomly assigned to 1 of 3 groups with different CPB-induced brain insults that involved I/R injury and SIRS (Figure 1A): (1) No surgery (control, no insult); (2) 34°C full-flow bypass for 60 minutes (mild CPB insult, CPB-induced SIRS); and (3) 25°C circulatory arrest for 60 minutes (severe CPB insult, CPB-induced SIRS with I/R injury). Five brains in each group were fixed at postoperative day 3 and week 4, respectively (Figure 1A). We performed all experiments in compliance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals." The study was approved by the Animal Care and Use Committee at Children's National Medical Center.

Surgery and Assessment of the Insult

CPB was established via ascending aortic perfusion and right atrial drainage. After initial normothermic perfusion, animals were cooled to a temperature of 25°C or 34°C, and then circulatory arrest or full-flow bypass was chosen according to the protocol (Figure 1A).[25] The hematocrit level of 30% was maintained, and a pH-stat strategy was performed to use the current standard CPB technique.[17,24] The tissue oxygen index (TOI) was measured by near-infrared spectroscopy (Figures 1B and 1C).[25] SIRS was identified by the leukocyte number and plasma interleukin-6 (IL-6) concentration (Figures 1D and 1E). Neurological and behavioral evaluations were performed blindly at 24-hour intervals beginning on postoperative day 1 (Figures 1F and 1G). A total deficit score of 400 indicates brain death, whereas a score of 0 is considered normal.[25]

Cellular Analysis

Cerebral WM was subdivided into 5 regions (Figure 1H): (1) Corpus callosum (CC); (2) medial periventricular WM (M-PVWM); (3) lateral periventricular WM (L-PVWM); (4) subcortical WM (SCWM); and (5) internal capsule (IC). Porcine oligodendrocyte lineage cells were successfully identified with specific antibodies for established oligodendrocyte developmental markers, including oligodendrocyte transcription factor 2 (Olig2), the basic helixloop-helix transcription factor mammalian achaete-schute Homolog 1 (Mash1; also known as Ascl1), the transmembrane chondroitin sulfate proteoglycan NG2, platelet-derived growth factor receptor-α (PDGFR-α), cell surface antigen O4, and monoclonal anti-adenomatous polyposis coli antibody clone CC1 (also known as APC) (Figures 1I through 1N).[12,28] Myelin basic protein (MBP) expression indicates myelination level (Figure 1I).[20,28] To determine cell density, the antibody-positive cells were blindly quantified in 6 microscopic fields from each WM region. MBP pixel intensity was compared with that of the corresponding brain region from control animals.[28]

Statistical Analysis

One-way ANOVA with Bonferroni post hoc comparisons was used to detect differences in the immunohistochemical analysis, TOI level, leukocyte number, IL-6 concentration, and neurological deficit score in the experimental groups. Two-group comparisons in the operative conditions, percentage of preoligodendrocytes, and caspase 3–positive (caspase-3^+) cell number in the cerebral cortex were performed by unpaired Student t test. Two-way ANOVA with Bonferroni comparisons with time as a factor was used to evaluate changes of cell numbers over time between WM regions and CPB groups. Changes in TOI in the 2 CPB groups were assessed by repeated-measures ANOVA. The Spearman rank correlation coefficient (ρ) was used as a measure of the association between the WM maturational stage and cellular damage. To evaluate the effects of the lowest TOI, plasma IL-6, and WM maturational stages on caspase-3^+ cells, ANOVA was applied with 2-tailed Bonferroni adjusted comparisons to determine whether caspase-3^+ cells differed between TOI $<45\%$ versus $>45\%$, as well as between IL-6 <250 pg/mL versus >250 pg/mL, for each of the 3 stages. To ascertain the effects of TOI and IL-6 for each stage on cellular damage, we used logistic regression analysis, in which we set cutoff values to define the damage based on 2 SDs above the mean caspase-3^+ cells for the controls at each stage.

Detailed Methods

Detailed methods are described in the online-only Data Supplement.

Results

Maturation of Postnatal Porcine WM Is Area Dependent

There were significant differences in CC width and cell number (Figures 2A–2D). MBP expression increased significantly with age in CC, whereas expression of this protein in SCWM and IC was not different between age groups (Figures 2E–2H). Interestingly, between the 2 PVWM regions, a significant increase in MBP expression with age was detected in M-PVWM but not L-PVWM (Figure 2H). The number of Mash1^+Olig2^+ OPCs decreased significantly with age, and by 12 weeks of age, only a small percentage of Mash1^+Olig2^+ OPCs were present in all regions (Figures 2I through 2L and 2Q), which indicates that the endogenous OPC pool was decreased significantly at this age.

To analyze changes in oligodendrocyte lineage cell development with age, the number of Olig2^+ oligodendrocyte lineage cells and CC1^+ mature oligodendrocytes was determined at different developmental stages. The number of Olig2^+ oligodendrocytes in CC and M-PVWM increased significantly with age; in contrast, in the IC, the number of these cells decreased with age (Figures 2M through 2P and 2R). No significant developmental difference was detected in L-PVWM and SCWM (Figure 2R). Similarly, CC1^+ mature

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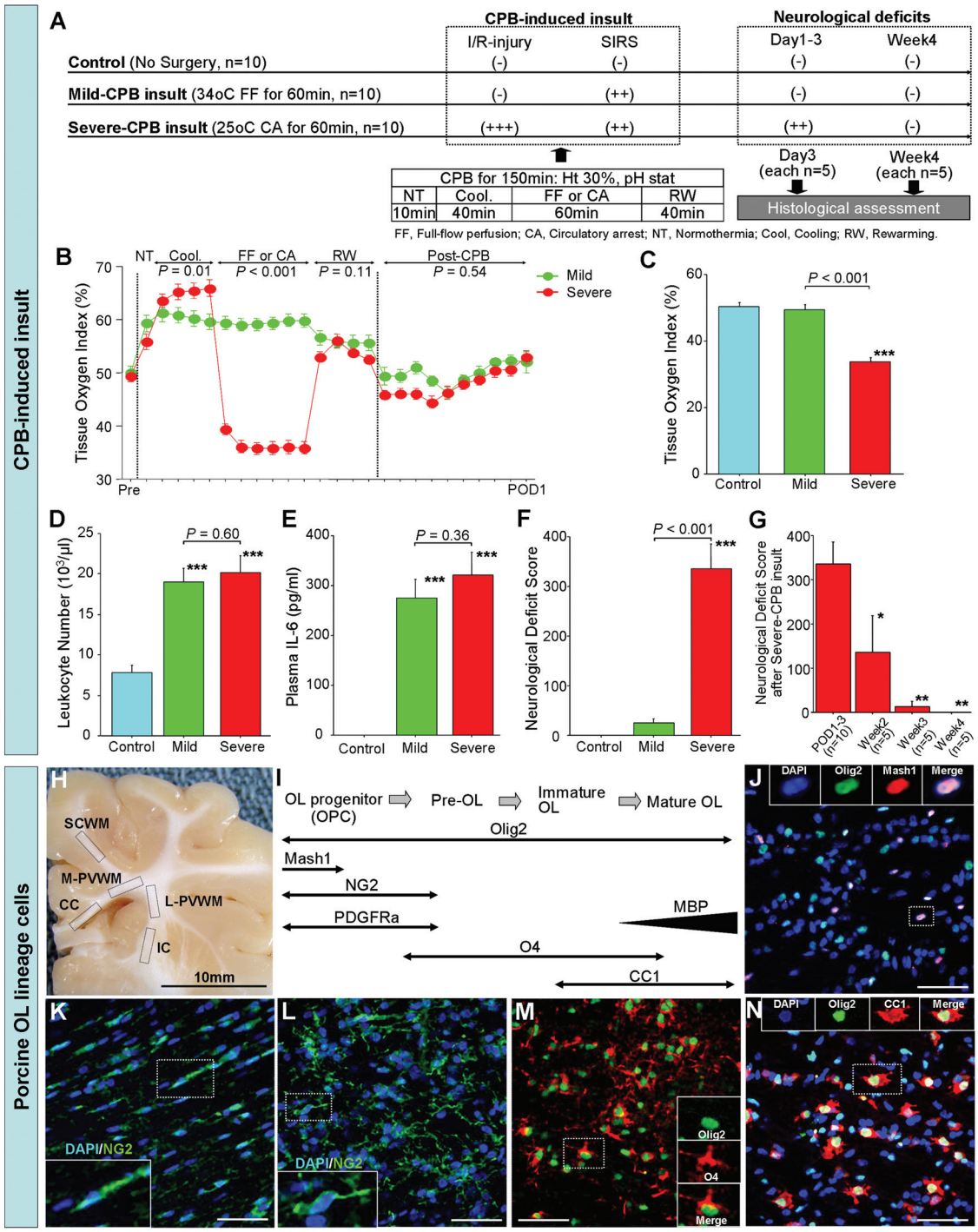

