



# Multi-Hit White Matter Injury-Induced Cerebral Palsy Model Established by Perinatal Lipopolysaccharide Injection

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### Specialty section:

This article was submitted to  
Pediatric Neurology,  
a section of the journal  
Frontiers in Pediatrics

Received: 01 February 2022

Accepted: 09 May 2022

Published: 06 June 2022

### Citation:

Liu L, Fang L, Duan B, Wang Y,  
Cui Z, Yang L and Wu D (2022)  
Multi-Hit White Matter Injury-Induced  
Cerebral Palsy Model Established by  
Perinatal Lipopolysaccharide  
Injection. *Front. Pediatr.* 10:867410.  
doi: 10.3389/fped.2022.867410

Cerebral palsy (CP) is a group of permanent, but not unchanging, disorders of movement and/or posture and motor function. Since the major brain injury associated with CP is white matter injury (WMI), especially, in preterm infants, we established a “multi-hit” rat model to mimic human WMI in symptomatology and at a histological level. In our WMI model, pups suffering from limb paresis, incoordination, and direction difficulties fit the performance of CP. Histologically, they present with fewer neural cells, inordinate fibers, and more inflammatory cell infiltration, compared to the control group. From the electron microscopy results, we spotted neuronal apoptosis, glial activation, and myelination delay. Besides, the abundant appearance of IBA1-labeled microglia also implied that microglia play a role during neuronal cell injury. After activation, microglia shift between the pro-inflammatory M1 type and the anti-inflammatory M2 type. The results showed that LPS/infection stimulated IBA1 + (marked activated microglia) expression, downregulated CD11c + (marked M1 phenotype), and upregulated Arg 1 + (marked M2 phenotype) protein expression. It indicated an M1 to M2 transition after multiple infections. In summary, we established a “multi-hit” WMI-induced CP rat model and demonstrated that the microglial activation correlates tightly with CP formation, which may become a potential target for future studies.

**Keywords:** cerebral palsy, perinatal infection, white matter injury, animal model, electron microscopy

## INTRODUCTION

Cerebral palsy (CP) is a permanent (not unchanging) motor dysfunction and postural abnormality caused by non-progressive disturbance or developing abnormality of the immature brain, with an average prevalence of 1.5–3.0‰ in neonates (1). In a meta-analysis of China, the prevalence of CP over the 32 years from 1988 to 2022 was 2.07‰ among 0–18 years old (2). Motor dysfunction, which is the main syndrome of CP, is often accompanied by intellectual disability, behavioral problems, sensation or perceptual disorders, epilepsy, etc. (3, 4). For now, there is no consensus on the upper

age limit of brain disturbances for the definition of CP. Children over 2 years of age who acquired brain damage may already have certain skills, and the resulting disability can be very different (5). Due to the high plasticity of the developing brain, therapies and rehabilitation of infants with CP should be started as soon as possible (6). For children with severe cerebral palsy, costly and long-term treatments are too burdensome for the whole family to carry on. Identifying the etiology can help diagnose CP as early as possible and even prevent it from occurring.

As a clinical syndrome, CP etiology varies, but the precise cause remains obscure. The risk factors affecting the central nervous system (CNS) in early development are mainly divided into the following categories: external environment, the physical condition of the mother, prenatal, perinatal, as well as postnatal risk factors (1). Premature birth, hypoxia-ischemia, placental insufficiency, and perinatal infection, such as chorioamnionitis, are well-studied causes of CP (7). Among the various CNS damages caused by risk factors mentioned above, white matter injury (WMI) is the major one that leads to CP. Especially in very premature infants born before 28 weeks, the incidence of CP is as high as 13–17% (5, 8). WMI is characterized by hypomyelination, astrogliosis, and microgliosis. Due to the vulnerability of myelin sheath, many risks including cerebral ischemia/reperfusion and perinatal infection could contribute to the pathogenesis of WMI, making it hard to maintain neural signaling stability (7, 9, 10). So, commonly used rat models of WMI mainly include perinatal hypoxia and inflammation. Lipopolysaccharide (LPS), the main component of the gram-negative bacterial cell wall, is widely used in the induction of inflammation, although inflammation is often induced prenatally, applied alone (11, 12) or in combination with hypoxia (13–15). Few studies of CP have applied LPS both prenatally and postnatally.

Microglia are immunocompetent cells that reside in the CNS representing 10% of brain cells (16). They have neuroprotective and neurotoxic effects and are considered “CNS macrophages” (17). They can be activated into “classical activation” M1 type and “surrogate activation” M2 type in different circumstances. M1 polarization results in the release of inflammatory factors such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , triggering an inflammatory cascade and aggravating brain damage, while M2 microglia secrete anti-inflammatory cytokines and neurotrophic factors to reduce the inflammatory response, protect neurons, and promote tissue repair (18, 19). When infection occurs, increased IL-6 and TNF- $\alpha$  induce microglial activation to the M1 type and release more pro-inflammatory factors, amplifying the inflammatory cascade by acting on nearby non-activated microglia, causing a vicious cycle that contributes to secondary brain injury (20, 21). Studies found that activated microglia impede oligodendrocyte lineage progression, reduce the production of myelin binding protein, and impair myelination (22–24). One of the theories showed that microglial activation would enhance the notch signaling pathway and inhibit oligodendrocyte differentiation and myelination (25–27). However, there are still many unknowns worth exploring.

As far as we know, microglia function as a double-edged sword. The role of microglial activation and polarization in the formation of cerebral palsy needs to be further studied. Here, we established a “multi-hit” WMI-induced CP rat model

to investigate the role of microglia activation and imbalance in the formation of CP, providing a potential target for the treatment of CP.

