Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes

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Reactive microglia in the CNS have been implicated in the pathogenesis of white matter disorders, such as periventricular leukomalacia and multiple sclerosis. However, the mechanism by which activated microglia kill oligodendrocytes (OLs) remains elusive. Here we show that lipopolysaccharide (LPS)-induced death of developing OLs is caused by microglia-derived peroxynitrite, the reaction product of nitric oxide (NO) and superoxide anion. Blocking peroxynitrite formation with nitric oxide synthase inhibitors, superoxide dismutase mimics, or a decomposition catalyst abrogated the cytotoxicity. Only microglia, but not OLs, expressed inducible NO synthase (iNOS) after LPS challenge; microglia from iNOS knockout mice were not cytotoxic upon activation. The molecular source for superoxide was identified as the superoxide-generating enzyme NADPH oxidase. The oxidase was activated upon LPS exposure, and its inhibition prevented microglial toxicity toward OLs. Furthermore, microglia isolated from mice deficient in the catalytic component of the oxidase, gp91^{phox}, failed to induce cell death. Our results reveal a role for NADPH oxidase in LPS-induced OL death and suggest that peroxynitrite produced by iNOS and NADPH oxidase in activated microglia may play an important role in the pathogenesis of white matter disorders.

inflammation | lipopolysaccharide | cerebral palsy | reactive nitrogen species | periventricular leukomalacia

Microglia are the resident macrophage-like cells of the CNS. They play a pivotal role in the innate immune response of CNS and are the first line of defense against microorganism invasion and injury (1). Localized activation of microglia, however, has also been implicated in the pathogenesis of a number of neurological diseases and disorders, including ischemia; AIDS-associated dementia (2); chronic neurodegenerative diseases (3), such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis; and white matter disorders, such as multiple sclerosis (4) and periventricular leukomalacia (PVL) (5). Microglia are extremely responsive to environmental or immunological challenges and are the predominant cell type producing inflammation-mediated neurodegeneration. Upon activation, microglia release proinflammatory molecules, such as cytokines and chemokines, and mediators of cell injury, such as proteases and reactive oxygen species (ROS)/reactive nitrogen species (6–8). Although necessary for normal functions, activation of microglia requires tight regulation to avoid bystander injury to other CNS cells. The identification of primary toxic factors, localization of their molecular sources, and the elucidation of signaling pathways associated with microglial overactivation are therefore of paramount importance to our understanding of cellular injury in the CNS.

PVL is the lesion of cerebral white matter that underlies most cases of cerebral palsy developing in premature infants (9). It is characterized by focal necrotic lesions in the deep white matter and by diffuse injury to premyelinating oligodendrocytes (preOLs) and possibly axons in the central cerebral white matter. The diffuse lesion is followed by subsequent hypomyelination, thought to be due to a loss of preOLs and subsequently mature myelin-producing OLs. The pathogenesis of PVL is believed to be initiated by two key upstream mechanisms, hypoxia/ischemia, and maternal-fetal infection/inflammation (10). Recently, it was reported that the cytotoxicity of lipopolysaccharide (LPS) to preOLs was completely dependent on microglia because of the activation of the microglial Toll-like receptor 4 signal transduction pathway (11). Astrocytes, OLs, or cortical neurons do not express this receptor in culture. Thus, the results of this study indicate that activation of microglia may contribute to oligodendroglial damage observed in white matter disorders and provide a mechanistic link between innate immunity and loss of OLs. The data are consistent with recent neuropathological findings showing that the diffuse white matter lesion in PVL is populated with activated microglia (5). Furthermore, other studies demonstrate a positive relationship between maternal-fetal infection, hypoxia/ischemia and white matter injury similar to PVL (12–14).

In this study, we demonstrate that it is peroxynitrite generated from LPS-activated microglia that mediates the death of OLs. We further determined the molecular sources of peroxynitrite. Our data indicate that up-regulation of inducible nitric oxide synthase (iNOS) in microglia but not in preOLs and activation of the microglial superoxide-generating NADPH oxidase contribute to the formation of peroxynitrite that is cytotoxic to developing OLs.

