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Murine model: maternal administration of stem cells for prevention of prematurity

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Abstract

OBJECTIVE—Using a mouse model of intrauterine inflammation, we have demonstrated that exposure to inflammation induces preterm birth and perinatal brain injury. Mesenchymal stem cells (MSCs) have been shown to exhibit immunomodulatory effects in many inflammatory conditions. We hypothesized that treatment with human adipose tissue-derived MSCs may decrease the rate of preterm birth and perinatal brain injury through changes in antiinflammatory and regulatory milieu.

STUDY DESIGN—A mouse model of intrauterine inflammation was used with the following groups: (1) control; (2) intrauterine inflammation (lipopolysaccharide); and (3) intrauterine lipopolysaccharide + intraperitoneal (MSCs). Preterm birth was investigated. Luminex multiplex enzyme-linked immunosorbent assays were performed for protein levels of cytokines in maternal and fetal compartments. Immunofluorescent staining was used to identify and localize MSCs and to examine microglial morphologic condition and neurotoxicity in perinatal brain. Behavioral testing was performed at postnatal day 5.

RESULTS—Pretreatment with MSCs significantly decreased the rate of preterm birth by 21% compared with the lipopolysaccharide group ($P < .01$). Pretreatment was associated with increased interleukin-10 in maternal serum, increased interleukin-4 in placenta, decreased interleukin-6 in fetal brain ($P < .05$), decreased microglial activation ($P < .05$), and decreased fetal neurotoxicity ($P < .05$). These findings were associated with improved neurobehavioral testing at postnatal day 5 ($P < .05$). Injected MSCs were localized to placenta.

CONCLUSION—Maternally administered MSCs appear to modulate maternal and fetal immune response to intrauterine inflammation in the model and decrease preterm birth, perinatal brain injury, and motor deficits in offspring mice.

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Keywords

intrauterine inflammation; mesenchymal stem cell; perinatal brain injury; preterm birth

Globally, there are approximately 15 million children who are born preterm each year.^{1,2} Preterm birth accounts for 75% of perinatal deaths and more than one-half of the long-term morbidity.³ Intrauterine inflammation is thought to lead to preterm birth and the subsequent development of fetal inflammatory response syndrome.⁴⁻⁶ A proinflammatory cytokine response in the context of intrauterine inflammation is central to perinatal brain injury and is associated with a spectrum of adverse neurobehavioral outcomes that include cerebral palsy, schizophrenia, and cognitive delay.⁷⁻¹³

Mesenchymal stem cell (MSC)-based therapy has been effective in regenerative medicine.¹⁴⁻¹⁶ MSCs have emerged as a promising resource to promote functional neurologic recovery from perinatal stroke and hypoxia-ischemia.^{14,17,18} The beneficial effect of MSCs is thought to occur, in part, through their immunomodulatory properties.¹⁹ These properties are thought to counteract inflammation via both soluble factors and cell-to-cell contact. Through these mechanisms, MSCs can induce division arrest anergy in dendritic cells, naïve and effector T and B cells, and natural killer cells.¹⁹⁻²³

With inflammation (lipopolysaccharide stimulation), MSCs are known to reprogram macrophages to produce lower amounts of tumor necrosis factor alpha (TNF- α) and interleukin (IL)-6 and to dampen the proinflammatory response.²⁴ The reprogrammed macrophages also produce increased amounts of the regulatory cytokine IL-10.²⁴

To date, no immunomodulatory effect of MSCs on intrauterine inflammation and its sequelae have been reported. Our group and others have shown that a significant increase of proinflammatory milieu in maternal and fetal compartments after intrauterine inflammation was associated with fetal neurotoxicity, perinatal brain injury, and neurobehavioral sequelae.^{8,25-32} On the basis of remarkable features of MSCs, we hypothesized that maternal pretreatment with MSCs may decrease preterm birth and perinatal brain injury that are induced by intrauterine inflammation (primary hypothesis). Furthermore, we hypothesized that MSCs act on maternal and fetal compartments by increasing antiinflammatory and regulatory cytokines and ameliorating effects of the proinflammatory cytokines (secondary hypothesis).