Figure 1. Cardiopulmonary bypass (CPB)–induced insult and porcine oligodendrocyte lineage cells. **A**, Study design of the CPB groups. FF indicates full-flow perfusion; CA, circulatory arrest; I/R injury, ischemia-reperfusion/reoxygenation injury; SIRS, systemic inflammatory response syndrome; Ht, hematocrit; NT, normothermia; and RW, rewarming. **B** and **C**, Tissue oxygen index during surgery and minimum values. **D** and **E**, Maximum leukocyte numbers and plasma interleukin-6 (IL-6) concentration. **F**, Neurological deficit score on postoperative days 1 through 3. **G,** Time course of deficit after severe CPB insult. POD1-3 indicates postoperative days 1 through 3. **H**, Subdivision of cerebral white matter at the level of precentral sulcus. SCWM indicates subcortical white matter; M-PVWM, medial periventricular white matter; L-PVWM, lateral periventricular white matter; CC, corpus callosum; and IC, internal capsule. **I**, Oligodendrocyte antibody markers used to immunostain distinct developmental stages of porcine oligodendrocyte (OL) lineage cells. MBP indicates myelin basic protein. **J**, Olig2$^+$Mash1$^+$ oligodendrocyte progenitor cells (OPCs). **K** and **L,** NG2$^+$ OPCs display spindlelike (**K**) or multipolar (**L**) process-bearing morphology. **M**, O4$^+$Olig2$^+$ preoligodendrocytes. **N**, Olig2$^+$CC1$^+$ mature oligodendrocytes. *$P<0.05$, **$P<0.001$ vs day 1 to 3, ***$P<0.001$ vs control by ANOVA with Bonferroni comparisons. Scale bar=50 μm. Data are shown as mean±SEM (B–F; n=10). More data are presented in online-only Data Supplement Figure III.

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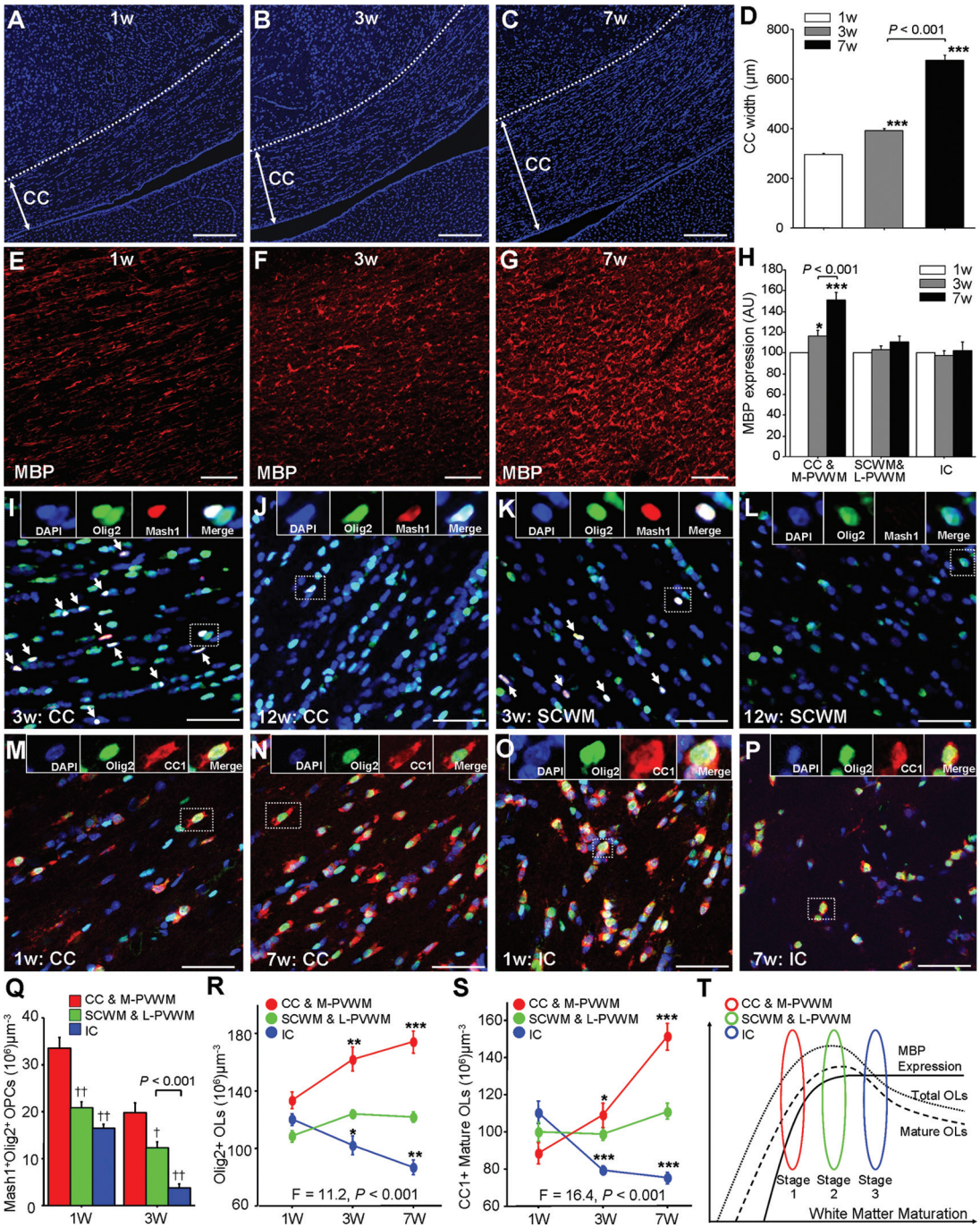

Figure 2. Porcine white matter (WM) maturation displays area-dependent progression. **A–D**, Corpus callosum (CC) width at 1, 3, and 7 weeks increases with age. **E–G**, Myelin basic protein (MBP) expression in CC in 3 age groups. **H,** Relative changes in MBP expression in 3 WM areas. AU indicates arbitrary units; M-PVWM, medial periventricular WM; SCWM, subcortical WM; L-PVWM, lateral periventricular WM; and IC, internal capsule. **I–L**, Olig2$^+$Mash1$^+$ oligodendrocyte progenitor cells (OPCs) at 3 and 12 weeks of age in CC and SCWM. **M–P**, Olig2$^+$CC1$^+$ mature oligodendrocytes at 1 and 7 weeks of age in CC and IC. **Q**, Mash1$^+$Olig2$^+$ OPC numbers in 3 WM maturational areas at 1 and 3 weeks of age. **R** and **S**, Changes in Olig2$^+$ oligodendrocyte lineage cells and in Olig2$^+$CC1$^+$ mature oligodendrocytes in 3 WM areas. OLs indicates oligodendrocytes. **T**, Porcine WM maturation based on time course of oligodendrocyte differentiation and MBP expression (stage 1, CC and M-PVWM; stage 2, SCWM and L-PVWM; stage 3, IC). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs 1week; †$P<0.01$, ††$P<0.001$ vs stage 1 by ANOVA with Bonferroni comparisons. Scale bar=50 μm. Data are shown as mean±SEM (n=5). Arrows indicate Olig2$^+$Mash1$^+$ OPCs. More data are presented in online-only Data Supplement Table I.

oligodendrocytes increased significantly in CC and M-SCWM with age but decreased significantly in IC (Figures 2M through 2P and 2S). Therefore, 3 distinct WM areas (CC and M-PVWM; L-PVWM and SCWM; IC) can be distinguished on the basis of developmental changes in Olig2$^+$ and CC1$^+$ cell numbers, respectively (Figures 2R and 2S).

