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LIPOPOLYSACCHARIDE-ACTIVATED MICROGLIA INDUCE DEATH OF OLIGODENDROCYTE PROGENITOR CELLS AND IMPEDE THEIR DEVELOPMENT

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Abstract—Damage to oligodendrocyte (OL) progenitor cells (OPCs) and hypomyelination are two hallmark features of periventricular leukomalacia (PVL), the most common form of brain damage in premature infants. Clinical and animal studies have linked the incidence of PVL to maternal infection/inflammation, and activated microglia have been proposed to play a central role. However, the precise mechanism of how activated microglia adversely affects the survival and development of OPCs is still not clear. Here we demonstrate that lipopolysaccharide (LPS)-activated microglia are deleterious to OPCs, that is, impeding OL lineage progression, reducing the production of myelin basic protein (MBP), and mediating OPC death. We further demonstrate that LPS-activated microglia mediate OPC death by two distinct mechanisms in a time-dependent manner. The early phase of cell damage occurs within 24 h after LPS treatment, which is mediated by nitric oxide (NO)-dependent oxidative damage and is prevented by N^G-nitro-L-arginine methyl ester (L-NAME), a general inhibitor of nitric oxide synthase. The delayed cell death is evident at 48 h after LPS treatment, is mediated by cytokines, and is prevented by blocking the activity of tumor necrosis factor-alpha (TNF-α) and pro-nerve growth factor (proNGF), but not by L-NAME. Furthermore, microglia-derived insulin-like growth factor-1 (IGF-1) and ciliary neurotrophic factor (CNTF) were significantly suppressed by LPS, and exogenous IGF-1 and CNTF synergistically protected OLs from death induced by LPS-treated microglia conditioned medium, indicating that a deficiency in trophic support may also be involved in OL death. Our finding that LPS-activated microglia not only induce two waves of cell death but also greatly impair OL development may shed some light on the mechanisms underlying selective white matter damage and hypomyelination in PVL.

Key words: nitric oxide, reactive oxygen species, myelin, TNF-α, cytokine.

Periventricular leukomalacia (PVL) is the most common form of brain injury in preterm infants and the leading cause of cerebral palsy. Studies increasingly indicate that oligodendrocyte (OL) progenitor cell (OPC), the dominant OL lineage at 24 to 32 weeks gestation (the high risk window for developing PVL), is the major cellular target of damage. Although the cause of PVL is still unknown, a number of studies implicate an etiological link between prenatal infection and PVL (Gilles et al., 1976; Grether and Nelson, 1997).

Microglia are the resident immune cells of the CNS and mount an immune response to brain injury, most commonly due to infection and hypoxia-ischemia. The activated microglia protect the brain from injury by many means, that is, destroying bacteria, clearing cell debris and repairing tissue. However, uncontrolled activation of microglia can cause damage to neighboring neurons and OPCs. In PVL, activated microglia have been found in both focal and diffuse forms of white matter lesions (Haynes et al., 2003). Our previous *in vitro* studies have demonstrated that the bacterial product lipopolysaccharide (LPS)-induced damage to OPCs was mediated by microglia (Pang et al., 2000). Later studies confirmed that the Toll-like receptor 4, a receptor that transduces LPS signaling, was expressed only by microglia but not OLs in the CNS (Lehnardt et al., 2002).

Activated microglia can release a variety of toxic molecules, including nitric oxide (NO), glutamate, and proinflammatory cytokines (Nakajima and Kohsaka, 2004). While intended to kill invading pathogens, these molecules can also lead to damage to neighboring glia or neurons. Indeed it has been shown that OLs at early developmental stages are particularly vulnerable to these microglia-derived factors (Follett et al., 2004; Baud et al., 2004; Horiuchi et al., 2006). As microglia-derived toxic factors range from simple chemicals (e.g., NO) to small proteins (e.g., proinflammatory cytokines), the time of synthesis, release, and turnover of those factors by microglia varies considerably (Nakamura et al., 1999). Therefore, damage to OPCs by activated microglia may occur in different phases (i.e., acute and delayed). A detailed study as how activated microglia cause OL damage in terms of a kinetic event, however, is never reported. Therefore, the first aim of this study is to test the hypothesis that activated microglia induce both an acute and a delayed pattern of OPC death.

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Abbreviations: 4-HNE, 4-Hydroxynonenal; BSA, bovine serum albumin; bFGF, basic fibroblast growth factor; CDM, chemically defined medium; CNTF, ciliary neurotrophic factor; GFAP, glial fibrillary acidic protein; IFCDM, insulin-free chemically defined medium; IGF-1, insulin-like growth factor-1; HBSS, Hank's buffered salt solution; iNOS, inducible nitric-oxide synthase; LDH, lactate dehydrogenase; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MBP, myelin basic protein; MCM, microglia-conditioned medium; MLPS, LPS-treated microglia-conditioned medium; NO, nitric oxide; OL, oligodendrocyte; OPC, oligodendrocyte progenitor cell; PDGF-AA, platelet-derived growth factor-AA; PI, Propidium Iodide; proNGF, pro-nerve growth factor; PVL, periventricular leukomalacia; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TNFab, TNF-α antibody; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide.

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Beyond cell death, activated microglia may also adversely affect OL biology. The number of myelinating mature OLs depends on the proliferation and differentiation of OPCs that are controlled by local growth/trophic factors secreted by neuronal and glial cells including microglia. Interference of OPC proliferation and differentiation will ultimately leads to hypomyelination, as recently shown by Segovia et al. (2008) that chronic hypoxia-ischemia-induced white matter damage is related to persistent arrest in OPC maturation and failure to differentiate and generate myelin. Therefore, the second aim of this study is to test the hypothesis that LPS-activated microglia alter OL development by arresting OPC differentiation.

EXPERIMENT PROCEDURES

Chemicals

LPS (055:B5) and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 and F15 medium, insulin-transferrin-selenium, bovine serum albumin (BSA), penicillin/streptomycin, 2.5% trypsin, and carboxy-H2DCFDA were purchased from Invitrogen (Carlsbad, CA, USA). 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) kit and lactate dehydrogenase (LDH) kit were purchased from Roche (Indianapolis, IN, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit was purchased from Serologicals (Norcross, GA, USA). FeTMPyP was from Alexis (Farmingdale, NY, USA). Antibodies against NG2, O4, myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), OX-42, *4-Hydroxynonenal* (4-*HNE*), active caspase-3, inducible nitric oxide synthase (iNOS) and BSA, and recombinant basic fibroblast growth factor (bFGF) were purchased from Millipore (Temecula, CA, USA). Neutralizing antibodies against TNF-α (TNFab), recombinant rat TNF-α, platelet-derived growth factor-AA (PDGF-AA), insulin-like growth factor (IGF-1) and ciliary nerve growth factor (CNTF) were obtained from R&D Systems (Minneapolis, MN, USA). Neutralizing antibody against pro-neurotrophin nerve growth factor (proNGF) was Alomone Labs (Jerusalem, Israel). The antibody against α-tubulin and the advanced ECL detection kit were purchased from GE Healthcare Biosciences (Piscataway, NJ, USA).