Materials and Methods

Mouse model of intrauterine inflammation and preterm birth

All animal procedures were approved by the Animal Care and Use Committee of Johns Hopkins University. A mouse model of intrauterine inflammation and preterm birth was used, as previously described.²⁹⁻³¹ Briefly, on embryonic day 17 of gestation (preterm), CD-1 timed-pregnant mice (Charles River Laboratories, Wilmington, MA) were placed under isoflurane anesthesia continuously, and a minilaparotomy was performed. Lipopolysaccharide (*Escherichia coli*, 055; B5; Lot 014M4029V; Sigma-Aldrich, St. Louis, MO) 50 μ g in 100 μ L of normal saline solution (NS) was infused between the first 2

gestational sacs in the lower right uterine horn. Routine closure was applied. *Preterm birth* was defined as delivery of the first pup within 24 hours after surgery.

A total of 72 dams were distributed into 3 groups: (1) control with NS, (2) intrauterine inflammation (lipopolysaccharide), and (3) intrauterine lipopolysaccharide plus intraperitoneal injection of human adipose tissue-derived MSCs (StemPro; Invitrogen, Grand Island, NY) 15 hours before the onset of intrauterine lipopolysaccharide injection. Pregnant mice were injected intraperitoneally with 400,000 MSCs (passage 2) in 100 μ L of NS per mouse (approximately 40 g). The dose of MSCs that was used was based on previously reported studies that indicated the neuroprotective role of MSCs in a rodent model and on pediatric clinical trial data.^{33,34} The best protective effect that was observed in rats was at a dose of 1 million MSCs per animal.³³ In the human pediatric transplantation cohort study, patients received, on average, 1.4 million/kg (range, 100,000/kg to 10 million/kg) intravenously injected MSCs without adverse effects.³⁴

After 6 hours of intrauterine inflammation or NS infusion and survival surgery, maternal serum, amniotic fluid, fetal brains and placentas were collected. Furthermore, fetal brains were processed for cortical neuronal primary culture. Additionally, after 24 hours of lipopolysaccharide administration, the maternal spleen, placenta, and fetal brain were collected and fixed.

Cell culture

MSC culture—MSCs were obtained (Invitrogen, Carlsbad, CA); their phenotype was confirmed by flow cytometry studies of cell-surface markers: positive for CD29, CD44, CD73, CD90, CD105, and CD166; negative for CD14, CD31, CD45, Lin1. MSCs were placed in 35-mm Petri dishes with complete Mesen-PRO RS Medium (Invitrogen) that was supplemented with 0.5 mmol/L L-glutamine and 1X penicillin-streptomycin antibiotics (Invitrogen). Every 3-4 days, the medium was replaced until cells were harvested for confirmatory immunocytochemistry. Consistent with previous literature, MSCs were used for in vivo experiments after 2 passages.³⁵

Cortical neuronal primary culture—Under sterile conditions,³⁶ cortical tissues were harvested from fetal brains. Primary cultures were created as per previously reported protocols.^{30,31} Cells were grown in 6- and 12-well tissue culture plates. For each experiment, 3-4 fetal brains from 1 dam constituted 1 culture. For each experimental trial, 3 dams per treatment group were used. The experiment was repeated 3 times. After 3 days of in vitro culture, the cells were fixed and analyzed by immunocytochemistry.