Cellular processes underling WM maturation include expansion of differentiated oligodendrocytes and upregulation

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of myelin protein expression,[29,30] followed by a decrease in mature oligodendrocyte number[28] with maintenance of myelination[21,31] (Figure 2T). Thus, our findings described above suggest that porcine WM development occurs through 3 main maturational stages that involve distinct WM areas: Stage 1, CC and M-PVWM; stage 2, L-PVWM and SCWM; and stage 3, IC (Figure 2T). This pattern is also consistent with the significant reduction observed in the number of Mash1$^+$Olig2$^+$ OPCs between these 3 stages at 1 and 3 weeks of age, respectively (Figure 2Q). Altogether, these results indicate that the porcine model allows investigation of selective vulnerability of distinct stages of the oligodendrocyte lineage to injury, as well as cellular and biochemical analysis of different maturational stages of distinct WM regions.

Maturation-Dependent WM Vulnerability to Bypass-Induced Brain Insult

There were no differences among CPB groups in preoperative and perioperative conditions (online-only Data Supplement Table II). The minimum TOI value in the severe CPB insult group was significantly lower than in the control and mild CPB insult groups, although mild CPB insult did not induce cerebral ischemia (Figures 1B and 1C). Both mild and severe CPB insults caused a significant increase in leukocyte numbers and plasma IL-6 concentration relative to control, but no difference was detected between the 2 CPB groups (Figures 1D and 1E). Severe CPB insult caused acute neurological symptoms, including seizures, whereas mild CPB insult did not (Figure 1F). Neurological symptoms improved with time and were not detected in postoperative week 4 (Figure 1G).

WM injury was first assessed by evaluating the total number of cleaved caspase-3$^+$ cells on postoperative day 3. Caspase-3$^+$ cell numbers increased significantly in all WM regions after severe CPB insult compared with control but not after mild CPB insult (Figures 3A through 3C). The numbers of caspase-3$^+$ cells after severe CPB insult varied in distinct WM regions ($P<0.001$), being highest in M-PVWM and lowest in IC, respectively (online-only Data Supplement Table III). The degree of injury in a given area was inversely correlated with the maturation stage as shown in Figure 2T (Figure 3D). To identify the stage of the oligodendrocyte lineage most vulnerable to severe CPB insult, we performed double immunostaining with different stage-specific antibodies. O4$^+$ preoligodendrocytes displayed significantly greater susceptibility than other developmental stages of the oligodendrocyte lineage (Figures 3E through 3L). This susceptibility of O4$^+$CC1$^-$ preoligodendrocytes was also demonstrated by terminal dUTP nick end-labeling (TUNEL) assay (Figures 3M through 3O). Preoligodendrocyte damage contributed to approximately 50% of the total cellular damage after severe CPB insult in immature WM areas (Figure 3P). Although we were unable to costain Olig2 or NG2 with caspase-3 because of secondary antibody cross-reactivity, we identified no significant changes in Mash1$^+$PDGFRα^+ OPCs colabeled with anti-caspase-3 (Figures 3E, 3F, and 3I) and no morphological changes in NG2$^+$ OPCs after severe CPB insults, respectively (Figures 3Q and 3R). This indicates that OPCs are significantly resistant to CPB-induced insults.

Proliferation of OPCs After CPB

The total number of Olig2$^+$ oligodendrocyte lineage cells on postoperative day 3 was significantly affected by CPB and varied between WM regions ($P<0.001$; online-only Data Supplement Table IV). Olig2$^+$ cell number was increased significantly in PVWM and SCWM after both mild and severe CPB insult (Figures 4A through 4C), most likely because of an increase in Olig2$^+$ cell proliferation, as demonstrated by immunostaining with the proliferation marker Ki67 and counting of Olig2$^+$Ki67$^+$ cells in both PVWM and SCWM (Figures 4A, 4B, and 4D). Although recent studies indicate that the transcription factor Olig2 is expressed in reactive astrocytes in rodent injury model,[32,33] we did not observe Olig2$^+$GFAP$^+$ cells in any of the WM regions analyzed (online-only Data Supplement Figure VI). Altogether, these findings suggest that CPB-induced brain injury causes oligodendrocyte lineage cell proliferation in certain WM regions. Finally, Olig2$^+$ cells proliferated more robustly in PVWM and SCWM after severe CPB insult than after mild CPB insult (Figure 4D). This is consistent with the finding that Olig2$^+$ cell number in this region after severe CPB insult was higher than after mild CPB insult (Figure 4C), which suggests that severe CPB insult resulted in a prolonged proliferative effect on oligodendrocyte lineage cells.

To identify the specific cell populations that responded to CPB-induced insult, we assessed the effect of CPB on NG2-expressing (NG2$^+$) OPCs, which represent a highly proliferative stage of the oligodendrocyte lineage.[22,23,28] In both PVWM and SCWM, NG2$^+$ cell number was increased significantly after severe CPB insult (but not after mild CPB) compared with control (Figures 4E through 4G). In PVWM, there also was a significant difference between mild and severe CPB insults (Figure 4G). In addition, NG2$^+$Ki67$^+$ cell number in PVWM and SCWM increased significantly after severe CPB insult but not after mild CPB insult (Figures 4E, 4F, and 4H). Finally, consistent with the findings in NG2$^+$ cells, the percentage of Olig2$^+$Mash1$^+$ OPCs increased significantly after severe CPB insult in PVWM compared with control (Figures 4I through 4L). Taken together, our findings suggest that CPB induced proliferation of OPCs in developing porcine WM.

Arrested Oligodendrocyte Maturation and Delayed Myelination After Severe Bypass Insult

To assess the long-term effects of CPB on WM development, we analyzed changes in oligodendrocyte lineage cells at 4 weeks postoperatively. The number of Olig2$^+$ oligodendrocyte lineage cells was not significantly different among CPB groups, but it differed according to WM region and developmental stage ($P<0.001$; online-only Data Supplement Table IV). MBP expression after severe CPB insult was reduced significantly in all WM regions compared with control and mild CPB insult; conversely, mild CPB insult did not affect MBP expression in CC, PVWM, and SCWM (Figures 5A through 5D). Interestingly, MBP expression in IC was slightly increased after mild CPB insult ($P=0.01$; online-only Data Supplement Table III). Consistent with these findings, severe CPB insult significantly reduced the number of CC1$^+$ mature oligodendrocytes compared with control and mild