Cell culture

Primary OPC cultures were prepared from neonatal rat optic nerves, as described previously (Pang et al., 2000, 2005). Briefly, optic nerves from the P7 rat were isolated and dissected into a Petri dish containing L15 medium. Cells were dissociated from the nerve tissue with 0.0625% trypsin/0.02% EDTA and cultured in 25 cm^2 uncoated Falcon flasks at 37 °C under 5% CO_2 in a humidified incubator. Cells were maintained in chemically defined medium (CDM), consisting of DMEM/F12, 0.1% BSA, 100 μM putrescine, 20 nM progesterone, 10 nM sodium selenium, 20 nM biotin, 5 μg/ml cysteine, 5 nM hydrocortisone, 5 μM insulin, 50 μM transferrin, 2 nM L-glutamine, and penicillin/streptomycin. OPCs were maintained in CDM supplemented with PDGF-AA and bFGF (10 ng/ml, each) and propagated as needed. The purity and phenotype of OPC culture were identified immunocytochemically using markers for astrocyte, microglia and OPC. As OPCs are the major cell population in P7 rat optic nerve, and they proliferate much quicker than astrocytes and microliga in the PDGF/bFGF-supplemented CDM, virtually no GFAP+ astrocytes or OX-42+ microglia remained in OPC culture after three passages. The phenotype of OPCs was identified as NG2+/O4+/O1−.

Microglia were separated from the mixed glial cultures as described previously (Pang, et al., 2000). Briefly, the cortices of P1 rat brain were separated from surrounding tissue, trypsinized, and maintained in DMEM/F12 with 10% fetal bovine serum. When cells reached confluence, the flasks were shaken in an orbit shaker at 180 rpm for 2 h. The floating cells were filtered through a cell strainer (40 μm pore size), centrifuged and plated in a non-coated flask. After 10 min, the flasks were gently shaken by hand. The supernatant was discarded. The attached microglia cells were then trypsinized and seeded on poly-lysine-coated coverslips or cell culture inserts in 12-well plates, depending on the purpose of treatment.

Microglia-OPC co-cultures were prepared using the Transwell Permeable Supports System (Costar, parts # 3462). Microglia were seeded on the clear polyester membrane-based cell culture inserts featuring 3.0 μm pore size and 1.12 cm^2 culture areas. OPCs were seeded on the poly-L-lysine-coated coverslips (3.79 cm^2 culture areas) in 12-well plates. The total number of microglia and OPCs was 1.5×10^5/well. Just before treatment, both microglia and OPC cultures were washed twice with 1×Hank's Buffered Salt Solution (HBSS), and the cell culture inserts with microglia were transferred to 12-well plates containing OPCs. The experimental procedure was approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and, in addition, was in accordance with the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Preparation of microglia conditioned medium

The CDM used as the maintaining medium for OPC culture contains high concentrations of insulin, which might interfere with some of the experiments; therefore, we used insulin-free CDM (IFCDM) to prepare the conditioned medium. Briefly, 48 h after plating, microglia in 12-well plates (1.5×10^5 cells/well)were washed twice with HBSS, and 1 ml of IFCDM with or without LPS (1 μg/ml) was added to each well. After 24 h, the conditioned medium was collected, filtered through a 0.2 μm syringe filter and stored at −80 °C until use. In the later studies, the term MCM refers to resting microglia-conditioned medium, and MLPS refers to LPS-activated microglia-conditioned medium. Unless otherwise stated, the conditioned medium used to treat OPCs was prepared at 24 h.

Cell survival/death and LDH assay

Cell survival in the short-term treatment (48 h) was measured using the XTT assay, which is based on the reduction of the tetrazolium salt XTT by mitochondrial dehydrogenase in viable cells to form an orange formazan product. Cell survival in the long-term treatment (5 days) was determined by cell counting and presented as a percentage of the number of live/total cells.

Cell death was determined by counting the number of pyknotic nuclei stained with Propidium Iodide (PI). For co-culture, cells were treated with LPS (1 μg/ml), LPS plus L-NAME (200 μM, pretreated for 1 h), LPS plus TNFab (2 μg/ml), or vehicle (Control) for 4, 16 and 48 h. For studies using the conditioned medium, OPCs were treated with MCM, MLPS or the IFCDM for 24, 48 and 72 h. After treatment, OPCs on coverslips were fixed with 4% paraformaldehyde (PFA) for 20 min, stained with PI (100 ng/ml in PBS) for 30 min, and viewed under a fluorescence microscope. PI is often applied to live cells to detect cell death as it only enters nuclei of dead cells and exclude by live cells, therefore, only dead cells can be stained. In the current study, however, PI was applied to cells after fixation so it enters the nuclei of all fixed cells. Therefore, all of the cell nuclei were visualized; however, the dead/dying cells were easily distinguished from healthy cells by their distinct nuclear morphology. The nuclei of dead/dying cells are brightly stained, irregular, condensed and/or fragmented (Sanvicens et al., 2006), while they were dimly but uniformly-stained in healthy cells. In a preliminary study, we

found that all of the dead cells identified with above-mentioned characteristics of PI staining were also positive for TUNEL; therefore, PI staining is used exclusively for detection of cell death in the co-culture study.

LDH was measured using an LDH kit. Because microglia also release LDH after LPS exposure, LDH was measured in both the co-culture (containing both OPCs and microglia) and a sister culture (containing microglia only, with the same density as in the co-culture system). The co-culture or the sister culture was treated with LPS for 4, 24 and 48 h. LDH in the medium as well as total cellular LDH (by lysing cells) was measured, and LDH release was calculated as a percentage of LDH in the medium to total cellular LDH. LDH released from OPCs was calculated by subtracting LDH in the co-culture medium (microglia+OPCs) from LDH in the medium of microglia culture (sister culture).

Detection of reactive oxidative species (ROS)

ROS were detected using a fluorescein dye carboxy-H2DCFDA, which only becames fluorescent after reacting with oxidative products. Briefly, before treatment, OPCs were loaded with 5 μM carboxy-H2DCFDA for 1 h. Cells were washed with HBSS and treated with the conditioned medium for 24 h. The optical density (OD) was obtained using a fluorescence multiplate reader (Synergy HT, Biotech, Winooski, VT, USA) at 492/520 nm. Cells being treated with 1 μM H_2O_2 were used as the positive control.

Immunocytochemistry

Cells were fixed with 4% PFA for 15 min at RT, blocked with a solution containing 5% normal serum/1% BSA and 0.1% Triton X-100 (except for O4 and O1) for 1 h, and were incubated with the primary antibodies for 1 h at RT (NG2 at 1:200, O4 at 1:300, MBP at 1:200, GFAP at 1:500, OX-42 at 1:200, and iNOS at 1:100), or overnight at 4 °C (4-HNE at 1:50). After washing, cells were incubated with biotin-conjugated second antibody (1:200) for 1 h, followed by incubation with fluorescence or Rhodamine-labeled streptavidin. Cells were counter-stained with PI or 4′,6-diamidino-2-phenylindole (DAPI), and viewed under a fluorescence microscope. Images were captured with a CCD camera (Oly-750, Olympus, Center Valley, USA), and superimposed using the Adobe Photoshop (version 7.0) software, if necessary.