Materials and Methods

Cell Cultures, Cell Treatment, Free Radical Accumulation Measurement, and NADPH Diaphorase Histology. Primary OLs and microglia were prepared from mixed glial cultures of newborn Sprague–Dawley rat forebrains by using a selective detachment procedure with modifications (15). Microglia were isolated by shaking flasks containing mixed glia for 1 h at 200 rpm on an orbital shaker (catalog no. 57018-754, VWR Scientific), maintained in DMEM containing 5% FBS and were used the next day. Purified OLs were cultured in a serum-free basal-defined medium for 7–9 days. The OL cultures were primarily progenitors and precursors ($A_2B_5^+$, O_4^+, O_1^-, and myelin basic protein$^-$) and are referred to as preOLs. Contamination by astrocytes and microglia was $<2\%$. Mouse microglia were isolated with the same method as above from mice deficient in *Nos2* (iNOS) and *cybb* (gp91^{phox}). See *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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Abbreviations: DPI, diphenyleneiodonium; FeTMPyP, 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphyrinato iron III; iNOS, inducible nitric oxide synthase; L-NMMA, *N*(G)-monomethyl-L-arginine monoacetate; LPS, lipopolysaccharide; MnTMPyP, manganese(III) tetrakis(*N*-methyl-2-pyridyl)porphyrin; OL, oligodendrocyte; preOL, premyelinating OL; PVL, periventricular leukomalacia; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Two different types of microglia plus preOL cocultures were used. Microglia were either plated together or placed into transwells above the preOL layer. Cell death was induced by exposure to LPS (*Escherichia coli* O111:B4, Sigma) in the presence of various reagents as specified in the figure legends. After 24–48 h, cell survival was evaluated. Accumulation of nitrite in the medium was determined by the Greiss reaction. Free radical generation was evaluated with dichlorohydrofluorescin diacetate and dihydrorhodamine 123 (15). NADPH diaphorase was stained as described in *Supporting Materials and Methods*.

Cell Survival Determination. Survival of preOLs was determined by counting $O4^+$ cells with normal nuclei. Briefly, cell cultures were treated in triplicate as specified in the figure legends for 24–48 h. After being washed with PBS and fixed with 4% paraformaldehyde, cells were immunostained for O4. Five random fields were counted in each coverslip under ×200 magnification, with a total of >1,000 cells counted under control conditions. Cell survival is expressed as mean ± SD.

Translocation of $p67^{phox}$ Assay. Cells were treated with or without LPS for 18–24 h and collected with a hypotonic buffer (20 mM Hepes, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM EGTA/1 mM EDTA/0.5 mM phenylmethanesulfonyl fluoride/1 μg/ml aprotinin/1 μg/ml pepstatin/2 μg/ml leupeptin). Cells were disrupted by five cycles of freezing–thawing with liquid nitrogen. After centrifugation at 10,000 × *g* for 10 min, plasma membranes were obtained by ultracentrifugation of the supernatant at 100,000 × *g* for 1 h at 4°C. The membrane pellets were gently washed with PBS, and the membrane proteins were solubilized with lysis buffer containing 1% Triton X-100. Translocation of cytosolic $p67^{phox}$ to plasma membrane upon LPS activation of microglia was determined by Western blot analysis.

Results

Microglia and Nitric Oxide Are Necessary for LPS-Induced Killing of preOLs. Primary microglia used in this study were >95% pure as determined by immunocytochemical staining for isolectin B4 or CD11 (Fig. 8*A*, which is published as supporting information on the PNAS web site). As reported in ref. 11, we confirmed that the presence of microglia was absolutely required for LPS-induced killing of preOLs (Fig. 8*B*). The death of preOLs was significantly blocked by the NOS inhibitor *N*(G)-monomethyl-L-arginine monoacetate (L-NMMA) and the iNOS specific inhibitor 1400W (Fig. 1). In contrast, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor antagonist NBQX had no protective effect (Fig. 1). These results demonstrated that production of NO upon LPS challenge but not receptor-dependent excitotoxicity was involved in the killing.