## MATERIALS AND METHODS

### Animals and Models

Healthy Sprague–Dawley rats of childbearing age were purchased from Beijing Vital River Animal Center, SCXK: (Jing) 2016-0006, including 10 males of 290–300 g and 20 females of 240–250 g. The experimental animals were housed in a standard animal center of (23  $\pm$  2) $^{\circ}$ C and 55% humidity, with free access to food and water, maintained on a diurnal rhythm of 12 h light and 12 h dark. After 1 week of adaptive feeding, rats were co-caged according to the ratio of male and female 1:2, lights off for 12 h, and the vaginal smears were examined the following day to count as gestational day 1 when sperm plugs were found. Fourteen pregnant rats were randomly divided into two groups, one group was intraperitoneally injected with 230  $\mu$ g/kg LPS (Sigma, 16511, Serotype typhimurium, United States) suspended in saline at 20 days of gestation (G20) and other group was injected with the same volume of saline. The LPS dose (230  $\mu$ g/kg) was chosen based on preliminary studies. Then, the respective offspring were treated as experimental and control groups (the mortality of the control group was 2.3%, while the experimental group was 24.6%). The pups in the experimental group received an intraperitoneal injection of 3 mg/kg LPS in saline at a postnatal age of 20 days (P20), while the control group received 3 ml/kg saline. To mimic the effects of repeated inflammatory infection in the early stage of development, we divided the experimental group into three groups at 40 days of age (P40), and the low-dose group was injected with saline, the middle dose group was injected with 3 mg/kg LPS, the high-dose group was injected with 5 mg/kg LPS, and the control group was injected with an equal amount of saline (**Figure 1**). The experiments were approved by the Experimental Animal Ethics Committee of Anhui Medical University: LLSC 20190126.

### Neurobehavioral Tests

#### Berderson Score

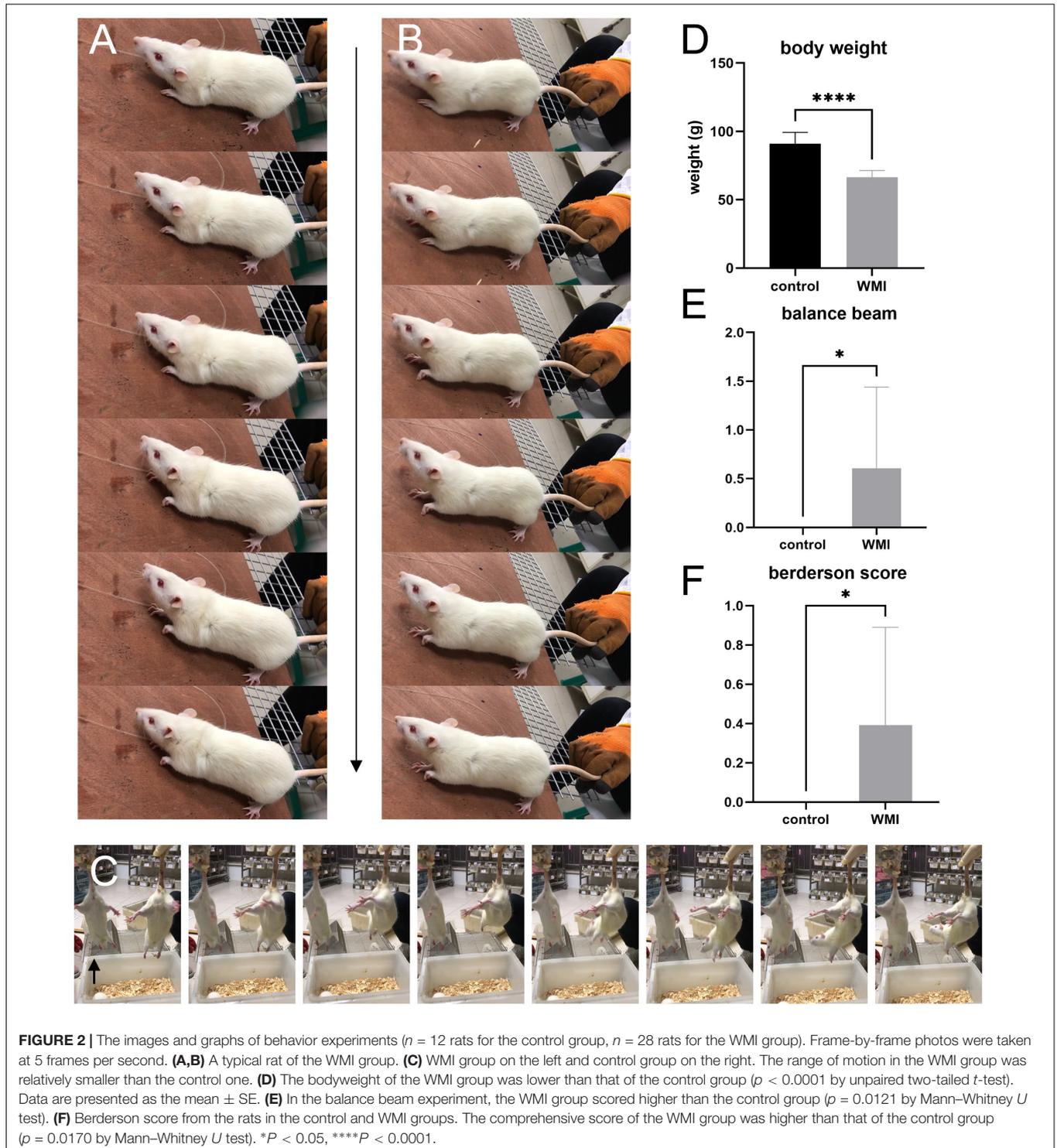
It is also known as the tail suspension test, and the postural reflex is observed by grasping a rat's tail at a certain distance from the ground.