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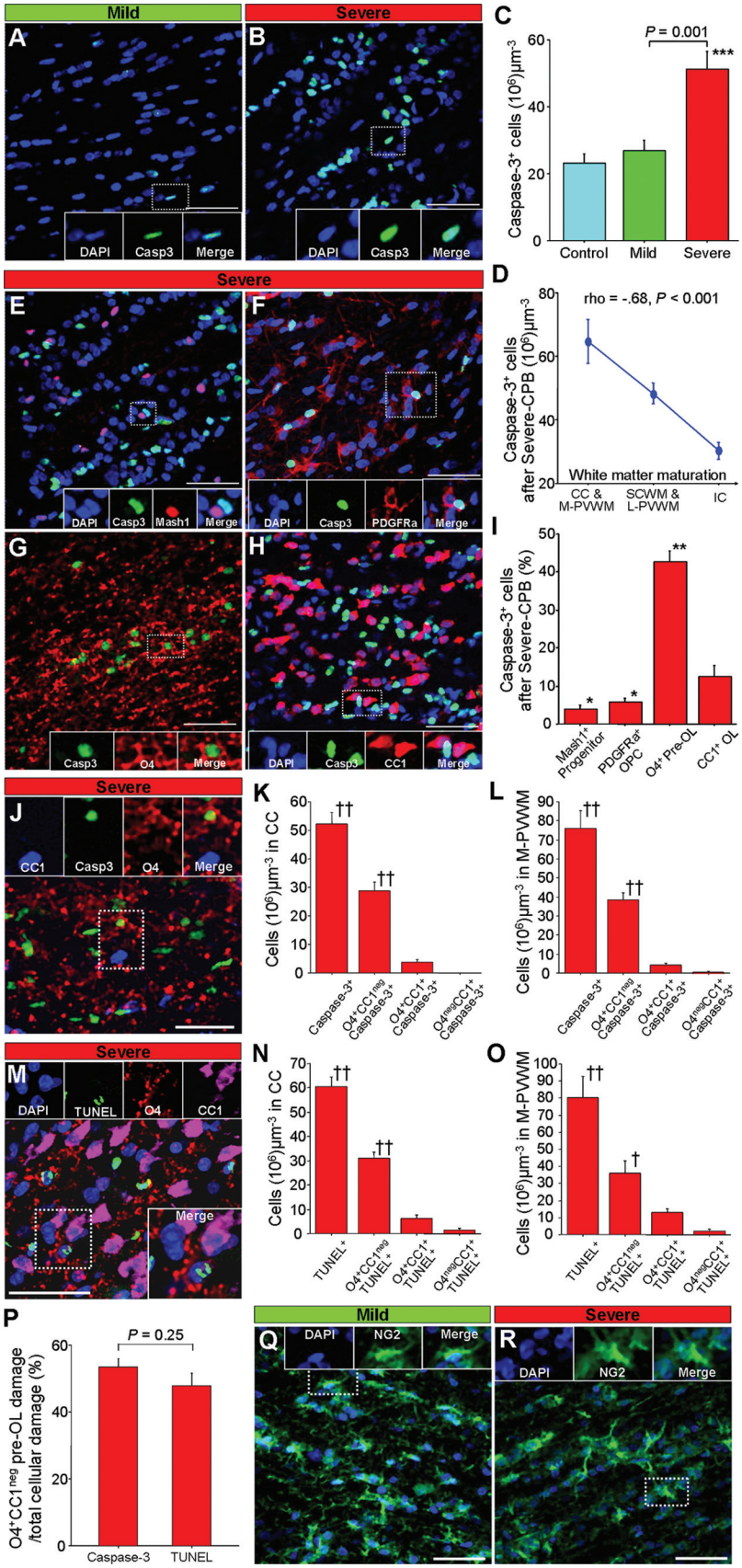

Figure 3. Developing porcine white matter (WM) displays maturation-dependent vulnerability to cardiopulmonary bypass (CPB)–induced insults. **A** and **B**, Caspase-3$^+$ cells (Casp3) in corpus callosum (CC) in different CPB groups. **C**, Caspase-3$^+$ cell number after CPB-induced insult. **D**, Relationship between caspase-3$^+$ cell number after severe CPB insult and WM maturation. M-PVWM indicates medial periventricular WM; SCWM, subcortical WM; L-PVWM, lateral periventricular WM; and IC, internal capsule. **E–H**, Coimmunostaining of caspase-3$^+$ with different oligodendrocyte lineage antibody markers, including Mash1, PDGFRa, O4, and CC1. **I**, Percentage of caspase-3$^+$ cells immunostained with oligodendrocyte antibodies. OPC indicates oligodendrocyte progenitor cell; OL, oligodendrocyte. **J–L**, Image of M-PVWM stained with caspase-3, O4, and CC1 on day 3 after severe CPB insult and numbers of different caspase-3$^+$ cells in CC and M-PVWM. **M–O,** Image of CC stained with terminal dUTP nick end-labeling (TUNEL), O4, and CC1 on day 3 after severe CPB insult and numbers of different TUNEL-positive cells in CC and M-PVWM. **P**, Percentage of apoptotic O4$^+$ CC1$^-$ preoligodendrocytes per total apoptotic cells in CC and M-PVWM after severe CPB insult, as determined by caspase-3 and TUNEL assays. **Q** and **R,** Images of NG2$^+$ OPCs in M-PVWM after mild and severe CPB insults. *$P<0.05$ vs O4$^+$ and CC1$^+$, respectively; **$P<0.001$ vs Mash1$^+$, PDGFRα^+, and CC1$^+$, respectively; ***$P<0.001$ vs control; †$P<0.05$, ††$P<0.001$ vs other 3 groups by ANOVA with Bonferroni comparisons. Data are shown as mean±SEM (n=5). More data are presented in online-only Data Supplement Table III and online-only Data Supplement Figure V.

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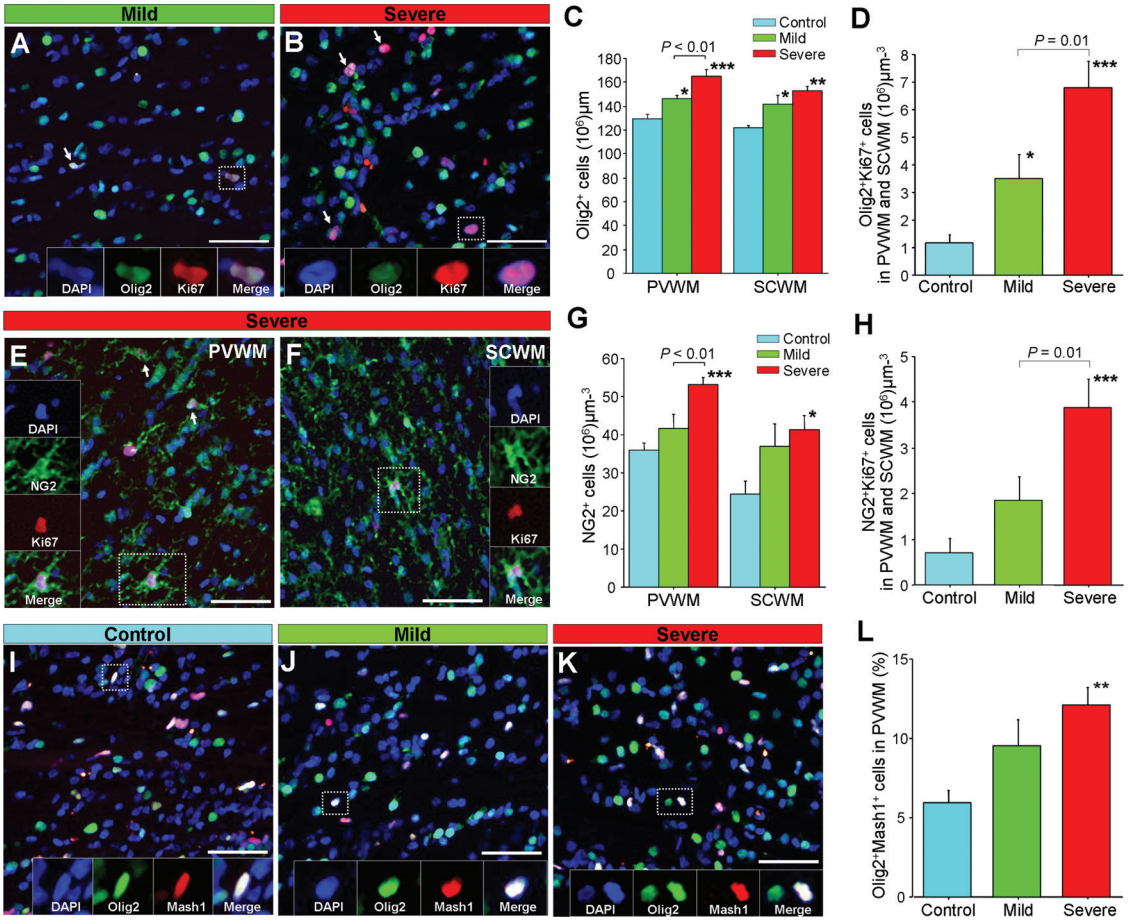