Immunofluorescent staining

Maternal spleen, placenta, and fetal brain were incubated with primary antibodies overnight at 4°C. Human-specific nucleus marker (HuNu) antibody was used because it stains human nuclei specifically and was used to distinguish human MSCs from mouse tissues.³⁷ MSC surface marker (CD44) is expressed widely by many stem-cell types and functions in cell adhesion, migration, homing, proliferation, survival, and apoptosis.^{38,39} Mouse anti-HuNu (MAB1281; Millipore, Billerica, MA) and rabbit anti-CD44 (HPA005785; Sigma-Aldrich)

were used to identify MSCs in tissues. The 4',6-diamidino-2-phenylindole (DAPI) was used to identify nuclei, regardless of species, because it stains DNA.

Fetal brains were stained with rabbit anti-Iba-1 antibody (Iba-1; 019-19741; Wako, Richmond, VA). Iba-1 is a microglia/macrophage-specific calcium-binding protein and identifies activated microglia.⁴⁰

Cortical cell cultures were fixed with 4% paraformaldehyde and stained after 3 days in vitro to assess morphologic changes between the treatment groups with the use of single staining of mouse microtubule-associated protein 2 (MAP2; Sigma-Aldrich). MAP2 is a neural marker that labels dendrites and cell body of neurons.⁴¹

Goat anti-rabbit or mouse Alexa Fluor 488 (Abcam, Cambridge, MA) and donkey anti-mouse Alexa Fluor 568 (Invitrogen) were used as secondary antibodies. Confocal microscopy (Leica SP2 Confocal System; Leica Microsystems Inc, Buffalo Grove, IL) was used for all of the immunohistochemical and immunocytochemical experiments.

Quantitative analysis of dendritic processes and microglia activation from cortical culture experiments and fetal brain

Fluorescent images at $\times 400$ magnification were obtained and with using image processing software (ImageJ, version 1.37; National Institutes of Health, Bethesda, MD). MAP2-stained dendritic processes of neurons in cortical culture were counted at 3 days in vitro, as per previously reported methods.^{31,32} Branches of microglial processes (ramifications), that were visualized by Iba-1 immunostaining, were counted at periventricular regions of brain sections.^{42,43} At least 30 clearly defined individual cells from each treatment group were evaluated.

Assessment of maternal serum, amniotic fluid, placenta, and fetal brain cytokines

Maternal serum, amniotic fluid, fetal brain, and placenta were obtained as described earlier and then were assessed using Luminex multiplex analysis (R&D Systems, Minneapolis, MO), according to the manufacturer's protocols, and read on a Luminex100 platform. Proinflammatory (IL-1 β , -6, and -12, TNF α , and INF γ), antiinflammatory (IL-4 and -5) and regulatory (IL-10 and -2 and granulocyte-monocyte colony stimulating factor) cytokines were analyzed. The experiment was repeated twice. All tests were performed in duplicate.

Quantitative analysis of MSCs in placenta and spleen

Quantification of HuNu⁺/CD44⁺ cells was performed for placenta and maternal spleen tissues. Cells were counted with the use of semi-automated counting software (ImageJ) from 10 random fields under $\times 40$ magnification for each treatment group. The frequency of HuNu⁺ cells was compared with the total of all cells (DAPI⁺) in the sections.

Behavioral evaluation

A developmental milestone scoring system⁴⁴ was used, with modifications at postnatal day 5, by previously reported methods.²⁹ The cliff aversion test measured the ability of the mouse to turn and crawl away from an edge; the surface righting test determined the ability

of the mouse to right itself after being placed on its back. This test had the most robust response to lipopolysaccharide in our previous studies.²⁹

Statistical analysis

Statistical analyses were performed with the use of the SigmaStat software program (Aspire Software International, Ashburn, VA). Preterm birth rates were compared using the χ^2 test. All data were tested for normality; 1-way analysis of variance (ANOVA) or ANOVA on ranks was used to compare the values between the treatment groups, where appropriate. Cytokine data were log-transformed before statistical analysis. Multiple comparisons were performed when there was a significant difference between the groups with 1-way ANOVA or ANOVA on ranks.