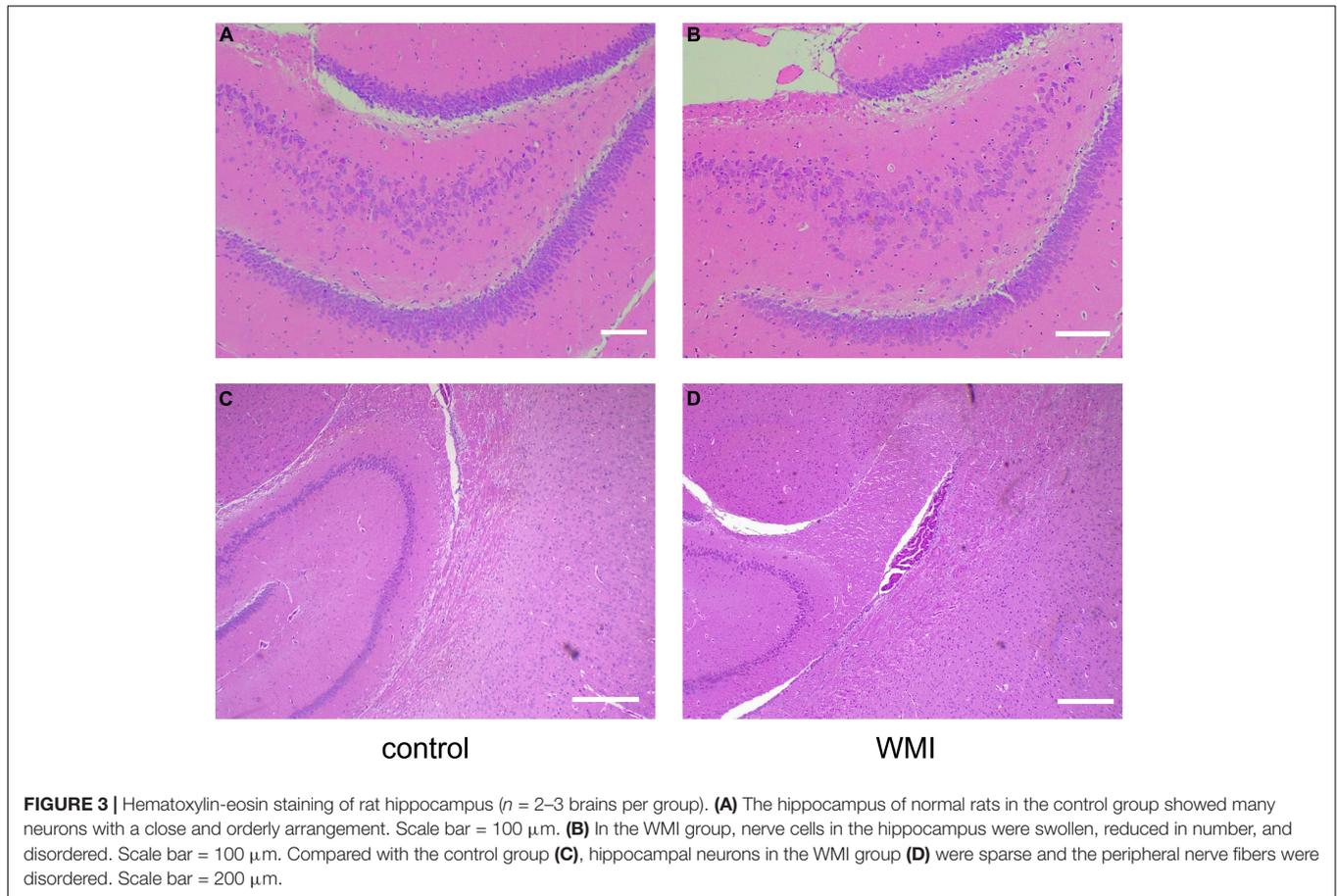
1. Hold the rat by the tail and keep it 0.5 m high above the table. Record the scores as follows: 0 = both limbs symmetrically extended forward; 1 = internal rotation of shoulder and adduction of limbs.
2. Place the animal on a rough surface. Record the scores as follows: 0 = a strong grasp on the rough surface with good resistance when pushed; 1 = a slight resistance was only seen in one paw; 2 = no resistance when pushed in one direction.
3. Place the rat's forelimbs on a metal net and lift the rat gently to check the muscle strength of the forelimbs. Record the scores as follows: 0 = both forelimbs grasp the net



**TABLE 1** | List of antibodies used in this study.

Primary/secondary antibody	Species	Sources	Catalog number	Usage	Dilution factor
IBA1	Rabbit	Abcam, United States	ab178846	WB/IHC	1:2000
Arg1	Rabbit	GenTex, United States	GTX109242	WB	1:2000
CD11c	Rabbit	Affinit, China	7853	WB	1:1500
$\beta$ -actin	Rabbit	Zenbio, China	380624	WB	1:5000





(Motic AE2000, Japan). Five regions of each specimen were randomly collected to calculate the average number and the average optical density of positive cells by Image J.

### Electron Microscopy

Three rats from each group were anesthetized intraperitoneally with 10% chloral hydrate, and the heart was fully exposed by thorax opening. We quickly removed the brain after fully injecting a mixture of paraformaldehyde and glutaraldehyde into the heart. Then the tissue of the frontal cortex was cut into  $1 \times 1 \times 1 \text{ mm}^3$  blocks on ice and fixed in a 2.5% paraformaldehyde glutaraldehyde solution. After rinsing, fixing, dehydrating, embedding, and sectioning into 70 nm, uranyl acetate and lead tartrate were double-stained and photographed under a transmission electron microscope (Thermoscientific Talos L120C G2, United States).