Figure 4. Cardiopulmonary bypass (CPB) induces proliferation of oligodendrocyte progenitor cells in white matter (WM) on postoperative day 3. **A** and **B,** Olig2$^+$Ki67$^+$ cells in medial periventricular WM (M-PVWM) after mild and severe CPB insults. **C**, Number of periventricular WM and subcortical WM (SCWM) Olig2$^+$ cells in different CPB groups. **D**, Number of Olig2$^+$Ki67$^+$ cells in PVWM and SCWM. **E** and **F**, PVWM and SCWM NG2$^+$Ki67$^+$ cells after severe CPB insult. **G**, PVWM and SCWM NG2$^+$ cell number in different CPB groups. **H**, Number of PVWM and SCWM NG2$^+$Ki67$^+$ cells in different CPB groups. **I–K**, M-PVWM Olig2$^+$Mash1$^+$ cells in different CPB groups. **L**, Percentage of PVWM Olig2$^+$Mash1$^+$ cells in different CPB groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control by ANOVA with Bonferroni comparisons. Scale bar=50 μm. Data are shown as mean±SEM (n=5). More data are presented in online-only Data Supplement Table III.

CPB insult; in contrast, the number of CC1$^+$ cells was not modified after mild CPB insult compared with control (Figures 5E through 5H). When Olig2$^+$CC1$^-$ immature oligodendrocyte number was assessed, severe CPB insult significantly induced an increase in their number compared with control and mild CPB insult in 4 of 5 WM regions analyzed (Figure 5I). This suggests that as previously observed in the rodent ischemia model,[34] severe CPB insult caused arrested oligodendrocyte maturation and/or delayed myelination in the porcine brain.

The change in Olig2$^+$ oligodendrocyte lineage cell numbers from day 3 to week 4 significantly and independently differed according to either WM region or type of CPB insult (Figures 5J and 5K). Severe CPB insult significantly reduced Olig2$^+$ oligodendrocyte lineage cell number from day 3 to week 4 in PVWM and SCWM, most likely through a decrease in Olig2$^+$CC1$^+$ mature oligodendrocyte numbers (Figure 5J). On the other hand, in IC, both mild and severe CPB insults increased the number of Olig2$^+$ oligodendrocyte lineage cells, as opposed to the decline seen under normal development (Figure 5K). However, distinct populations of oligodendrocyte lineage cells in IC differed in their response to the 2 types of insults (Figure 5L). Mild CPB insult significantly increased Olig2$^+$ cells and CC1$^+$ mature oligodendrocytes but had no effect on Olig2+CC1$^-$ immature oligodendrocytes (Figures 5K and 5L). In contrast, severe CPB insult caused an increase in Olig2$^+$ oligodendrocyte lineage cells, which likely resulted from a significant expansion of Olig2$^+$CC1$^-$ immature oligodendrocytes (Figures 5K and 5L). When taken together, these findings suggest that the oligodendrocyte lineage cellular response after CPB varies according to WM region.

Acute Neuronal Damage in Cortex and Long-Term Axonal Injury in WM

To address the effect of CPB on WM axon and neuron-axonal elements, we next assessed axonal injury by morphological analysis using the pan-neurofilament marker SMI-312. A previous study in an ovine model demonstrated acute morphological changes after ischemic WM injury.[35] In the present study, however, we did not find any morphological changes in either CPB group on postoperative day 3 (Figures

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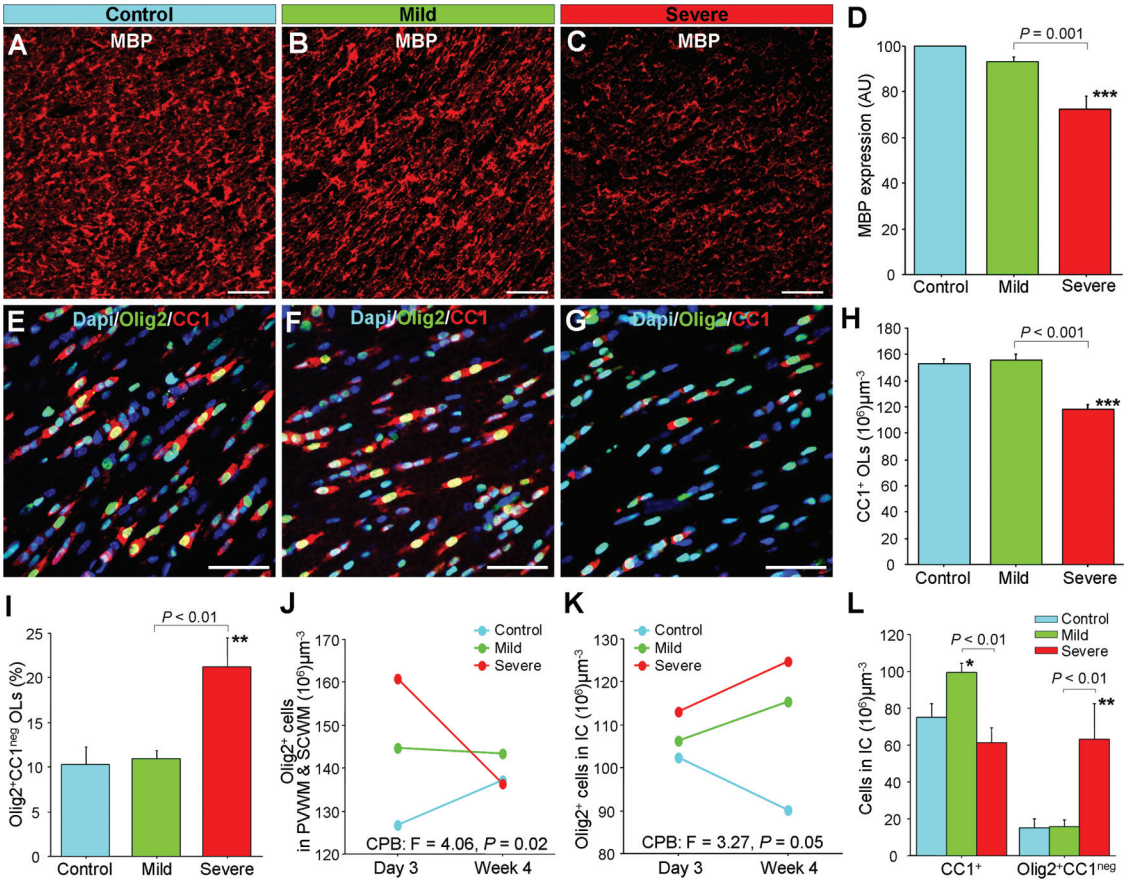

Figure 5. Arrested oligodendrocyte maturation and delayed myelination occur at 4 weeks postoperatively in severe cardiopulmonary bypass (CPB) insult. **A–D**, Myelin basic protein (MBP) expression in corpus callosum (CC) of different CPB groups and relative changes within these groups. **E–H**, CC Olig2$^+$CC1$^+$ cells in different CPB groups and density of CC CC1$^+$ mature oligodendrocytes within these groups. **I**, Olig2$^+$CC1$^-$ immature oligodendrocyte percentage in CC. **J** and **K**, Change of Olig2$^+$ cell density in periventricular white matter (PVWM) and subcortical white matter (SCWM; **J**) and in internal capsule (IC; **K**) after CPB. **L**, IC CC1$^+$ and Olig2$^+$CC1$^-$ cell density in different CPB groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control by ANOVA with Bonferroni comparisons. Scale bar=50 μm. Data are shown as mean±SEM (n=5). More data are presented in online-only Data Supplement Table III.