Results

Human MSCs express human and stem-cell markers

To confirm that cultured human MSCs maintain the characteristics of stem cells before in vivo experiments, immunocytochemistry was performed. Cultured MSCs displayed typical spindle-shaped fibroblast morphologic condition and expressed the human nucleus marker HuNu (Figure 1, A) and MSC surface protein CD44 (Figure 1, B). Cells were counterstained with DAPI (Figure 1, C) to identify DNA material. Colocalization of HuNu and CD44 validated the undifferentiated status of MSCs before intraperitoneal injection.

MSCs decrease preterm birth rate

Exposure to intrauterine lipopolysaccharide resulted in 64% preterm delivery within 24 hours. As expected, there was no preterm delivery in NS-exposed dams. Pretreatment with MSCs significantly decreased the preterm birth rate to 43% (21% reduction; $P < .05$, χ^2 test).

MSC treatment alleviates lipopolysaccharide-induced morphologic changes in neurons

We previously demonstrated that exposure to intrauterine inflammation decreases neuronal arborization and leads to morphologic changes that are consistent with neurotoxicity.^{31,32,45} To examine whether MSC treatment can ameliorate neurotoxicity, immunohistochemistry of MAP2 (cytoskeletal probe) was used for morphologic observation of cortical neurons. MAP2 is enriched in dendrites of neurons and plays an essential role in neurogenesis.^{46,47} In our current study, similar to our previous reports,^{26,30,31,45} there was a significant decrease in the number of dendritic processes in the lipopolysaccharide group, compared with the control group (Figure 2; $P < .05$, Student-Newman-Keuls test). In contrast, the number of dendrites was increased significantly in that MSC treatment group, as compared with lipopolysaccharide-exposed group ($P < .05$, Student-Newman-Keuls test) and was similar to the control group ($P > .05$, Student-Newman-Keuls test).

MSCs decrease microglial activation

As the main form of active immune response in the central nervous system, microglial cells exert a pivotal role in reacting to inflammation by releasing multiple inflammatory mediators.⁴⁸ Activation of microglia is thought to play a role in long-term adverse

neurologic sequelae.²⁹ To access whether MSCs ameliorate immune response in fetal brain, we examined microglial morphologic condition, which is an indicator of microglia activation, using Iba-1 immunohistochemistry in periventricular regions.

There were distinct differences in microglial morphologic condition between groups. Microglia in the control group were ramified highly, in contrast to the amoeboid-shaped microglia of the lipopolysaccharide-exposed group, which indicates its activation (Figure 3, A). After quantitative analysis, there were significantly fewer branches in the lipopolysaccharide group as compared with the control group ($P < .001$, 1-way ANOVA); MSCs increased microglial ramification compared with the lipopolysaccharide group ($P < .01$, 1-way ANOVA), which suggests a decrease in its activation (Figure 3, B).

MSCs improved behavioral performance of pups that were exposed to intrauterine lipopolysaccharide stimulation

To evaluate the functional consequences of MSCs in the treatment of intrauterine lipopolysaccharide-exposed pups, behavior was assessed by cliff aversion test. Similar to our previous data,²⁹ lipopolysaccharide-treated pups showed a deficit in the cliff aversion test on postnatal day 5 (Figure 4; $P < .001$, 1-way ANOVA). These significant motor behavioral deficits were not observed in MSCs-pretreated lipopolysaccharide offspring ($P < .001$, 1-way ANOVA).

MSCs regulated the maternal and fetal response of proinflammatory/antiinflammatory/regulatory cytokines

To determine a possible mechanism by which MSCs decrease preterm birth and improve perinatal brain injury, we examined cytokine profiles in both maternal and fetal compartments. As shown in the Table, on stimulation with lipopolysaccharide, increases in proinflammatory cytokines, which include IL-1 β , -6, and -12 and TNF α , were observed in maternal serum, amniotic fluid, and placenta. Interestingly, maternal administration of MSCs significantly decreased IL-6 levels in fetal brain ($P < .05$) compared with the lipopolysaccharide group. There was a significant difference between groups ($P < .05$) in regulatory cytokine IL-10 and proinflammatory IL-12 in maternal serum. MSCs treatment showed a trend of increase in type 2 helper T cell (Th2) cytokine, IL-4, in the placenta.