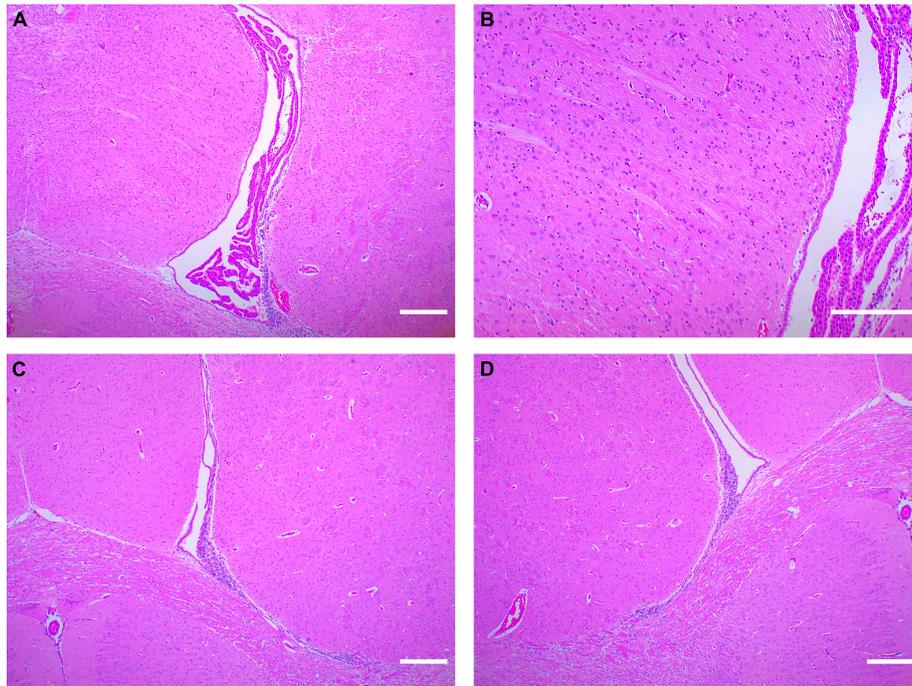
### Statistical Analyses

Statistical analysis of mean differences between groups was performed by using one-way ANOVA (followed by Bonferroni test) or unpaired Student's  $t$ -test. Non-normal distribution data were analyzed by Mann-Whitney  $U$  test. We used GraphPad Prism (v.9.1.0.221) for all the statistical analyses.  $P < 0.05$  was considered significant and all  $p$ -values and  $n$  values are indicated in figure legends.

## RESULTS

### Behavior Tests on Experimental Rats

After 3 days of intraperitoneal injection of neonatal rats, we weighed the rats and ran behavior tests on both WMI and control groups ( $n = 12$  rats for the control group,  $n = 28$  rats for the WMI group). The average bodyweight of the control group was  $91.98 \pm 2.41 \text{ g}$ , and the WMI group was  $66.51 \pm 0.91 \text{ g}$ , the difference indicating that the rats in the WMI group grow slower than normal ones (**Figure 2D**). In the balance beam test, WMI rats showed weaker body control and exhibited more retardation on how to balance but just hug the beam, and so scored higher than the control group ( $P = 0.0121 < 0.005$ ; **Figure 2E**). In the Berderson score, some rats in the WMI group showed spasms/paresis of the upper limb and limited movement. When we placed them on the rough table, a slight resistance was only seen in one paw. The rat had mild paresis of the left upper limb, whereas the right forepaw and both hind paws grasped forcefully. The left forepaw spasticity was evident on movement (**Figures 2A–B**). We held the rats by the tail and kept them 0.5 m high above the ground. It was noticed that the rat in the control group symmetrically extended both its limbs forward, but the rat in the WMI group showed a slight internal rotation of the left shoulder and adduction of the left upper limb (arrow in **Figure 2C**). It also moved rigidly and showed an incoordinate



**FIGURE 4 |** Hematoxylin-eosin staining around the lateral ventricle in rats ( $n = 2\text{--}3$  brains per group). **(A)** The rats in the control group (P20) had normal lateral ventricles, developed choroid plexus, and abundant and orderly nerve fibers in the white matter adjacent to the lateral ventricles. **(B)** Periventricular white matter at  $4\times$  magnification. **(C,D)** In the WMI group (P20), lateral ventricle development was slower, choroid plexus atrophy and nerve fibers were sparse. Scale bar =  $200\ \mu\text{m}$ .

postural reflex, while the rat in the control group showed normal spontaneous movement (**Figure 2C**). Taking these tests together, the total Berderson score was higher than that of the control group ( $P = 0.0170 < 0.05$ ; **Figure 2F**).

## Histopathological Results of the Brain Tissue

We took the brain tissue of rats for hematoxylin-eosin staining and observed the changes in cerebral structure in the experimental group from the histopathologic level. Compared with the control group, the WMI group had a fewer and looser arrangement of neurons, unclear cell boundaries, irregular arrangement of peripheral white matter nerve fibers, and massive infiltration of inflammatory cells (**Figure 3**). In normal rats of the control group, the choroid plexus was well developed in the lateral ventricle, and periventricular nerve fibers were orderly arranged, while in the WMI group, the white matter of the same part was looser, and nerve fibers were more scattered and less in number (**Figure 4**).