Immunoblotting

To quantify MBP expression, OPCs were treated in the conditioned medium for 5 days. At the end of the treatment, cells were washed once with ice-cold PBS, and detached from flasks using a cell scraper. Cells were pelleted by centrifugation, washed twice with PBS, and lysed in the buffer containing 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF and protease inhibitor cocktail (EMD Chemicals, Gibbstown, NJ, USA) for 30 min on ice with vortexes at 10 min intervals. Total protein was determined using the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA). To detect TNF-α and proNGF in the conditioned medium, the conditioned medium was concentrated 50× using a micron membrane (Millipore, Temecula, CA, USA) with a cut size at 3 kDa. Samples from cell lysis or conditioned medium were denatured and subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blocked with 5% non-fat milk in PBS for 1 h at RT. The membranes were incubated with primary antibodies (MBP at 1:500, proNGF 1: 200) overnight at 4 °C, or 2 h at RT (TNF-α at 1:500). Following washing, the membranes were incubated with horseradish peroxidase-conjugated second antibody. Signals were detected using the ECL plus or advanced ECL system (GE healthcare, Piscataway, NJ, USA). The membranes were stripped and reprobed for α-tubulin (1:20000, for cell lysis) or BSA (1:2000, for conditioned medium) to normalize protein loading. The intensity of target bands was determined by scanning with a densitometer and quantified using the ImageQuant software (Molecular Dynamics, Bio-Rad, Hercules, CA, USA).

ELISA

The content of IGF-1, CNTF and PDGF-AA in the microglia-conditioned medium was measured by ELISA kits (R&D Systems, Minneapolis, MN, USA). The conditioned medium was prepared at 24 after LPS treatment.

Statistics

Unless otherwise indicated, results are represented as Mean±SEM of at least three separate experiments. One Way Analysis of Variation followed by Student–Newman–Keuls test was used to determine statistical significance among treatments, and the level of significance was set at $P<0.05$.

RESULTS

LPS-activated microglia-induced acute OPC death was associated with NO

To investigate the interaction between OPCs and microglia after LPS exposure, we first used a microglia-OPC co-culture system that physically separates microglia and OPCs but allows free exchange of molecules between the two culture compartments. As shown in Fig. 1 A and 1 B, no cell death was detected in the control culture over the 48 h period. However, a large amount of OPCs directly underneath the cell culture insert (Fig. 1A: LPS center, also shown in lower magnification in Fig. 1B) died in the LPS-treated wells by 16 h. The dead cells detached from the coverslips and floated into the medium so few cells remained on the coverslips. By 48 h, the number of OPCs at the center of the coverslip was further reduced; however, OPCs outside the edge of the cell culture insert (LPS periphery, Fig. 1A) showed a delayed pattern of cell death. No significant cell death was detected at 16 h, but the nuclei of most cells showed a characteristic of apoptosis with condensed and/or fragmented morphology at 48 h.

Cells detached from the center of coverslip floated into the medium and were counted 24 h after LPS treatment, as shown in Fig. 2A. The significantly increased number of detached cells indicates that it is probably a necrotic cell death and the cell membrane was primarily damaged. To test this idea, we measured LDH released into the medium by OPCs in a time-dependent manner. LDH release started as early as 4 h, peaked at 24 h, but no further significant increase was observed after 24 h (Fig. 2B). In a separate experiment with OPC+LPS (without microglia), no changes of LDH was noticed. Treatment of microglia with L-NAME significantly suppressed LDH release by OPCs at 24 h (Fig. 2C), and also prevented cell detachment from the center of coverslips (Fig. 1A, LPS+L-NAME center), indicating that the acute cell death was mediated by NO. Immunocytochemistry study confirmed that L-NAME indeed suppressed iNOS expression by microglia at 24 h (Fig. 2D).

Cell death in the periphery of the coverslips showed a characteristic of apoptosis that only became detect-

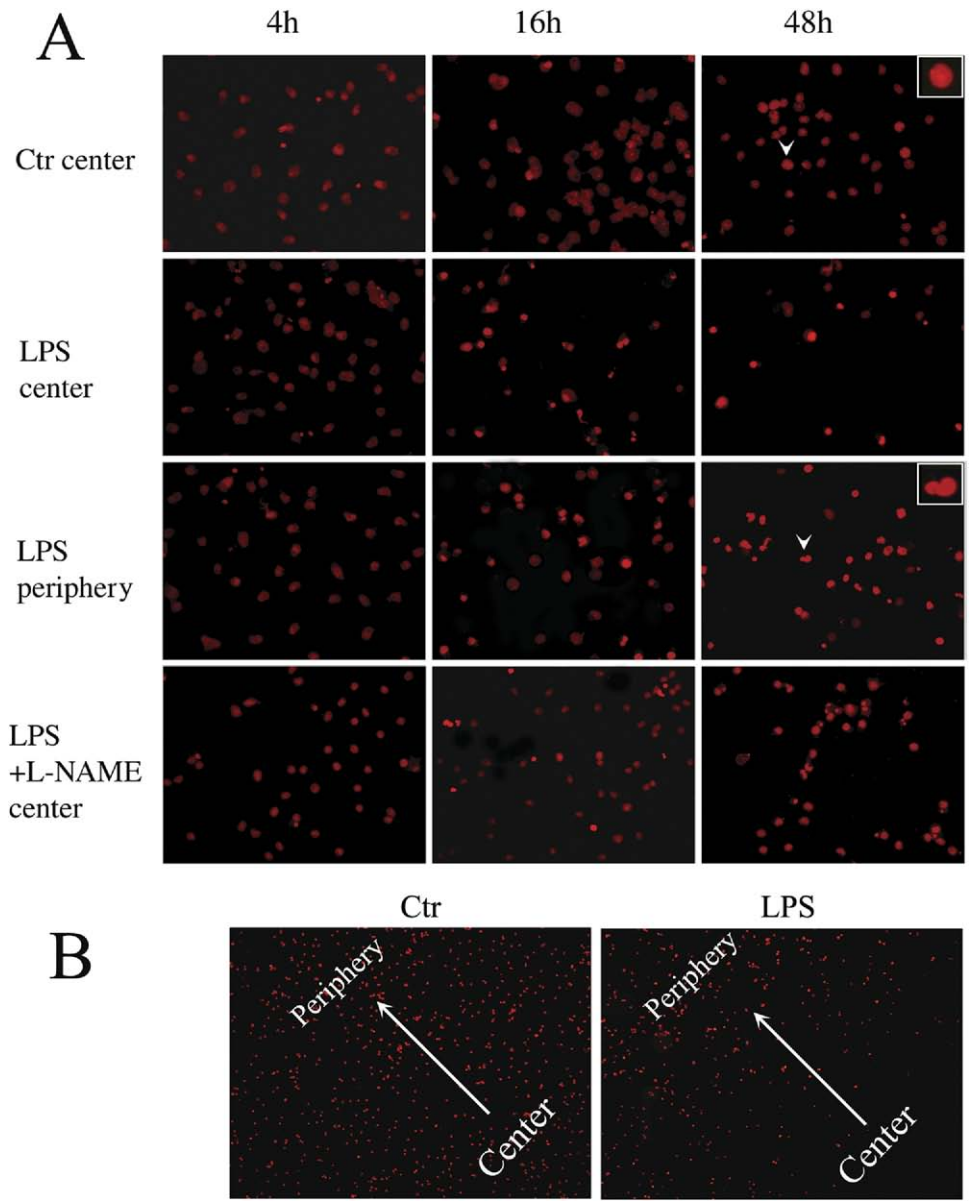

Fig. 1. LPS-activated microglia induced acute OPC death. (A) The OPC-microglia co-culture was treated with IFCDM only (Ctr), LPS or LPS+L-NAME (pre-treatment for 1 h) for 4, 16 and 48 h. Cells were fixed and stained with PI. Cells in the control show normal nuclear morphology (indicated by an arrow head in the panel of Ctr center 48 h, a representative normal nucleus is also shown in a high magnification in a white box). The acute OPC death was detected at 16 h after LPS treatment and was found most prominent at the center of coverslips which was directly underneath the cell culture inserts containing microglia. Some OPCs were already detached from the center. By 48 h, few OPCs remained at the center of coverslip, while cells at the periphery still attached to the culture surface but showed characteristics of apoptosis (arrow indicated in panel of LPS periphery at 48 h). (B) A low-power view shows the gradual decrease of cell density from the center to the periphery of coverslips at 48 h after LPS treatment. Magnification: A: 100×, B: 10×. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