MSCs localize to maternal spleen and placenta, but not fetal brain

To localize MSCs, the maternal spleen, placenta, and fetal brain were examined with the use of immunohistochemistry (HuNu, CD44, and DAPI; Figure 5, A). MSCs were located in the maternal side of the placenta. Significantly greater numbers of MSCs were found in placenta as compared with maternal spleen (Figure 5, B; $P < .05$). MSCs were not detected in any of the fetal compartments within 24 hours of administration (data not shown). MSCs were not present in control placentas, which were not exposed to lipopolysaccharide.

Comment

For the first time, our study demonstrates that maternal administration of MSCs reduces preterm birth and ameliorates perinatal brain injury in the mouse model of intrauterine

inflammation. We speculate that the decrease in prematurity and an amelioration of perinatal brain injury were mediated through MSC regulation of antiinflammatory/proinflammatory/regulatory milieu balance.

It has long been established that shifting the cytokine profile contributes to successful maintenance of pregnancy.^{49,50} Proper balance among proinflammatory, antiinflammatory, and regulatory cytokines provides self-tolerance and fetal tolerance and protects against pathogen invasion to avoid preterm birth and fetal inflammatory response syndrome.⁵¹⁻⁵⁴ The magnitude of the immune response towards inflammation is determined by the balance between antigen-driven activation of lymphocytes and regulatory influences that dampen the response.⁵⁵ Regulatory T-cell influence may occur at recognition, activation, or effector phases of an immune response.⁵⁴

MSCs are known to prevent immune activation by acting on the regulatory mechanism and by interfering with cell cycle.⁵⁶⁻⁵⁸ Batten et al⁵⁹ revealed a distinctly different cytokine profile after coculture of T cells with MSCs than with other cell types: proinflammatory type 1 helper T-cell cytokines IL-1 β , interferon- γ , and TNF- α were down-regulated, whereas antiinflammatory Th2 cytokines IL-3, -5, and -10 were up-regulated. After coculture with MSCs, T cells exhibited a regulatory phenotype.

Furthermore, MSCs are characterized by their ability to self-renew, proliferate, migrate, and differentiate into various specialized tissue types on different stimulations.^{60,61} Because of their properties, MSC treatment has been tested in clinical trials of diseases and animal studies.⁶²⁻⁶⁶ In clinical trials, when transplanted to recipients, MSCs are used to treat graft-vs-host disease.⁶⁷⁻⁶⁹ In animal studies, this low immunogenicity is used for cross-species experiments, because researchers have displayed more interest in these immunomodulatory effects of MSCs.⁷⁰⁻⁷² Their effects are thought to occur through Toll-like receptor 4 and multiple cytokine receptors that are possessed by MSCs.⁷³⁻⁷⁵

Lipopolysaccharide, a component of the cell wall of gram negative bacteria, acts by way of its Toll-like receptor 4, which is expressed highly in placenta and on macrophages.^{7,32,76-78} In our study, the activation of proinflammatory response to lipopolysaccharide was evidenced by increases of IL-1 β (placenta), IL-6 (placenta and fetal brain), TNF- α (amniotic fluid), and IL-12 (maternal serum). Maternal administration of MSCs resulted in increased levels of regulatory and antiinflammatory cytokine IL-10 (maternal serum), Th2 anti-inflammatory cytokine IL-4 (placenta), and decreased proinflammatory cytokine IL-6 in the fetal brain.