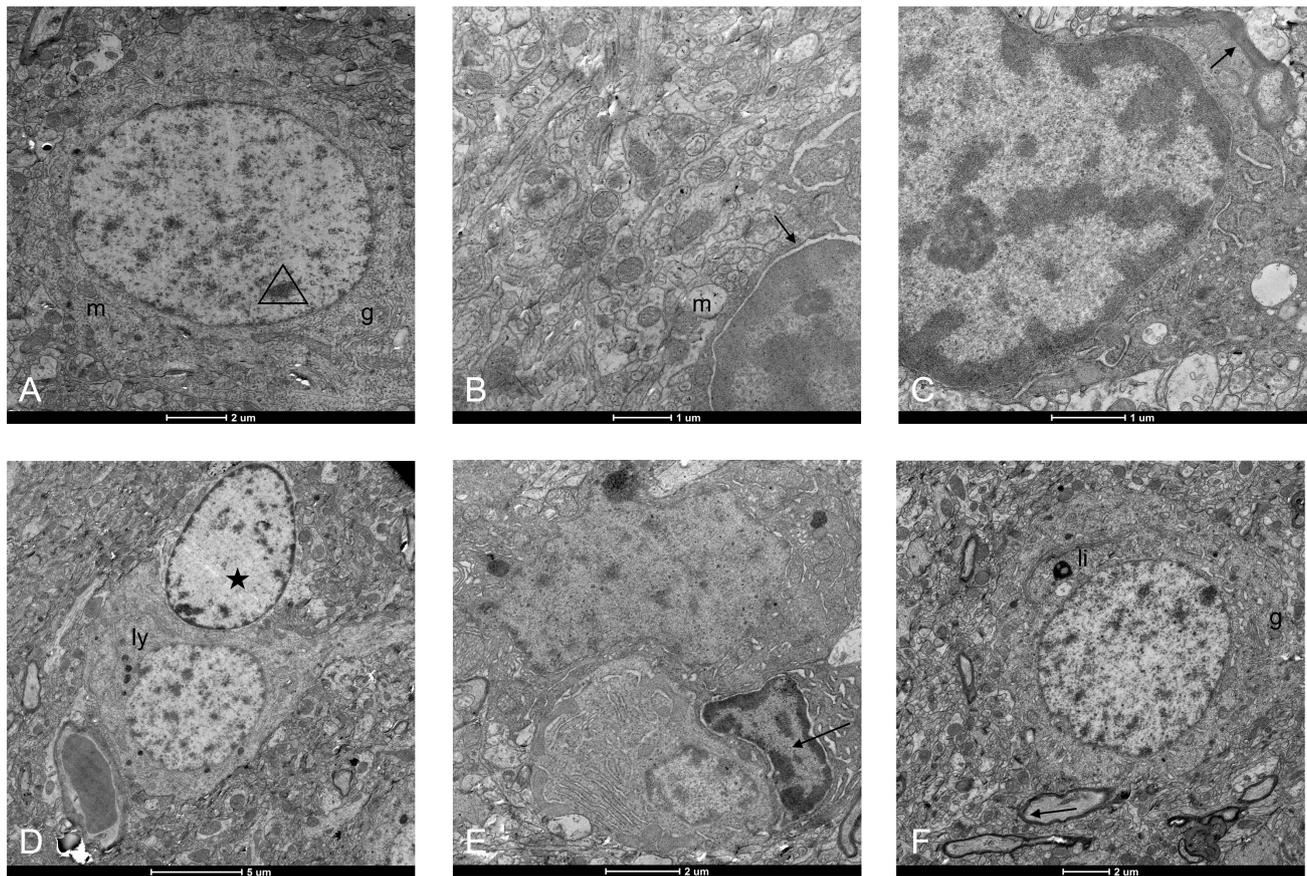
## Scanning Electron Microscopy Observations

Deep into the cellular level, the neural cells of the WMI group were damaged. Cells started showing chromatin condensation with little changes in the organelles (**Figure 5A**). The karyotheca disintegrated, chromatin highly condensed, mitochondria were swollen, and even vacuolated degeneration appeared

(**Figures 5B,C**). We captured an astrocyte surrounding the degenerating neuron and tried to repair it (**Figure 5D**). In the late phase of tissue injury, some microglia exhibit active phagocytic capacity, processing the necrotic neural cells (**Figure 5E**). After microglial activation, jugged1 expression is upregulated and its interaction with notch1 receptors on oligodendrocyte precursor cells is enhanced, which in turn inhibits oligodendrocyte maturation and myelination, hampering the normal conduction of nerve fibers (26). We could see a degenerating neuron with mildly condensed chromatin and partly fragmented mitochondria. The surrounding myelin sheath was slightly damaged (arrow), and the thickness of the peripheral myelin sheath was thinner than normal (**Figure 5F**).

## Abnormal Activation of Microglia

Microglia, as the resident immune cells in the brain, we speculate that it will be activated during cerebral white matter injury, and different degrees of infection will cause different degrees of cell activation. We selected a specific protein, ionized calcium-binding adaptor molecule1 (IBA1) (30), to trace the microglia. In the WMI group, microglia were numerous and morphologically diverse, with most cells extending processes. However, in the control group, microglia appeared less active (**Figures 6A–D**). The average number of IBA1 + cells was  $56.2 \pm 3.1$  in the WMI group and  $16.8 \pm 1.3$  in the control group ( $P < 0.0001$ ), which illustrates a greater number of activated microglia in the experimental group (**Figure 6E**). The expression of IBA1 was also higher as compared to that of the control group (**Figure 6F**).