able>16 h. TNF-α is one of the most important proinflammatory cytokines released by LPS-activated microglia (Nakamura et al., 1999; Facchinetti et al., 2003), and has been shown to induce apoptosis in OPCs (Scurlock and Dawson, 1999; Pang et al., 2005). Therefore, we tested if blocking TNF-α activity would protect against cell death in the periphery. Pretreatment of co-culture with a TNFab (2 μg/ml), but not L-NAME (200 μg/ml) significantly prevented OPC death in the periphery after 48 h (Fig. 2E). A separate experiment confirmed that TNFab at 2 μg/ml completely abolished recombinant rat TNF-α (100 ng/ml)-induced OPC death (Fig. 2F).

The acute OPC death was mediated by ROS

It is well known that LPS-activated microglia release a large amount of NO through iNOS, and NO is readily oxidized to form ROS (mostly peroxynitrite). To test the hypothesis that the acute OPC death was mediated by NO-derived ROS, we investigated oxidative products in

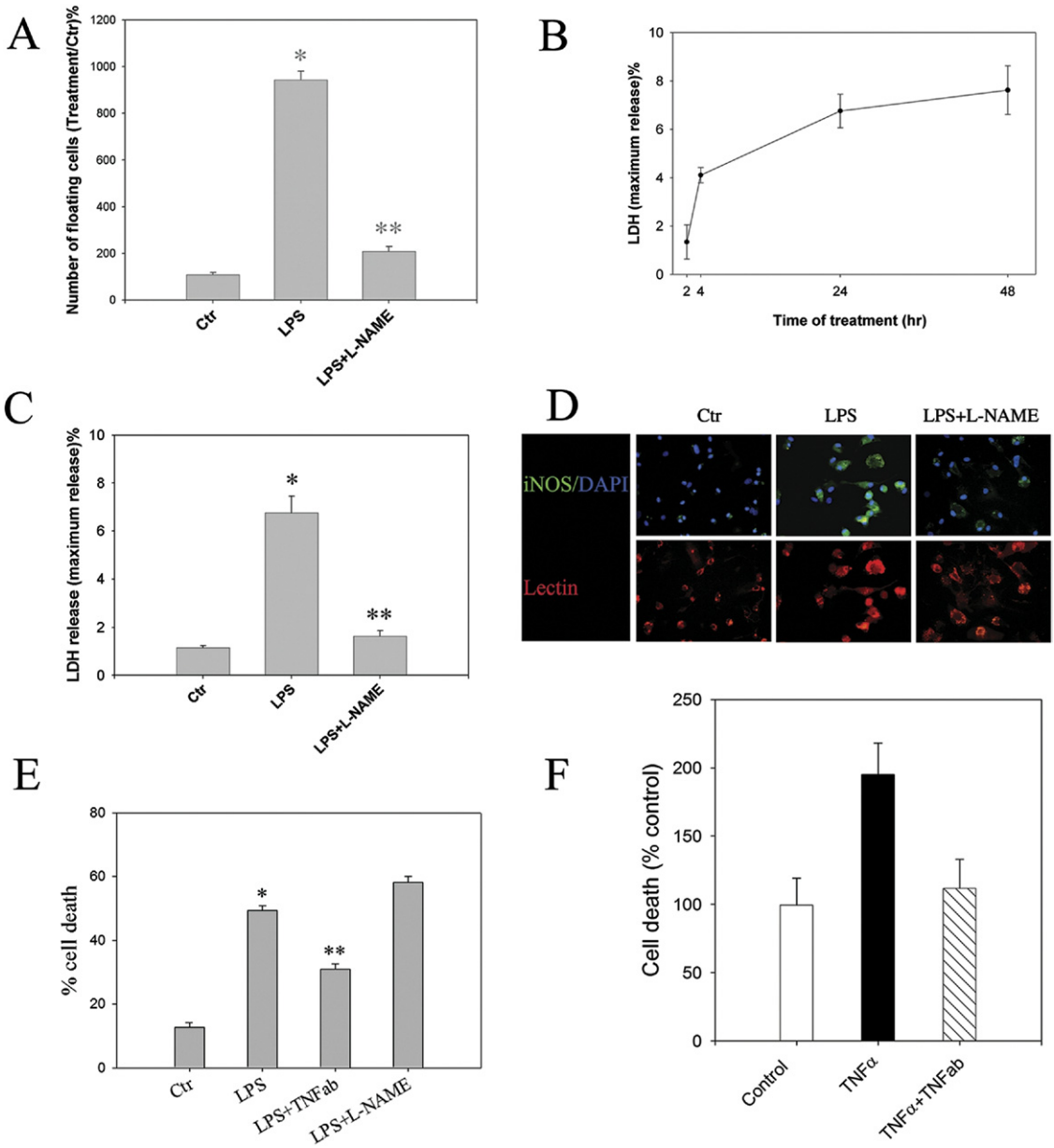

Fig. 2. L-NAME prevented OPC death in the center, while TNFab prevented OPC death in the periphery of coverslips that been co-cultured with microglia and treated with LPS. (A) L-NAME significantly reduced the number of the detached cells 24 h after LPS treatment. (B) LDH release from OPCs being co-cultured with microglia started at 4 h and peaked at 24 h after LPS treatment. (C) L-NAME significantly suppressed LDH release from OPCs 24 h after LPS treatment. (D) A double-labeling study showed that L-NAME indeed blocked iNOS expression in microglia by 24 h. (E) TNFab, but not L-NAME prevented cell death in the periphery by 48 h. (F) The specificity of TNFab was confirmed as TNFab (2 μg/ml) completely abolished TNF-α (100 ng/ml)-induced OPC death after 48 h exposure. Magnification in D: 100×. * $P<0.001$ vs Ctr, ** $P<0.01$ vs LPS.

OPCs in the co-culture system. First we examined if 4-HNE, an oxidative product located in the cell membrane, was induced in OPCs. 4-HNE was undetectable in either OPCs co-cultured with microglia, or without microglia (OPCs in IFCDM). However, strong 4-HNE signals were detected by 24 h in OPCs co-cultured with microglia when treated with LPS. Consistent with the ability in preventing cell death, L-NAME reduced 4-HNE production in OPCs (Fig. 3A). Further study showed that ROS production increased in OPCs co-cultured with microglia 24 h after LPS treatment (Fig. 3B). Pretreatment of microglia with L-NAME significantly prevented ROS production. Correspondingly, L-NAME and FeTMPyP (a cell-permeable catalyst for peroxynitrite decomposition) greatly enhanced cell survival at 48 h after LPS treatment (Fig. 3C).