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6A–6C). On the other hand, we did observe that severe CPB insult induced a significant increase in cortical neuronal injury, as assessed by NeuN$^+$caspase-3$^+$ cell number in cortex layer II/III and V/VI, compared with control and mild CPB insult, respectively (Figures 6D through 6F, 6J, and 6K).

Myelinated axons normally contain phosphorylated neurofilaments, whereas under pathological conditions, axons display increased levels of nonphosphorylated neurofilaments, as identified by the marker SMI-32.[36] In our porcine model, we did not observe an increase in axonal SMI-32 at 3 days after CPB in any of the groups analyzed; however, at 4 weeks after severe CPB insult, there was a significant increase in SMI-32$^+$ neurofilaments in SCWM and PVWM compared with control and mild CPB insult (Figures 6G–6I and 6L), which indicates that severe CPB insult caused long-term axonal injury.

Effects of I/R Injury and Systemic Inflammation at Different Stages of WM Development

Finally, we investigated the effects of CPB-induced I/R injury and SIRS in different WM maturational stages, as shown in Figure 2T. Ischemia, as indicated by a low TOI (<45%), had a significant effect on caspase-3$^+$ cell number ($P<0.001$); however, this effect was only observed for stages 1 and 2 and not for stage 3 (Figure 7A). In addition, when TOI was less than 45%, significant differences in the number of caspase-3$^+$ cells were observed between distinct maturational stages of the WM, with stage 1 being higher than stage 2 and 3, and stage 2 higher than stage 3 (Figure 7A). Conversely, when TOI was higher than 45%, there were no significant differences in caspase-3$^+$ cell number between the 3 maturational stages (Figure 7A).

We also investigated the effects of inflammation on WM injury at the cellular level. A high level of inflammation, as indicated by an IL-6 level >250 pg/mL, had a significant effect on caspase-3$^+$ cell number ($P<0.001$); however, this effect was observed only for stage 1, and not for stages 2 or 3 (Figure 7B). In addition, when IL-6 was >250 pg/mL, there were significant differences in caspase-3$^+$ cell numbers between distinct maturational stages (Figure 7B). However, when IL-6 was <250 pg/mL, no significant differences in caspase-3$^+$ cells were detected (Figure 7B). Furthermore, the present results demonstrated that CPB management under higher values of TOI (>45%) and lower IL-6 levels (<250 pg/mL) resulted in significant decreases in caspase-3$^+$ cell number not only in WM but also in cerebral cortex (Figures 7C and 7D).

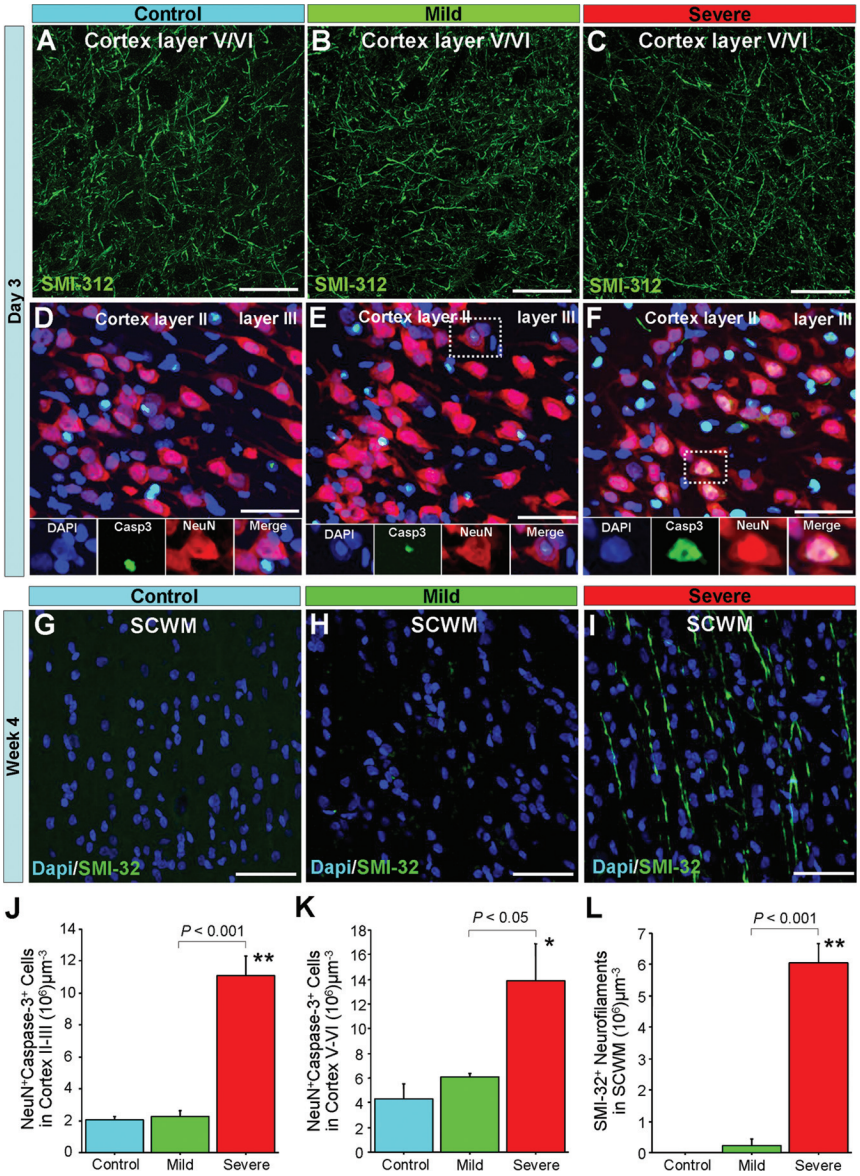

Figure 6. Severe cardiopulmonary bypass (CPB) insult results in acute cortical neuronal injury and long-term white matter axonal injury. **A–C,** SMI-312$^+$ neurofilaments in cortex layer V/VI on postoperative day 3 in different CPB groups. **D–F,** NeuN$^+$caspase-3$^+$ cells in cortex layer II/III on postoperative day 3 in the CPB groups. **G–I,** SMI-32$^+$ neurofilaments in subcortical white matter (SCWM) at week 4 after surgery in the CPB groups. **J** and **K,** NeuN$^+$caspase-3$^+$ cell number in cortex layer II/III (**J**) and V/VI (**K**). **L,** Density of SMI-32$^+$ neurofilaments in SCWM in the CPB groups. *$P<0.01$, **$P<0.001$ vs control by ANOVA with Bonferroni comparisons. Data are shown as mean±SEM (n=5). More data are presented in online-only Data Supplement Table III and online-only Data Supplement Figure VIII.