Mature myelin basic protein expressing OLs were also sensitive to LPS. However, they died at a slower rate (survival, 45 ± 13%; 72 h) than preOLs (survival 3 ± 2%; 48 h; $n = 2$) in response to LPS-activated microglia. This finding is consistent with previous observations that preOLs are more sensitive to oxidative and nitrative stress than mature OLs (16, 17).

Peroxynitrite Is the Diffusible, Short-Lived Factor That Is Toxic to preOLs. To identify the mechanism underlying the killing of preOLs by LPS-activated microglia, we next asked whether cell–cell contact was required for the toxicity. To pursue this question, we performed experiments by using porous transwells in which microglia were cultured in transwells and placed into 24-well culture plates containing preOLs. The distance between the microglia layer and the preOLs was 1 mm, and the pore size of the transwell membrane was 0.4 μm. Thus, there was no physical cell–cell contact between the two cell types but soluble/secreted factors could move across the transwell. In the absence

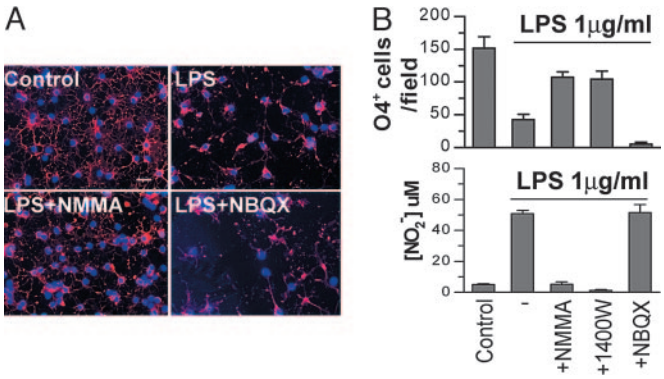

Fig. 1. LPS-activated microglia were cytotoxic to preOLs. LPS-induced, microglia-dependent killing of preOLs in preOL plus microglia cocultures was blocked by the NOS inhibitor L-NMMA but not by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor antagonist NBQX. preOL plus microglia cocultures were treated with or without 1 μg/ml LPS in the presence of 1 mM L-NMMA, 10 μM 1400W, or 100 μM NBQX for 48 h. (*A*) Survival of preOLs was evaluated by immunocytochemistry (red, O4; blue, bisbenzamide). (Scale bar, 10 μm.) (*B*) Survival of preOLs was evaluated by cell counting (mean ± SEM). NO production was assayed by the Greiss reaction. Results represent one of three independent experiments with similar results.

of microglia, LPS did not have any effect on preOL survival. However, in the presence of transwells containing microglia, LPS led to massive preOL death (Fig. 2*A*). Interestingly, only preOLs directly beneath the transwell died. preOLs that were outside the perimeter of the transwell rim were unaffected (Fig. 2 *A* and *B*). This observation suggested that diffusible and short-lived factors were responsible for the cytotoxicity. As expected, an NOS inhibitor, L-NMMA, prevented cell death (Fig. 2 *A* and *C*).

Because NO is known to react with superoxide anion at a diffusion-limited rate to form the short-lived, potent oxidant peroxynitrite, we next examined whether peroxynitrite was involved in the toxicity. Indeed, a peroxynitrite decomposition catalyst, 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphyrinato iron III (FeTMPyP), completely blocked LPS-induced cell death (Fig. 2*C*). Consistently, a cell permeable superoxide dismutase (SOD) mimic, manganese(III) tetrakis(*N*-methyl-2-pyridyl)porphyrin (MnTMPyP), was equally effective in protecting preOLs without affecting the level of NO produced (Fig. 2*D*). Exogenous SOD had no effect, probably because of its inability to enter cells. Treatment of cells with SOD plus catalase had a partial protective effect, probably because catalase, in addition to removing hydrogen peroxide, can potentially decrease peroxynitrite formation by consuming NO catalytically in the presence of hydrogen peroxide (18). Taken together, these results suggested that peroxynitrite was the toxic factor produced by LPS-activated microglia.