The delayed death of OPCs was mediated by proinflammatory cytokines

To determine the mechanism underlying the delayed OPC death observed in the periphery of the coverslip, OPCs were treated with the conditioned medium from LPS-

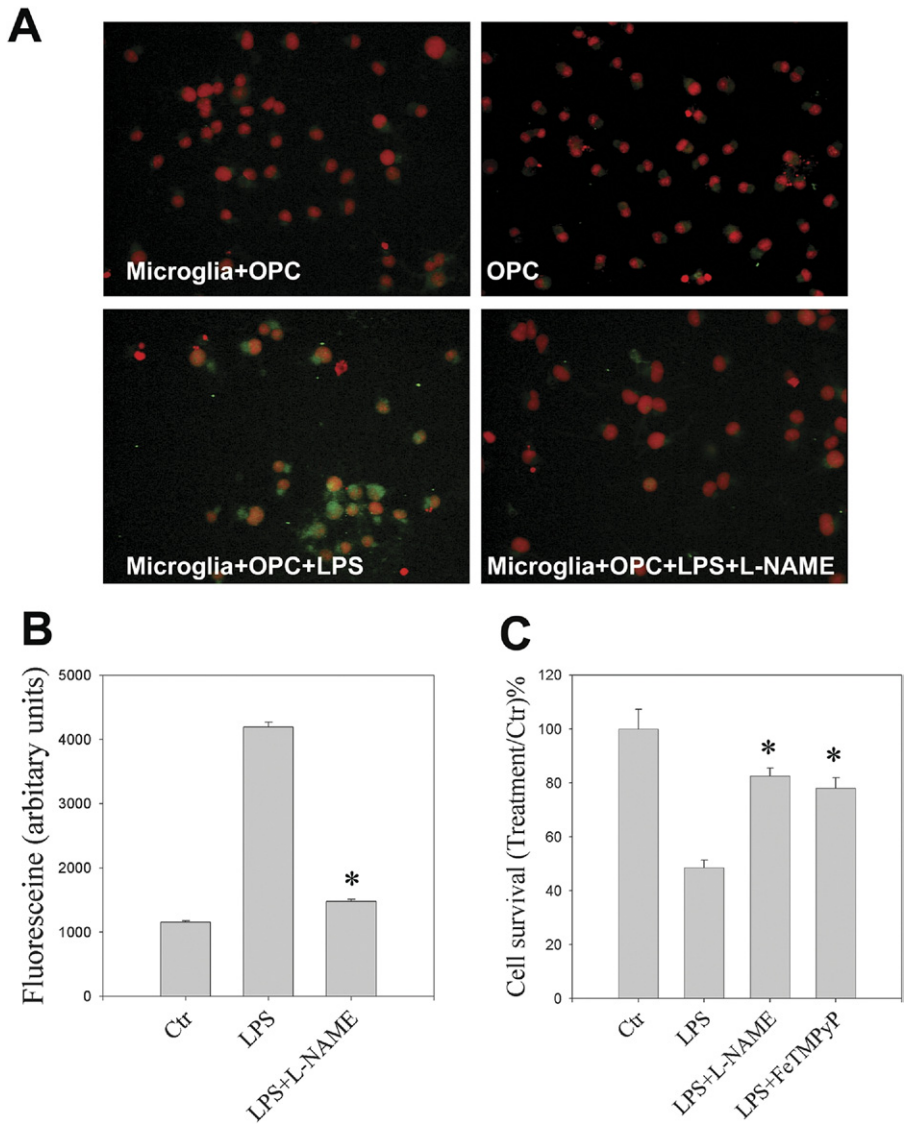

Fig. 3. Oxidative damage to OPCs by LPS-activated microglia. (A) 4-HNE immunostaining (green fluorescence) was detected in OPCs co-cultured with microglia and treated with LPS (Microglia+OPC+LPS) for 24 h, but not in the control culture (microglia+OPC, no LPS added) or in OPCs without microglia (OPC). L-NAME significantly reduced 4-HNE immunoreactivity (Microglia+OPC+LPS+L-NAME). Cell nuclei were counterstained with PI (red). (B) ROS production was significantly increased by 24 h following LPS treatment, as measured using H2DCFDA as a probe. L-NAME significantly reduced ROS production in OPCs. (C) LPS treatment resulted in 50% reduction of cell survival. L-NAME, as well as the ROS decomposition catalyst FeTMPyP, significantly increased cell survival. Magnification in A: 100×. * $P<0.05$ vs LPS.

treated microglia. At 24 and 48 h of treatment, there were no signs of cell death in the MCM or vehicle-treated cells, while some MLPS-treated cells showed damaged nuclei at 48 h of treatment (Fig. 4A). At 72 h, substantial cell death was detected in both IFCDM and MLPS-treated, but not MCM-treated cells. The dead cells were also positively stained with TUNEL (Fig. 4B). As TNFab protected against cell death at the periphery in the co-culture system, we next tested if TNFab also remained effective in preventing MLPS-induced OPC death in the conditioned medium. Consistent with the observation in the co-culture system, TNFab partially but significantly prevented MLPS-induced caspase-3 activation (Fig. 5A) and decrease of OPC survival (Fig. 5B, XTT survival assay), while MLPS from microglia pretreated with L-NAME did not. Both a small (17 kDa) and a larger (26 kDa) TNF-α proteins were detected in MLPS but not in MCM, by Western blot. However, only the 26 kDa protein was detected in LPS-treated microglia cell lysis (Fig. 5C). Although significant, blocking TNF-α only partially protected OPCs from death, suggesting that there might be other contributing factors. Interestingly, neutralizing proNGF activity, which has been shown to mediate mature OL death by activated microglia (Yune et al., 2007), significantly prevented caspase-3 activation