Logistic regression confirmed a significant increase in the risk of WM damage for lower TOI values according to both type of bypass insult and developmental stage. The probability curves illustrate that TOI was much more predictive of WM damage for less mature stages, in which there was greater vulnerability to damage (Figure 7E). Differences in the probability of damage between maturational stages were more pronounced when TOI levels were very low, whereas for higher TOI levels, the probability of damage was lower, and the differences in risk between stages were much smaller (Figure 7E). Interestingly, for stage 1, the probability of WM damage was high even for higher values of TOI >45%.

The statistical model that includes both IL-6 and maturation stage indicated a positive relationship between IL-6 and probability of damage and an inverse relation between damage and maturational stage (Figure 7F). Interestingly, the damage probability due to inflammation was similar between stage 1 and 2, with stage 3 showing lower probability of damage compared with earlier stages given the same levels of IL-6 (Figure 7F).

Discussion

This study is the first to reveal region-specific WM development in the porcine brain and to define acute and long-term cellular responses of WM oligodendrocyte lineage cells and neuron-axonal elements to CPB using this animal model, which is anatomically and physiologically close to humans. The present analysis identifies a uniquely susceptible cellular target of CPB-induced WM injury within the oligodendrocyte lineage, as well as an oligodendrocyte developmental stage resistant to the injury. Finally, our studies also define an optimal strategy of CPB management for WM protection, thus contributing to reduced neurological injury in CHD patients.

Because of reduced mortality after complex pediatric cardiac surgery, a current challenge is to reduce or eliminate long-term sequelae, particularly neurodevelopmental delays.[4] Clinical studies suggest that achieving this goal will require an improved understanding of the effects of CPB on developing WM.[4,8–10,19] However, lack of laboratory-based inves-

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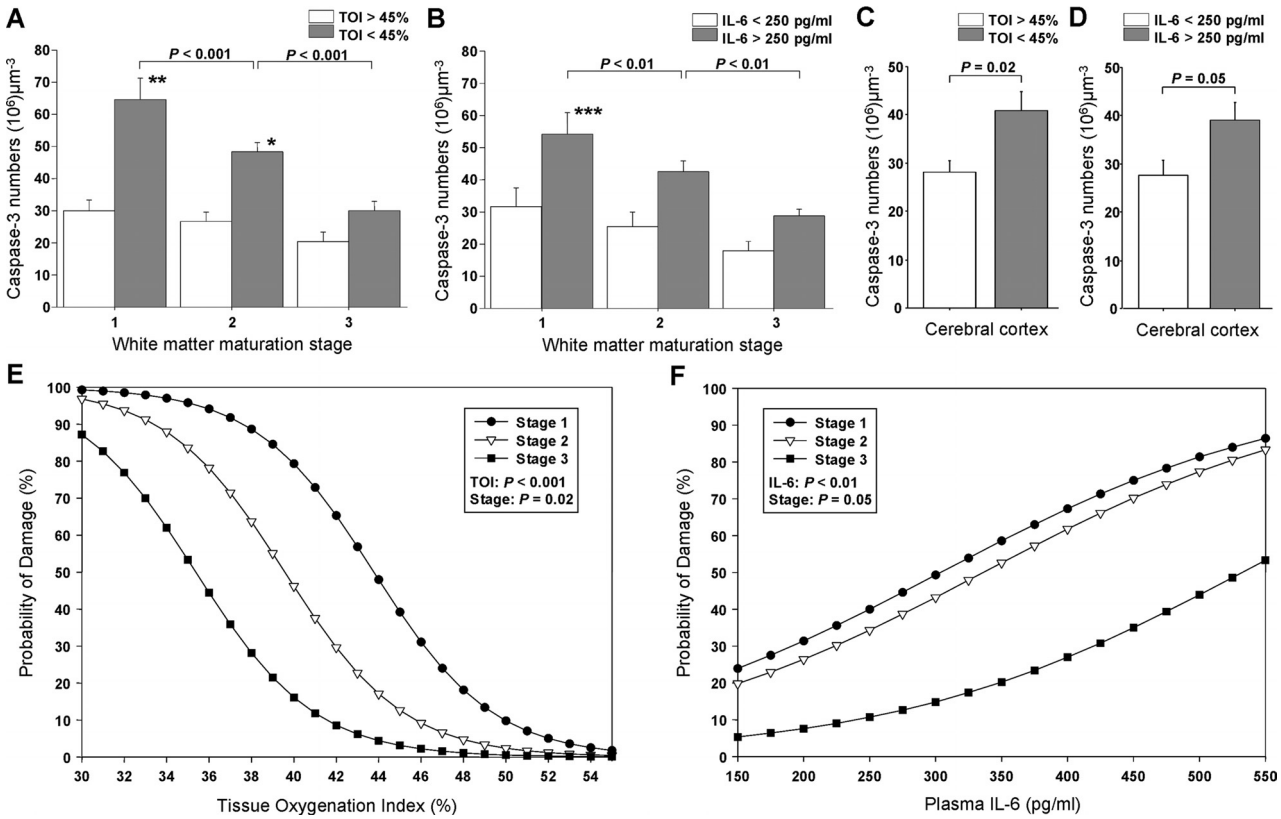

Figure 7. Maintaining high tissue oxygen index (TOI) and reducing inflammation minimize the risk of cardiopulmonary bypass (CPB)–induced injury at immature stages of white matter (WM) development. **A** and **B**, Effects of minimum TOI and maximum plasma interleukin-6 (IL-6) concentration on total cell death (caspase-3$^+$ cells) at 3 different stages of WM maturation. **C** and **D,** Effects of minimum TOI and maximum plasma IL-6 level on cell death in cerebral cortex. **E** and **F**, Damage probability curves as a function of TOI and IL-6 level at 3 different stages of WM maturation. These stages are defined as shown in Figure 2T. Stage 1 corresponds to corpus callosum and medial periventricular WM; stage 2, subcortical WM and lateral periventricular WM; and stage 3, internal capsule. For example, a bypass corresponding to a TOI of 40% is associated with a probability of damaging nearly 80% for stage 1, 45% for stage 2, and only 15% for stage 3 (**E**). IL-6 level of 400 pg/mL is associated with a probability of 68% for stage 1 and only 25% for stage 3 (**F**). *$P<0.05$, **$P<0.001$ for TOI >45% vs <45%; ***$P<0.05$ for IL-6 <250 pg/mL vs >250 pg/mL by ANOVA with Bonferroni comparisons. Data are shown as mean±SEM (n=10).

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tigation has prevented the identification of the causes of WM injury and of the cellular and molecular substrates altered by CPB. These questions need to be investigated in an animal model that replicates the clinically relevant effects of CPB on brain development.[17,25] Our findings indicate that porcine WM development displays area-dependent maturation similar to the human WM.[30] This suggests that the porcine brain not only has an anatomic structure similar to the human brain but also parallels its developmental progression. A lack of cellular investigations in postnatal human brain because of technical and ethical difficulties, however, prevented us from defining the epochs in human WM development that correspond to our model. Future studies using, for example, magnetic resonance imaging, will be necessary to identify corresponding developmental time windows between humans and this model. The relevance of this model to human development will allow the application of the tremendous advances in cellular and molecular methods derived from rodent studies to clinically relevant questions regarding neurodevelopmental disorders.

The present results demonstrate that severe CPB insult together with I/R injury and SIRS poses a significant risk of WM damage and that vulnerability to this injury varies according to WM maturational stage. Interestingly, this finding is consistent with other types of developmental brain injuries, such as periventricular leukomalacia in premature birth, which also display maturation-dependent WM vulnerability.[29,37] Our results in porcine brain demonstrate that O4$^+$ preoligodendrocytes exhibit significant vulnerability to bypass-induced insults and identify this oligodendrocyte developmental stage as a target for cellular protection. The susceptibility of O4$^+$ preoligodendrocytes to oxidative stress and hypoxia has been well recognized in animal models such as rodents or fetal sheep.[35,38] Although the expression level of glutamate receptors on preoligodendrocytes is a possible cause of this susceptibility,[39] the mechanism of damage is not fully understood. An understanding of the cellular and molecular mechanisms of preoligodendrocyte susceptibility to damage will assist in developing novel and adjunctive brain protection approaches during CPB.