Our results are similar to those of others, which document that regulatory IL-10 modulates resistance to inflammatory stimuli by down-regulating proinflammatory cytokines.^{24,79,80} We speculate that these inhibiting effects occur by way of the reprogramming of lipopolysaccharide-stimulated macrophages and microglia in the fetal brain. Furthermore, activation of microglia, as demonstrated by Iba-1 immunohistochemistry, was ameliorated by MSC pretreatment. We believe these changes may have occurred through soluble mediators and through cell-to-cell contact, which ultimately lead to a decrease in preterm birth (maternal side) and a decrease in perinatal brain injury (fetal side).

Interestingly, MSC pretreatment led to further increases in proinflammatory cytokines such as IL-12 in maternal serum, IL-1 β , IL-12, TNF- α in amniotic fluid, and IL-6 in placenta. We believe that these changes in cytokine profile were associated with MSC interaction with dendritic cells.⁸¹ It is known that, in addition to inhibitory effects in macrophages, MSCs may exert promoting effects on lipopolysaccharide-induced dendritic cells.⁸² Ultimately, the shift in cytokine profile allowed for decreases in detrimental maternal and fetal phenotypes.

Similar to previous studies, MSCs localized to maternal spleen.⁸³ However, our study for the first time examined MSC administration in pregnancy and localized cells to the maternal side of placenta. Furthermore, there were more MSCs in placenta than in maternal spleen, even though the cells were delivered systemically, which indicated a possible targeted protective role in prevention of effects that were exerted by intrauterine (localized) inflammation.

When MSCs are administered systemically to humans and animals, they are found to migrate specifically to damaged/inflamed tissue sites.^{84,85} Local inflammatory factors, such as transforming growth factor beta and sphingosine 1-phosphate, may provide cues to mobilize MSCs to tissue sites with damage (homing signal).⁸⁶⁻⁸⁸ It has also been shown that the immunomodulatory function of human MSC is enhanced when the cells are exposed to an inflammatory environment.⁸⁹ These properties may explain the reason that MSCs localized to the placenta after lipopolysaccharide treatment, but not in the control group. Further studies will identify the exact layer of the placenta to which stem cells home on the maternal side. Postnatal MSC administration studies also are needed urgently in this model to delineate the possible distribution of MSCs and their effect after exposure to intrauterine inflammation.

The fate of MSCs after transplantation is not understood fully. In our study, MSCs still expressed the stem-cell surface protein marker CD44 and preserved its phenotype at the time of harvest at 24 hours after lipopolysaccharide exposure. Similarly, others have demonstrated short-term survival of MSCs.⁹⁰⁻⁹² Long-term, MSCs are thought to differentiate into a variety of cell types, such as chondrocytes, adipocytes, and bone marrow stromal cells, in a site-dependent manner.^{93,94} Future characterization of the microenvironments to which MSCs migrate would enhance our understanding of MSCs trafficking, phenotype, and changes and would improve targeted therapies.

In conclusion, MSC pretreatment decreased preterm birth and ameliorated perinatal brain injury in the mouse model of intrauterine inflammation (a scenario that most closely mimics that of preterm birth in humans). These effects likely occurred through changes in the balance of proinflammatory/anti-inflammatory/regulatory cytokines. Our study also demonstrated the complexity of MSC immune regulatory mechanisms. MSCs are capable of avoiding xeno-/allorecognition and generating a local immunomodulatory microenvironment. The effectiveness of the MSC response is dependent on the timing of the administration, the administration route, the cell sources, and the dosage. Further studies are needed to address these challenges and precise mechanisms of MSC action in pregnancy and in the postnatal period.

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Human mesenchymal stem cell markers

FIGURE 1. Mesenchymal stem cells before intraperitoneal injection

Mesenchymal stem cells expressed human and mesenchymal stem-cell markers before intraperitoneal injection. Cultured mesenchymal stem cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with Triton X-100. Cells were **A**, incubated with human-specific nucleus marker and **B**, mesenchymal stem-cell surface marker, followed by **C**, secondary fluorescent antibodies incubation and 4',6-diamidino-2-phenylindole counterstaining. **D**, Panel shows merged staining of HuNu, CD44, and DAPI. Mesenchymal stem cells maintained their phenotype and expressed both HuNu and CD44 before injections.