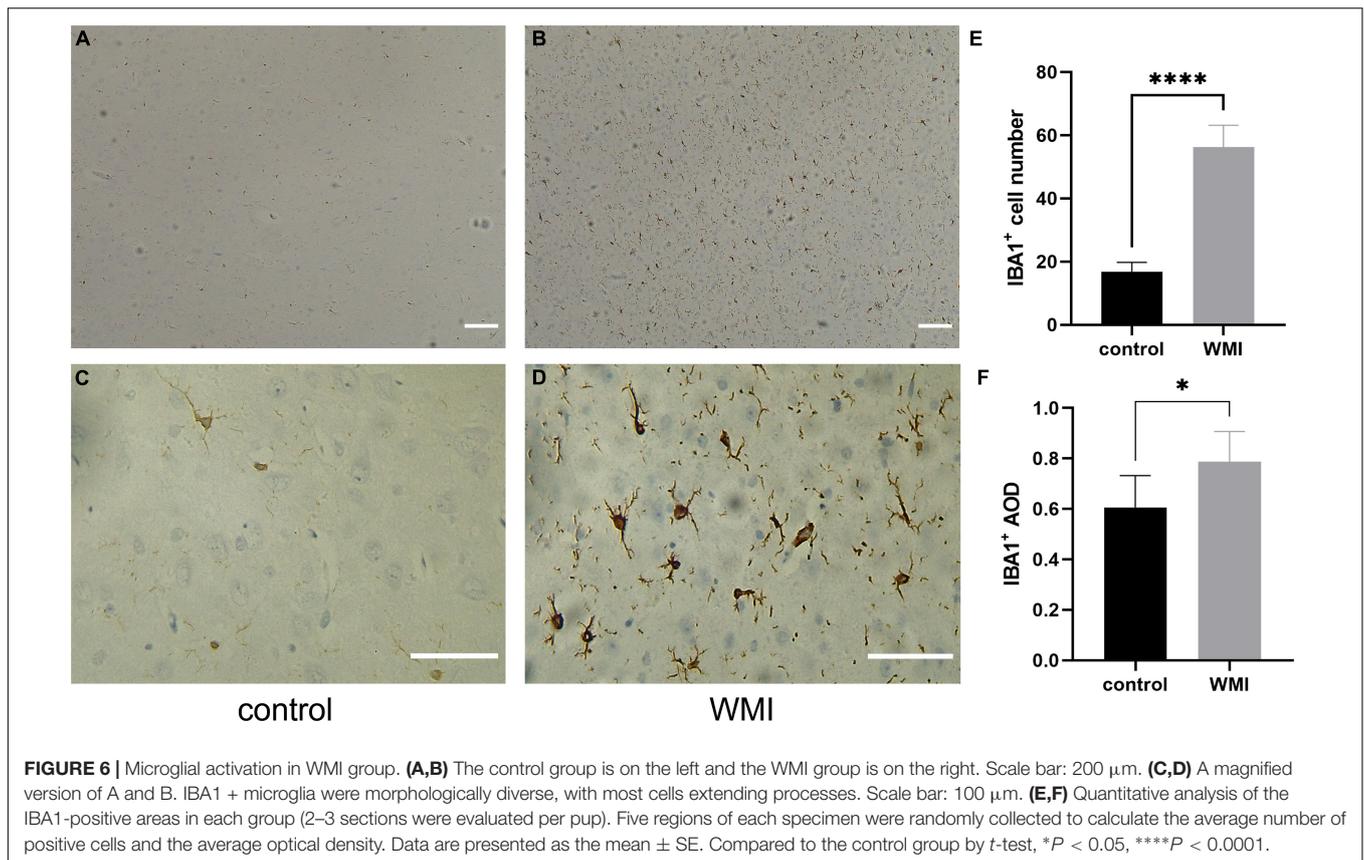


**FIGURE 5 |** Ultrastructure of neural cells under an electron microscope in the WMI group. **(A)** Neuronal cell injury initiates programmed cell apoptosis, and chromatin in the nucleus begins to condense. Organelles such as mitochondria (“m”) and Golgi apparatus (“g”) are normal. Scale bar = 2  $\mu$ m. **(B)** Nucleolus pyknosis, nuclear membrane, and nucleolar space enlarges (arrow), and mitochondrial swelling. Scale bar = 1  $\mu$ m. **(C)** The oligodendrocyte has a darker cytoplasm than the surroundings and is in attachment to a myelin sheath (arrow). Its nucleus was deformed, and chromatin was highly condensed. Mitochondria appeared vacuolated with excessive swelling, even some floccule appeared in the lumens. Scale bar = 1  $\mu$ m. **(D)** A neuron in contact with the blood vessel was degenerative with more intracytoplasmic lysosomes (“ly”). An astrocyte came to repair it (pentacle). Scale bar = 5  $\mu$ m. **(E)** The microglia (arrow) phagocytized a neural cell and a plasma cell. The specific cell morphology of the nerve cell above disappeared, but the plasma cell below was still visible with relatively intact cell morphology, and there was also abundant Golgi in the cytoplasm. Scale bar = 2  $\mu$ m. **(F)** Lipofuscin (“li”) appeared within the cytoplasm of an injured neuron and there was some stunted myelin in the periphery. Myelin damage (arrow). Scale bar = 2  $\mu$ m.

Then, we divided WMI mice into three groups and again intraperitoneally injected LPS to simulate the effects of multiple infections after birth on WMI-induced cerebral palsy (**Figure 7A**). One-way ANOVA was used to compare the three experimental groups. The differences between the groups were significant in Arg1 ( $F = 177.6$ ,  $df = 3$ ,  $P < 0.0001$ ), IBA1 ( $F = 94.78$ ,  $df = 3$ ,  $P < 0.0001$ ), and CD11c ( $F = 114.7$ ,  $df = 3$ ,  $P < 0.0001$ ). Then, the mean of every group was compared by the Bonferroni test. The expression levels of Arg1 and IBA1 in the LPS2 and LPS3 groups were higher than those in the control group, while both LPS1 groups showed no significant differences when compared with the control group (Arg1:  $P = 0.2581 > 0.05$ ; IBA1:  $P = 0.0861 > 0.05$ ). But there were significant differences among different dose groups. The expression levels of Arg1 and IBA1 in the LPS3 group were higher than LPS2 group, and in the LPS2 group, it was higher than in the LPS1 group. It showed that the expression level was increased with the elevating concentration of

LPS, suggesting a certain dose-dependent effect (**Figures 7B,C**). The expression level of CD11c in LPS groups was lower than that in the control group. With the increase in LPS dosage, the expression of CD11c decreased gradually (**Figure 7D**). In different microenvironments, microglia can be activated into M1 and M2 types, respectively, and normally, the conversion between the two maintains a balance. The expression of CD11c, an M1-specific protein, gradually decreased with an increase in LPS concentration, while Arg1, an M2-specific protein, showed a gradual increase. It was seen that when WMI rats were infected early in development, microglia were not only activated but also polarized toward M2 type cells, more toward anti-inflammation to protect cerebral white matter.