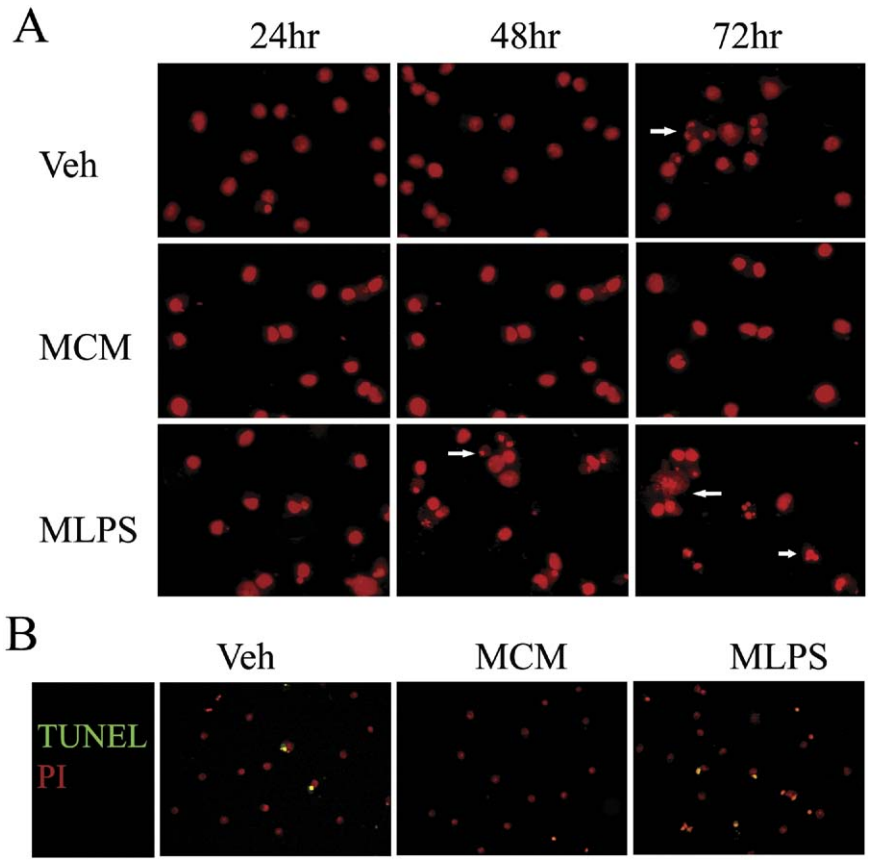

Fig. 4. Delayed OPC death induced by MLPS. OPCs cultured in coverslips were treated with vehicle, MCM or MLPS for 24, 48 and 72 h. Cells were fixed and stained with PI to visualize their nuclei. Some coverslips were also stained with TUNEL. (A) No cell death was detected in MCM-treated OPCs over 72 h period, while some dead cells were present in the vehicle-treated OPCs at 72 h. Significant cell death was detected in MLPS-treated OPCs at 48 h, and was more prominent at 72 h (arrows). (B) The dead cells identified by PI-staining were also positively stained by TUNEL. Magnification A: 150×, B: 100×.

(Fig. 5A) and OPCs from death (Fig. 5B). Western blot confirmed that proNGF was released into the conditioned medium by LPS-activated microglia (Fig. 5D).

LPS-activated microglia lost ability to support OL survival and development in long-term cultures

We further investigated OPC development using several well-defined OL markers. At 48 h post treatment, OLs in the MCM developed fine processes, while MLPS-treated cells had fewer and shorter processes, as showed by O4 immunostaining (Fig. 6A, low panel). After 5 days, most cells in MCM appeared to be healthy and intensively immunostained with the mature OL marker MBP, while the majority of cells died and only a few cells were found MBP+ in either IFCDM or MLPS. The surviving cells appeared to be unhealthy, as shown by much shorter/fewer processes when immunostained with MBP (Fig. 6B, upper panel) and seen on the phase-contrast microscope (Fig. 6B, lower panel). Further immunoblotting demonstrated more than 90% reduction in the quantity of MBP expressed in MLPS-treated OLs, as compared to that of MCM (Fig. 6C). Interestingly, neutralizing TNF-α activity, which was previously shown to protect cells from death, did not increase MBP expression.

Suppression of microglia-derived growth factors by LPS is associated with OL death and developmental retardation in the long-term culture

OL survival and differentiation are highly dependent on growth/trophic factors locally secreted by microglia and astrocytes. Suppression of growth/trophic factors for OPCs survival and differentiation from microglia may indirectly contribute to death and developmental retardation of OPCs. Therefore, we measured the contents of several growth/trophic factors that have been shown to be critical for OL survival and development. At 24 h, the concentration of both IGF-1 and CNTF in MLPS was significantly decreased as compared to that in MCM, while PDGF-AA was not affected (Fig. 7A).

To further investigate if the decrease of IGF-1 and CNTF plays a role in OL death and developmental retardation induced by MLPS in the long-term culture, OPCs were treated with MLPS or MLPS supplemented with recombinant IGF-1 and CNTF individually or in combination (both at 1 ng/ml). Cell survival and differentiation were determined after 5 days. No effects were observed for both IGF-1 and CNTF if used individually. However, a combination of IGF-1 and CNTF significantly increased both cell survival (Fig. 7B) and the

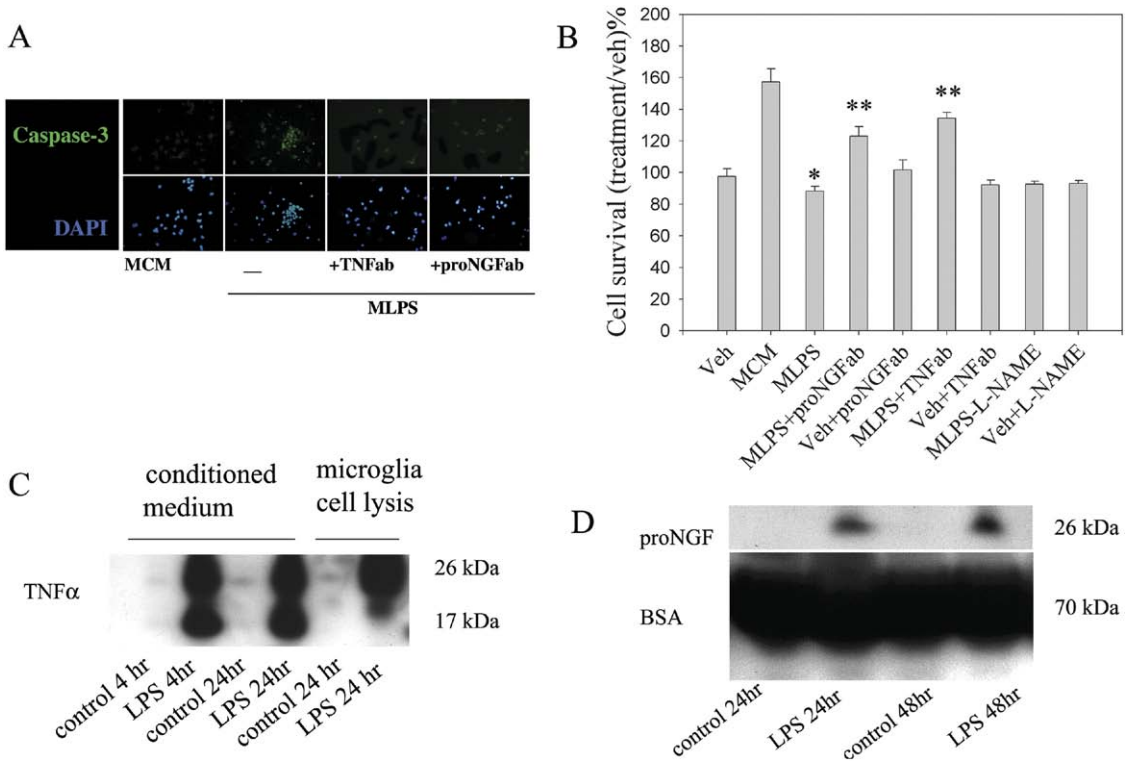

Fig. 5. Blocking TNF-α and proNGF activity protected against MLPS-induced OPC death. (A) Activation of caspase-3 in OPCs by MLPS was prevented by neutralizing TNF-α and proNGF after 24 h treatment. (B) Treatment with TNF-α and proNGF neutralizing antibodies, but not L-NAME, protected against cell death. (C) and (D) Detection of TNF-α and proNGF proteins in LPS-treated microglia conditioned medium by Western blot. Both 17 and 26 kDa TNF-α proteins were detected in the conditioned medium, but only the 26 kDa protein was detected in cell lysis. * $P<0.01$ vs MCM, and ** $P<0.05$ vs MLPS. Magnification in A: 100×.

number of MBP+ cells in the culture as compared to MLPS treatment (Fig. 7C).

DISCUSSION

Here we provide evidence that LPS-activated microglia are harmful to OPC. This finding is consistent with many studies showing that activated microglia were deleterious to developing OLs (Pang et al., 2000; Sherwin and Fern, 2005; Domercq et al., 2007; Li et al., 2005). The novel finding of the current study, however, is that we demonstrated that OPC died in two distinct time frames.