Microglia Are the Sole Source for LPS-Induced NO Production. Next, we investigated the molecular sources of NO and superoxide responsible for the production of peroxynitrite. It has been controversial whether OLs are capable of expressing iNOS and producing NO under inflammatory conditions (19, 20). By using microglia plus preOL cocultures treated with LPS, we found that only microglia were positive for NOS activity as determined by NADPH diaphorase histochemistry (Fig. 3*A*). Immunocytochemical staining revealed that expression of iNOS always colocalized with activated microglia (Fig. 3*B*). Furthermore, LPS induced iNOS expression and NO production in a concentration-dependent manner in cocultures but failed to do so in OL cultures (Fig. 3*C*), reinforcing the observation that only microglia but not preOLs are capable of expressing iNOS. To further determine whether physical contact between microglia and

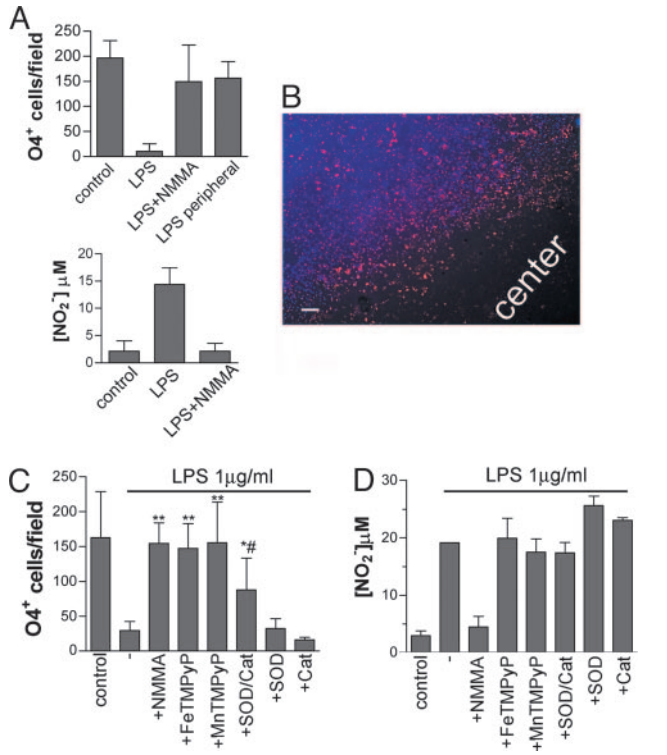

Fig. 2. Peroxynitrite was responsible for LPS-induced, microglia-dependent toxicity to preOLs. (*A*) preOLs and microglia were cocultured in separate compartments by using transwells, and treated as indicated for 48 h (LPS 1 μg/ml, L-NMMA 1 mM). Surviving preOLs underneath the transwells were counted and the nitrite level in the media was assayed. preOLs in fields outside of the insert region were unaffected (LPS peripheral). (*B*) Photomicrograph of O4 immunocytochemistry of preOLs beneath a microglia insert treated with LPS. Only preOLs positioned directly beneath the transwell containing microglia died, indicative of the presence of a diffusible and short-lived toxic molecule. Center indicates the center of the area under the transwell. (Scale bar, 50 μm.) (*C* and *D*) Peroxynitrite was the diffusible factor toxic to preOLs. preOLs and microglia in transwell were cocultured and treated with a peroxynitrite decomposition catalyst, 5 μM FeTMPyP, cell-permeable superoxide scavenger, 10 μM MnTMPyP, and 500 unit/ml SOD and 100 unit/ml catalase (Cat) for 48 h. Cell survival (*C*) and NO production (*D*) were then analyzed. *, $P < 0.01$; **, $P < 0.001$; compared with LPS treatment. #, $P < 0.05$ when compared with control. Results are representative of three experiments.