Combined with the above microscopic examination and behavior experimental observation, the experimental group showed behavioral abnormalities, delayed neural development, and periventricular injury histologically. In the present results,



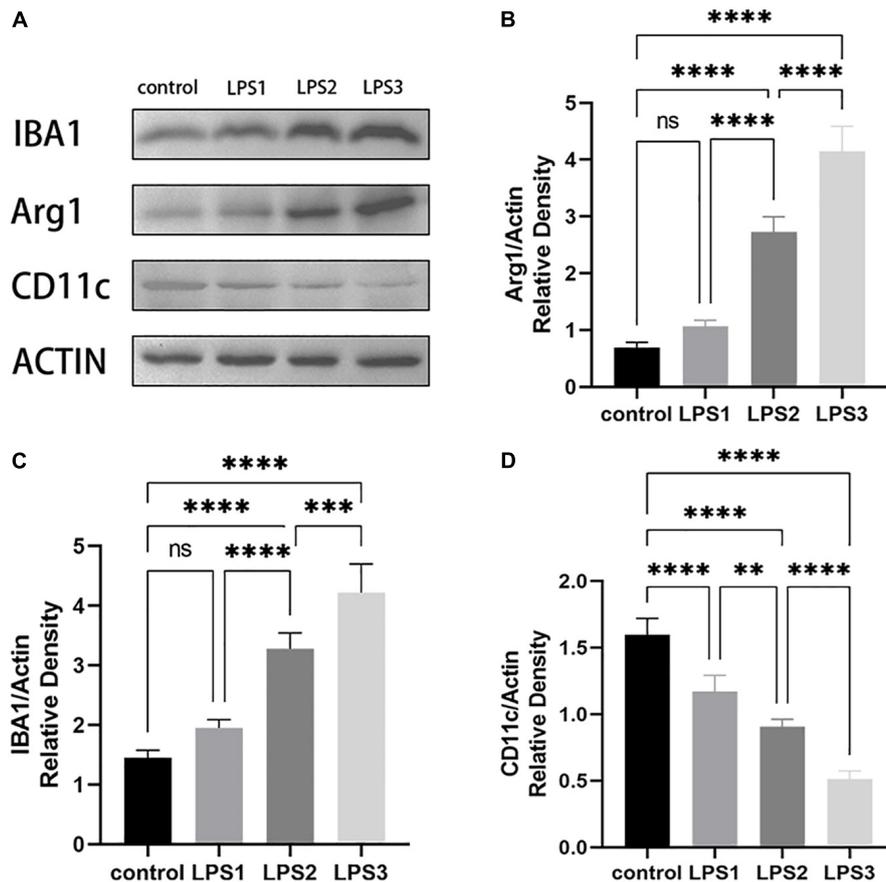
we successfully established an animal model of WMI-induced cerebral palsy. Substantial microglial activation and a trend of microglia protecting the neural tissue after multiple injections of LPS were also observed. However, the specific roles of microglia require further studies to explore.

## DISCUSSION

There are many patients with CP who did not manifest significant limb paralysis symptoms in the primary injury stage but gradually appeared in the subsequent development stage. For example, dyskinetic CP caused by bilirubin encephalopathy, usually appears as paralysis clinical symptoms 6 months after birth; mild CP caused by premature birth and intrauterine hypoxia need to be diagnosed at over 2 years old, some even reach 4 years old (31); and not all the WMI patients will finally develop CP based on clinical practice (32). Brain injury from a single etiology does not seem to be enough to cause CP, and the post-injury effects may play an important role. In animal studies, LPS-preconditioning on neonates may prime the brain to be more sensitive to subsequent inflammatory hits (33, 34). An observational study in humans also supports this possibility (35). An acute (non-progressive) brain interference in development is one of the causes that lead to brain damage, but two or more interferences are associated with a higher risk of damaging the brain in preterm newborns (36–38). It suggests that the onset of

cerebral palsy is the result of “multi-hit” (39), including cerebral injury and biological or environmental adverse factors (such as inflammatory infections early in development) during the perinatal period. In the present study, we injected maternal rats with LPS intraperitoneally in the early stage of pregnancy and again administered two LPS in neonatal rats at P20 and P40 to create a WMI-induced cerebral palsy animal model, referring to the experimental methods of Felipe Stigger and Yanrong Hu et al., with some modifications (13, 14). Under some special circumstances, an inflammation-induced environment protects the brain from a secondary hit (40). Researchers applied a harmless dosage of LPS 24 h before a large dosage of LPS administration to make it resistant to brain injury (41). Repeated LPS administration would attenuate when the time interval is 24–72h, and less than 6 h or more than 72h prior to a second LPS exposure would exacerbate brain injury, even though the dose is not enough to cause injury alone (34, 41). Based on the “multi-hit” theory, we applied LPS injection both prenatally and postnatally to get a brain injury model more easily and more clinically. These models manifested paresis of limbs/tail, incoordination, and reduced activity, which mimic the symptoms of CP. It indicates that WMI would trigger CP under multiple effects, such as perinatal infection.