Activated microglia rapidly respond as the first line of defense to protect against brain damage. During activation, microglia transform from a ramified to an amoeboid shape. This process occurs rapidly within minutes to a few hours after the original injury (Davalos et al., 2005). It is increasingly recognized that activated microglia are involved in many CNS diseases (Barger and Basile, 2001; Minghetti, 2005) including PVL (Rezaie and Dean, 2002; Huleihel et al., 2004). Activated microglia can release or up-regulate a plethora of cytokines, chemokines and enzymes such as IL-1β, TNF-α, IL-6, transforming growth factor-β1 (TGF-β1), macrophage-colony stimulating factors (M-CSF), and iNOS (Bal-Price and Brown, 2001). The synthesis and secretion of these molecules varies from minutes to hours. It is important to note that while microglial activation has been repeatedly documented in the context of brain injury *in vivo*, the exact cause and effect relationships between microglial activation and OPC injury have not been completely understood. Indeed, much attention has been paid to neuronal death by activated microglia through NO. iNOS is not normally expressed in the brain, but can be induced by LPS and cytokines. Once sustained high levels of NO being generated by iNOS, it can damage neighboring neurons by mechanisms which include inhibition of mitochondrial respiration and release of glutamate from neurons and glia (Uehara et al., 1999; Bal-Price and Brown, 2001). An alternative way for NO to cause cell damage is that NO is oxidized to form ROS. Interestingly, OLs are highly susceptible to ROS-induced damage (Fragoso et al., 2004; Baud et al., 2004). Recently, Li et al. (Li et al., 2005) have shown that NO-derived peroxynitrite is the major toxic factor in mediating OL damage by LPS-activated microglia. In line with this, the current study clearly demonstrated a central role of NO-derived ROS in mediating OPC damage by LPS-activated microglia, by the following evidences: (1) the acute death of OPCs directly underneath the culture insert with microglia and the delayed cell death in the periphery suggests that the harmful molecules secreted by microglia have a relatively short half-life and do not easily diffuse to the periphery of the coverslip; (2) the acute OPC death could be prevented by the NOS inhibitor L-NAME and the ROS decomposer FeTMPyP which further suggests the involve-

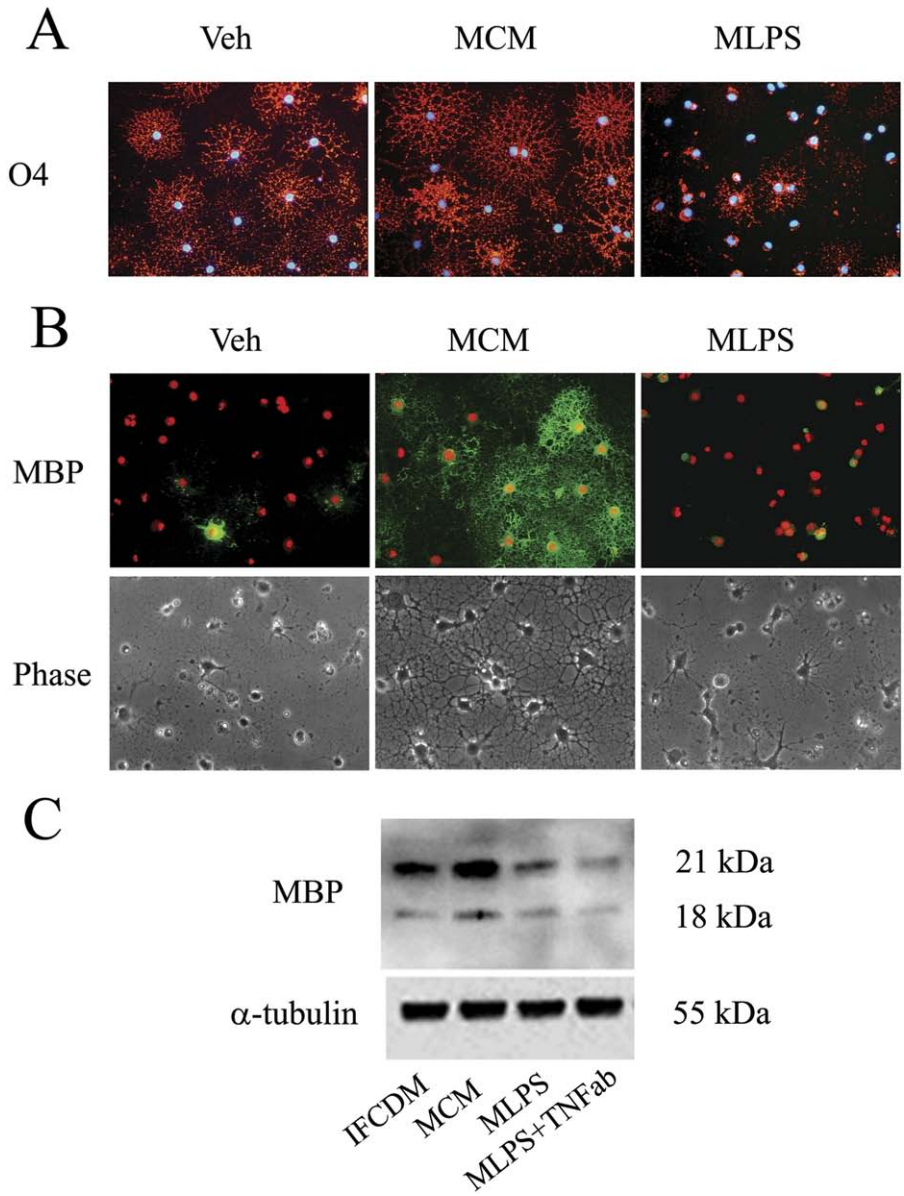

Fig. 6. LPS-activated microglia impeded OL development. OPCs were cultured in the vehicle, MCM or MLPS, cell development was assessed using stage-specific markers. At 48 h, OPCs cultured in MLPS showed great reduction in the number and length of cell processes (immunostained with O4) as compared to that in both the vehicle and MCM (A). After 5 days, only a few cells in the vehicle expressed MBP, while most of cells in MCM were intensively immunostained with MBP at both cell bodies and processes. Cells in MLPS expressed very limited amount of MBP and it was only weakly stained in cell bodies (B, upper panel). The corresponding phase-contrast images showed that cells cultured in MCM developed fine processes, while cells either in the vehicle or MLPS exhibited shorter and stunted cell processes (B, low panel). Western blot confirmed that the content of MBP was significantly reduced in OLs cultured in MLPS, as compared to that of MCM treatment (C). Blocking TNF-α activity failed to increase MPB expression. Magnification: 100×.

ment of NO-derived ROS; and (3) oxidative products increased in OPCs co-cultured with microglia after LPS treatment. The evidence of acute damage to the cell membrane, that is, LDH leakage and cells detachment from the culture surface, are also characteristics of ROS damage. However, the ROS-mediated OPC damage as detected by LDH leakage, occurs in a relatively short time period, by 24 h, no further significant increase of LDH was detected.