FIGURE 2. Mesenchymal stem-cell treatment

Mesenchymal stem cell treatment prevented morphologic changes in neurons that were exposed to lipopolysaccharide. After 3 days of *in vitro* culture, primary cortical fetal neurons were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with Triton X-100. Microtubule-associated protein 2 antibody was applied to visualize dendrites of neurons. Lipopolysaccharide stimulation significantly decreased the number of dendrites ($P < .05$; analysis of variance, Student-Newman-Keuls test). Morphologic findings and organization of cortical neurons was recovered by mesenchymal stem cells and was similar to the control group (normal saline solution). The *asterisk* represents a probability value of $< .05$; analysis of variance, Student-Newman-Keuls test). There were 3 dams per treatment group.

Iba-1 expression in microglial cells

FIGURE 3. Decreased microglial activation

Mesenchymal stem-cell treatment decreased microglial activation after exposure to in utero inflammation. Fetal brains were fixed with 4% paraformaldehyde overnight and permeabilized with Triton X-100. Iba-1 antibody was used to visualize microglial cells. **A**, Microglial cells showed high activation (amoeboid shape, *circle*) in the lipopolysaccharide-treated group, as indicated by large cell bodies and shorter and thicker processes. In control (normal saline solution) and mesenchymal stem-cell-treated groups, the morphologic condition was similar, with small cell bodies and longer, thinner processes (*arrows*). **B**, On quantitative analysis, lipopolysaccharide-exposed microglia had fewer branches as compared with the control group (*triple asterisks* indicate a probability value of < .001; analysis of variance, Student-Newman-Keuls test). Mesenchymal stem-cell-treated microglia had more branches than the lipopolysaccharide group (*double asterisks* indicate a probability value of < .01; analysis of variance, Student-Newman-Keuls test). There were 3 dams per treatment group.

FIGURE 4. Motor activity in neonates

Mesenchymal stem cell treatment improved motor activity in neonates that were exposed to uterine inflammation at postnatal day 5. Cliff aversion test was performed to assess neurodevelopment in offspring. Lipopolysaccharide-exposed pups had significantly slower completion times for cliff aversion at postnatal day 5 compared with the control group (normal saline solution; $P < .001$; analysis of variance Student-Newman-Keuls test). The motor deficit was improved significantly by mesenchymal stem-cell pretreatment in lipopolysaccharide offspring ($P < .001$; Student-Newman-Keuls test).

FIGURE 5. Stem cells localize to placenta

Mesenchymal stem cells localized to placenta in lipopolysaccharide-exposed dams. Maternally administered mesenchymal stem cells localized to murine placenta. **A**, Immunohistochemistry of murine placentas revealed cells that were positive for both human nuclei (anti-HuNu) and mesenchymal stem-cell surface marker (*CD44*), specific for human-derived mesenchymal stem cells. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (*DAPI*). All 3 panels demonstrated that human mesenchymal stem cells that are administered intraperitoneally localized to murine placenta (*arrows*). **B**, Quantification of HuNu⁺/DAPI cells demonstrated a greater number of mesenchymal stem cells in lipopolysaccharide-exposed placenta than in maternal spleen or in control (normal saline solution) placenta. The *asterisk* indicates a probability value of < .05 (χ^2 test).