We hypothesized that microglia activation and polarization play an important role in the pathogenesis of WMI-induced CP. Known as CNS macrophages, microglia show similar bio functions but different cellular origins with the macrophage.



**FIGURE 7 |** Western Blot of Arg1, CD11c, and IBA1 ( $n = 2-3$  brains per group). **(A)** WB bands of the control group, low dose, medium dose, and high dose WMI + LPS groups. **(B-D)** The histograms represent the normalized relative density of Arg1(B), CD11c(C), and IBA1(D) between the control group and experimental groups. Data are presented as the mean  $\pm$  SE. Compared the mean of each group with the mean of every other group by the Bonferroni test. ns > 0.05, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

When infection occurs, the activated microglia transform toward an amoeboid morphology, phagocytose the broken neural cells, and release cytokines, or oxidases to mediate the apoptosis of pre-oligodendrocytes (19, 42, 43). Microglial activation can also inhibit the differentiation and myelination of oligodendrocytes by enhancing the notch signal-pathway (25–27). Microglia clusters near periventricular axonal crossroads of premature infants, help prune the axonal bundles (44–46); some even colonize the subplate and synchronize with the synaptogenesis and growth of subplate neurons (7, 47). These signs reveal that microglia not only regulate the microenvironment of CNS but also participate in the formation of neural fibers in the early developmental stage. Through immunohistochemistry and western blotting, we screened tremendous activation of microglia in the experimental group, which is in line with the existing research points.

As mentioned before, activated microglia can be classified as pro-inflammatory M1 type and anti-inflammatory M2 type. Some researchers called “resting microglia” as M0 type or attenuated M2 type, but the debate remains (18). Based on the high plasticity and rapid transition between different phenotypes, others propose calling it “never-resting microglia” may be better

(48). Anyway, such an M1/M2 classification helps researchers judge microglial states and microenvironment around more conveniently. In our experiments, the protein marked M1 got a lower expression in the WMI + LPS group while M2 specific protein showed an increased expression. The results showed an M2 polarization in P40 rats. D.M. Norden et al. demonstrated that repeated LPS injections (at regular intervals) did not magnify the inflammatory reaction, but instead stimulated the neuroprotective effect of microglia, suggesting that the protective effect of microglia may be time-dependent (49). A brain injury could surely activate microglia, and the M2-to-M1 microglial transition only occurred in the acute phase, but after a certain time, M2 started to dominate (27, 50). We speculate that in our WMI-induced cerebral palsy model, such a time demarcation also exists. Before the time point, risk factors such as infection can aggravate white matter damage and microglia mainly exert neurotoxic effects. After the time point, the microglia mainly exerts anti-inflammatory effects; however, brain damage at this moment is already irreversible. So, finding this time demarcation would play a key role in the future treatment of brain injury, which needs further studies.

In recent years, many researchers were keenly aware of the link between gender and cerebral inflammation. Both in animals and humans, it has been found that males are more likely to show increased expression of inflammatory indicators and corresponding neurobehavioral symptoms in inflammatory encephalopathy, while females show resilience to neuroinflammation and less severe phenotype (51–53). Lin-Chao Yu et al. used male mice when studying the therapeutic effect of IL-4 on periventricular leukomalacia to avoid the bias of gender in the experiment (50). In a detailed study of mice microglia, researchers found that a small cluster of extremely specific microglia is enriched in females, which is different from males, and sex only had little effect on microglia diversity (16). Further research should focus on the specific gender-related microglia and the mechanisms behind them.

Nowadays, treatments for cerebral palsy are mainly physiotherapy and rehabilitation. Neural stem cell transplantation seems to be a better choice for patients with CP recently. Researchers have applied stem cell therapy in neurodegenerative diseases or brain injury for its multi-differentiation potential and migration ability. Hoping that stem cells could reach the lesion, proliferate, differentiate, and finally reshape neurons binding (54–56). Besides, trophic factor supplement and immunomodulation are two critical keys for effective neural stem cell therapy (57). Since microglia are immunocompetent cells and important regulators of the cerebral microenvironment, our findings will shed light on the combination of immunomodulation and NSCs transplantation.

Our study also has certain limitations. The first limitation is that we only chose one marker protein for each type of microglia. There are other markers such as iNOS/CD16/CD86 for M1-type microglia and CD163/CD106 for M2-type microglia (27, 50). Perhaps different markers can be applied in a future study to improve experimental accuracy. The other limitation is that we only captured M2 polarization because of the long interval between LPS injections. In further research, by adding more experimental groups and shortening the interval between LPS injections, the critical time of the transition from M1 polarization to M2 polarization could be found.

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In conclusion, the perinatal infection can induce WMI to CP based on our “multi-hit” animal model and microglia play a critical role in cerebral inflammation. The establishment of this model supports the “multi-hits” theory of CP formation. Our findings may shed some light on further mechanism research of microglia in CP and future treatment discovery.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Experimental Animals, Anhui Medical University.

## AUTHOR CONTRIBUTIONS

LL and DW: study design. LL, BD, LF, YW, and ZC: study implementation. LF and LL: data analysis and figures preparation. LF, LL, DW, and LY manuscript drafting. All authors approved the final version of the manuscript.

## FUNDING

This study was supported by the National Natural Science Foundation of China, Nos. 81472167 (to DW).

## ACKNOWLEDGMENTS

Thanks to the teachers and seniors of the Pathogen Microbiology Laboratory of Anhui Medical University for their guidance on experiments.

advances in diagnosis and treatment. *JAMA Pediatr.* (2017) 171:897–907. doi: 10.1001/jamapediatrics.2017.1689

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