In contrast to the susceptibility of preoligodendrocytes, we observed that OPCs are highly resistant to CPB-induced insult compared with other oligodendrocyte developmental stages. Furthermore, we also found that CPB induced OPC proliferation in porcine PVWM and SCWM. It is well known that OPCs mediate endogenous myelin repair after WM

injury.[22,23] Interestingly, the number of OPCs in 12-week-old piglets was significantly reduced compared with age 1 and 3 weeks, respectively, which suggests that the capacity for regenerative response mediated by OPCs after injury[22,23] decreases with age.[40] Thus, immature porcine WM is vulnerable to CPB-induced injury but also retains a significant endogenous cellular potential for recovery. Many of the molecular pathways that induce and sustain the OPC regenerative response after WM injury have been identified,[21,23,28,41,42] and it will be important to determine whether these molecular mechanisms are also conserved in porcine OPCs to design pharmacological approaches that promote WM repair through mobilization of endogenous OPC pools.

On the basis of clinical magnetic resonance imaging studies that have demonstrated a high incidence of WM injury after neonatal cardiac surgery,[13–15] the current controversy is whether surgery should be avoided at this age.[4] This is a question of great significance, because surgical repair of CHD in the newborn (primary repair) has enormous advantages not only for organ maturation other than the brain in the early years of life,[43–45] but also for the family[46] and costs to society[47] compared with delayed 2-stage repair (palliative surgery). It has been recognized recently that abnormal cerebral circulation due to CHD results in significant brain abnormalities in utero.[14,48] These observations imply that we should pursue early normalization of cerebral circulation by primary repair. The present results suggest that the optimal time window for CHD repair to allow appropriate WM maturation is the period during which the WM contains the largest number of endogenous OPCs, which are highly resistant to CPB-induced brain insults and are available to promote endogenous myelin repair. Furthermore, in contrast to palliative surgery, early normalization of cerebral circulation by primary repair has the potential to promote recovery of the neurodevelopmental delay detected in term newborns with CHD.[19,48,49]

One of the major issues raised by the present study is how to manage surgery with CPB in the neonate and young infant, who are uniquely susceptible to the deleterious effects of CPB on WM development. Indeed, the present results indicate that immature WM regions are more vulnerable to both bypass-induced I/R injury and SIRS than mature regions. A recent clinical study has also identified that immature brain is associated with brain injury after CPB.[15] With higher TOI (>45%) and lower levels of IL-6 (<250 pg/mL), however, no significant differences in caspase-3$^+$ cell numbers were detected during WM maturation. This suggests that when higher cerebral oxygenation and lower inflammation are maintained, the maturation stage is not a crucial determinant of CPB-induced WM injury. Furthermore, logistic regression confirmed that maintenance of high TOI and low levels of inflammation were effective in minimizing the risk of injury in the early stages of WM development. In the present study, we defined maturational stage 1 as a developmental time window in which both MBP expression and mature oligodendrocyte numbers are increasing. In human WM, MBP expression has been detected at approximately 30 weeks of gestation and increases during the third trimester, continuing throughout childhood.[11,29] Although term newborns with CHD have a delay in brain development of approximately 1 month,[48,49] the period described as stage 1 likely corresponds with the age at which neonatal cardiac surgery is necessary. Thus, the present results suggest that cardiac surgery in the neonate and young infant should avoid I/R injury, and efforts should be made to maintain high cerebral oxygenation and reduce inflammation.

In the present study, we demonstrated that hypomyelination occurs at 4 weeks after surgery in the group with CPB-induced I/R injury with SIRS. At 3 days after surgery, we identified WM oligodendrocyte and cortical neuronal injury, as well as increased WM OPC proliferation in the severe CPB group. However, the expansion of the endogenous OPC pool on day 3 did not result in rescue of long-term CPB-induced hypomyelination, which suggests arrested oligodendrocyte lineage maturation. These findings are consistent with preoligodendrocyte accumulation after perinatal I/R injury in a rodent model.[34] Interestingly, Segovia et al[34] demonstrated that arrested oligodendrocyte maturation conferred enhanced susceptibility to recurrent hypoxia. Complex CHD patients are at greater risk of ischemia and inflammation followed by recurrent hypoxia.[6] In addition, hypoxia experienced by CHD babies in utero[6,19] potentially results in arrested oligodendrocyte maturation before birth. This might help to explain at a cellular level the recent clinical findings that brain injury after CPB is impacted by preoperative brain maturation.[15] In the present study, we were unable to test this hypothesis, because our model was based on animals without CHD. Therefore, other models and methods will be necessary to investigate the effects of CPB on WM with arrested oligodendrocyte maturation. Understanding the mechanisms of arrested oligodendrocyte maturation and the effects of CPB on the brain will contribute to further development of treatment strategies in CHD patients.

Recently, endogenous WM repair mechanisms, including promotion of remyelination by enhanced signaling systems[28] and functional recovery of WM axons by OPCs migrating from the subventricular zone,[50] have been described in rodent models.[22,23,41] For successful WM development in CHD patients, it is imperative to understand how CHD and subsequent surgery affect these endogenous repair mechanisms and developmental cellular processes. Future studies using our porcine model will provide novel insights of cellular and molecular mechanisms in human brain development and allow for the design of targeted therapies and conditions to improve neurodevelopmental disorders.

Acknowledgments

We are thankful to Dr Li-Jin Chew for critically reading this manuscript and to all our colleagues at the Center for Neuroscience Research for discussion and for their support. We thank colleagues of the Children's National Medical Center Research Animal Facility for their support with porcine survival studies.

Sources of Funding

This work was supported in part by National Institutes of Health grants R01HL060922 (to Dr Jonas), R01HL104173 (to Dr Jonas), R01NS045702 (to Dr Gallo), R01NS056427 (to Dr Gallo), P01NS0626860 (to Dr Gallo), and K08NS073793 (to Dr Scafidi) and by National Institutes of Health Intellectual & Developmental Disabilities Research Center grant P30HD40677 (to Dr Gallo).

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Disclosures

None.

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CLINICAL PERSPECTIVE

The most common neurological deficits in children after surgery for congenital heart disease are fine and gross motor deficits. Recent magnetic resonance imaging studies have demonstrated a significant number of newly developed white matter (WM) lesions in infants after surgery. The present study describes region-specific WM development in the juvenile porcine brain, which is similar in developmental stage to the human newborn. Acute and long-term cellular responses to cardiopulmonary bypass in oligodendrocyte lineages and neuron-axonal elements, which are the most prominent cell populations in WM, have been observed. A uniquely susceptible cellular target of cardiopulmonary bypass–induced WM injury in the oligodendrocyte lineage, as well as maturation-dependent vulnerability of developing WM, was found. Oligodendrocyte progenitor cells, which mediate WM recovery function, are highly resistant to cardiopulmonary bypass–induced injury. Interestingly, oligodendrocyte progenitor cell number decreases with age, which suggests that immature WM is vulnerable but also retains a significant endogenous cellular potential for recovery. Therefore, the optimal time window for congenital heart disease repair is the period during which WM contains the largest number of oligodendrocyte progenitor cells. Importantly, it was identified that under conditions of higher cerebral oxygenation and lower inflammation, the maturation stage was not a crucial determinant of cardiopulmonary bypass–induced WM injury. Together with the recent clinical finding that newborns with congenital heart disease have delayed brain development due to abnormal cerebral circulation in utero, the present study suggests that earlier normalization of cerebral circulation by primary congenital heart disease repair using higher cerebral oxygenation and lower inflammation should improve WM development in this population.

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