TABLE

Cytokine concentrations in maternal and fetal compartments

Cytokine concentration	Maternal serum			Placenta		Amniotic fluid		Fetal brain	
	Normal saline solution (n = 6)	Lipopolysaccharide (n = 6)	Lipopolysaccharide + mesenchymal stem cells (n = 11)	Normal saline solution (n = 5)	Lipopolysaccharide (n = 5)	Lipopolysaccharide + mesenchymal stem cells (n = 10)	Normal saline solution (n = 8)	Lipopolysaccharide (n = 8)	Lipopolysaccharide + mesenchymal stem cells (n = 14)
Interleukin									
1 β	30.38 $\pm$ 9.21	21.67 $\pm$ 7.10	26.49 $\pm$ 6.33	6.22 $\pm$ 0.43 ^a	25.06 $\pm$ 5.33 ^{a,b}	30.82 $\pm$ 8.24 ^{a,c}	7.97 $\pm$ 2.20 ^a	21.18 $\pm$ 11.36 ^a	144.29 $\pm$ 67.11 ^{a,d}
2	24.24 $\pm$ 5.05	47.93 $\pm$ 13.11	30.84 $\pm$ 7.72	19.38 $\pm$ 5.01	26.34 $\pm$ 5.11	31.64 $\pm$ 3.83	56.42 $\pm$ 12.53	60.19 $\pm$ 12.20	78.60 $\pm$ 16.90
4	62.25 $\pm$ 15.33	55.20 $\pm$ 13.95	42.39 $\pm$ 13.67	10.61 $\pm$ 3.26	14.74 $\pm$ 5.92	28.90 $\pm$ 7.00	78.75 $\pm$ 29.23	51.71 $\pm$ 11.95	72.96 $\pm$ 22.16
5	—	—	—	—	—	—	—	—	—
6	20.90 $\pm$ 9.55	819.27 $\pm$ 769.69	870.75 $\pm$ 598.55	5.82 $\pm$ 4.62a	54.68 $\pm$ 19.49 ^{a,b}	133.79 $\pm$ 72.24 ^{a,c}	62.47 $\pm$ 50.54	759.16 $\pm$ 675.40	1214.57 $\pm$ 594.23
10	8.20 $\pm$ 3.00 ^a	9.80 $\pm$ 5.59 ^a	49.97 $\pm$ 20.30 ^a	—	—	—	—	—	—
12	2.30 $\pm$ 1.16 ^a	48.35 $\pm$ 20.95 ^{a,b}	119.52 $\pm$ 40.36 ^{a,e}	2.89 $\pm$ 1.61	5.03 $\pm$ 2.33	6.40 $\pm$ 1.39	36.59 $\pm$ 10.42 ^a	51.68 $\pm$ 15.23 ^a	116.57 $\pm$ 19.44 ^{a,d}
Granulocyte-macrophage colony stimulating factor	3.64 $\pm$ 1.28	9.90 $\pm$ 7.84	13.46 $\pm$ 6.16	5.36 $\pm$ 0.86	15.00 $\pm$ 6.25	35.69 $\pm$ 14.06	134.08 $\pm$ 48.91 ^a	155.16 $\pm$ 51.80 ^a	186.48 $\pm$ 62.33 ^a
Interferon- γ	—	—	—	—	—	—	30.70 $\pm$ 15.21	27.10 $\pm$ 16.60	38.75 $\pm$ 12.05
Tumor necrosis factor- α	—	—	—	23.87 $\pm$ 3.39	50.27 $\pm$ 11.71	64.10 $\pm$ 18.46	—	75.68 $\pm$ 46.92	154.88 $\pm$ 66.25 ^d

Data are presented as mean $\pm$ SEM (picograms per milliliter); N represents the number of litters.

^a $P < .05$, analysis of variance on log-transformed data

^b $P < .05$, lipopolysaccharide vs normal saline solution

^c $P < .01$ lipopolysaccharide + mesenchymal stem cells vs normal saline solution

^d $P < .05$ lipopolysaccharide + mesenchymal stem cells vs lipopolysaccharide

^e $P < .001$ lipopolysaccharide + mesenchymal stem cells vs normal saline solution

^f $P < .05$ lipopolysaccharide + mesenchymal stem cells vs lipopolysaccharide.