In contrast to the acute cell death, the delayed cell death (>24 h) is mediated by a distinct mechanism that independent of NO. Considering the vulnerability of OPCs to TNF-α as shown in our previous study (Pang et al., 2005), we first investigated if TNF-α is involved in OPC death in the current system. The protection against cell death in both co-culture systems and conditioned medium by neutralizing TNF-α activity confirmed that TNF-α played a critical role in the delayed OPC death. In contrast, L-NAME was unable to prevent the delayed OPC death in either the co-culture system (cells in the periphery of coverslip) or the LPS-treated microglia-conditioned medium, suggesting cy-

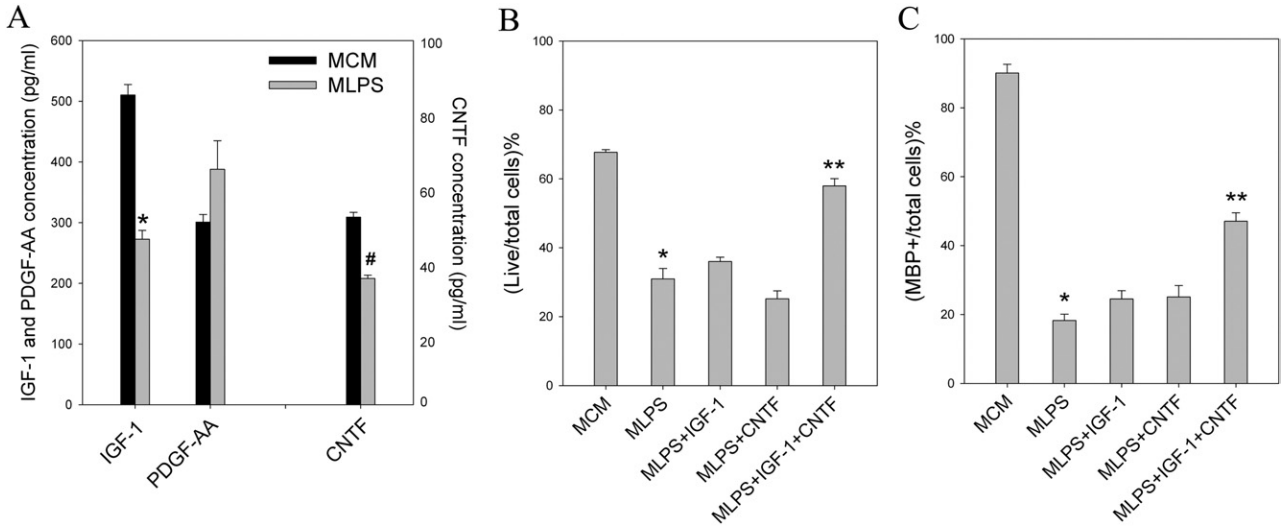

Fig. 7. Suppression of microglia-derived IGF-1 and CNTF by LPS was associated with OL death and developmental retardation in a long-term culture. The contents of IGF-1 and CNTF but not PDGF-AA in the microglia conditioned medium were significantly suppressed by LPS at 24 h (A, * $P<0.01$ and # $P<0.05$, compared to IGF-1 and CNTF in the MCM, respectively). Neither recombinant IGF-1 nor CNTF alone (both at 1 ng/ml) prevented the decrease of cell survival or the number of MBP+ cells by MLPS (B, C). However, when used in combination, both cell survival and the number of MBP+ cells were significantly increased. * $P<0.01$ vs MCM; ** $P<0.05$ vs MLPS.

tokines rather than ROS are responsible for the delayed cell death. Although TNF-α released by LPS-activated microglia could be detected within 1 h (Nakamura et al., 1999), our previous *in vitro* study showed that TNF-α-induced OPC death was only evident after 24 h (Pang et al., 2005). Characteristically, the dead cell bodies and fragmented nuclei were still attached to the coverslips 48 h after TNF-α treatment. It is interesting to note that the clearance of extracellular TNF-α seems to be different between *in vitro* and *in vivo*. Intracerebral injection of LPS induced a rapid increase of TNF-α in neonatal rat brain, peaking at 2 h but remaining non-detectable at 24 h (Pang et al., 2003); however, the TNF-α level was still high in the LPS-treated microglia conditioned medium which was collected 24 h later. This may suggest that TNF-α in the brain is primarily cleared by reuptake by other cells, rather than degradation. It is important to note that TNF-α cytotoxicity to OLs is a developmentally related event, as clearly shown by our previous study that the sensitivity of OL to TNF-α decreased following cell maturation. Specifically, OPCs are vulnerable while mature OLs are quite resistant to TNF-α cytotoxicity (Pang et al., 2005). More recently, Miller et al. (2007) reported that in a mixed microliga-OL culture, TNF-α produced by LPS-activated microglia reduced the survival of OPCs while increased the survival of mature OLs.

Interestingly, proNGF, a precursor form of the neurotrophin NGF, is also involved in the delayed OPC death. To our knowledge, this is the first report showing that proNGF is responsible for OPC death by LPS-activated microglia, although it has been recently demonstrated that proNGF could be released by activated microglia and induce mature OL death through activation of P75 neurotrophin receptor after spinal cord injury (Yune et al., 2007). The P75 neurotrophin receptor belongs to the TNF receptor superfamily, and has been shown to be expressed by OPCs (Petratos et al., 2004).

Beyond OPC death, abnormal myelination is a major pathological feature of PVL (Leviton and Gilles, 1996). Our previous studies showed that hypomyelination is common in both maternal and neonatal rodent models of white matter damage induced by LPS (Cai et al., 2000; Pang et al., 2003). Hypomyelination can be the result of either a reduced number or a dysfunction of mature OLs. Arrest in OPC differentiation may underlie the main mechanism. A recent study in human PVL brain showed that the number of OLs was not reduced but the quality of myelin was abnormal; in addition, O4+ OPCs in the white matter lacked processes (Billiards et al., 2008). In line with this, we found that the differentiation of OLs was greatly reduced by MLPS. The shorter and fewer processes, and the failure to produce MBP by OLs after treatment with MLPS may suggest a direct damage to cells by cytokines, but could also be a result from decrease in growth/trophic factor(s) that are normally secreted by resting microglia. Both the survival and differentiation of OL depend on local growth/trophic factors produced by glia and neurons (Barres et al., 1993). Our further studies indicate that suppression of microglia-derived growth/trophic factors may also contribute to the observed adverse effects by LPS in the long term. Microglia secrete many growth/trophic factors, for example, IGF-1, CNTF, brain-derived neurotrophic factor, bFGF, NGF and TGF-β (Nakajima and Kohsaka, 2004), and most of these factors also support OL survival and differentiation. Among these microglia-derived factors, IGF-1 and CNTF are two of the most potent growth factors that have been shown to support both OL survival and differentiation (Palacios et al., 2005; Talbott et al., 2007). Our result showed that the concentration of both factors was reduced in MLPS. Furthermore, exogenous supple-

mentation of recombinant IGF-1 and CNTF at concentrations close to those found in the MCM synergistically increased OL survival and differentiation. This is in accordance with an earlier study by Barres et al. (1993), showing that growth factors from different classes synergistically support long-term survival of OLs. Since neutralizing antibody for TNF-α protected OL from death but failed to promote MBP expression, it suggests that reduction of MBP in mature OLs by MLPS is not due to TNF-induced cytotoxicity, but likely results from other mechanisms such as lack of trophic support by microglia.

CONCLUSION

This study provides new insight into the mechanisms of OL damage by showing a dynamic OPC death mediated by LPS-activated microglia. Further, the deficiency in trophic support by LPS-activated microglia may contribute to the decrease in OL development.

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