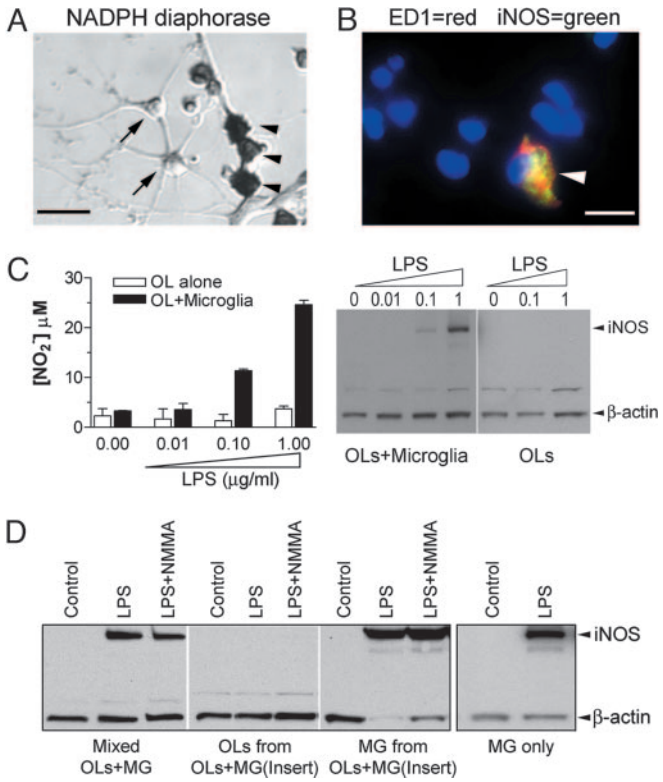

Fig. 3. Microglia were the sole source for LPS-induced NO production. (*A* and *B*) Microglia expressed iNOS upon activation by LPS. preOLs and microglia cocultures were treated with LPS for 24 h. (*A*) Cocultures were evaluated for NADPH diaphorase activity. Arrows indicate preOLs, and arrowheads indicate NOS-positive microglia. (Scale bar, 20 μm.) (*B*) Cocultures were evaluated by immunocytochemistry for iNOS. Arrowhead indicates one microglial cell expressing iNOS and its marker ED1. (Scale bar, 10 μm.) (*C*) Nitrite production and Western blot analysis of preOLs and preOL plus microglia coculture treated with increasing concentrations of LPS. (*D*) Western blot analysis of iNOS of the indicated cell cultures treated with 1 μg/ml LPS in the presence or absence of L-NMMA for 24 h confirmed that only microglia (MG) challenged with LPS expressed iNOS. Data are representative of three independent experiments.

preOLs was required for iNOS induction, we then tested the effect of LPS on iNOS expression by using cocultures containing microglia in transwells and preOLs residing underneath. Western blot analysis demonstrated that microglia in transwells but not the preOLs beneath expressed iNOS upon LPS exposure and that microglia cultured alone also responded robustly to LPS with expression of iNOS (Fig. 3*D*). As expected, L-NMMA, which inhibited NO production by inhibiting iNOS enzymatic activity, had no effect on LPS-induced iNOS expression (Fig. 3*D*).

To further test our hypothesis that LPS-activated microglia produce NO by means of iNOS up-regulation and that NO plays an essential role in LPS toxicity, we isolated microglia from mice deficient in the iNOS. First we confirmed that, in contrast to wild type, microglia isolated from iNOS-deficient mice failed to express iNOS and to produce NO (Fig. 4 *A* and *B*). Whereas LPS challenge of microglia isolated from wild-type mice led to death of preOLs, microglia deficient in iNOS were not effective in killing (Fig. 4*C*). There was no statistical difference in preOL survival in the presence of iNOS-deficient microglia treated with and without LPS (Fig. 4*C*).

Identification of Microglia as the Cellular Source for ROS Production. To identify where superoxide was generated, we first overexpressed CuZnSOD and MnSOD in preOLs via viral infection as described in ref. 21. Overexpression of mitochondrial MnSOD has been shown to inhibit glutathione depletion-induced oxidative death of preOLs (21). However, overexpression of CuZnSOD or MnSOD in preOLs had no protective effect against LPS-activated microglia (data not shown), indicating that the main source for superoxide production was likely to be microglia.

By using a redox sensitive dye, dihydrorhodamine 123, which becomes fluorescent after oxidation by ROS and/or peroxynitrite (22), we observed strong fluorescence in LPS-activated microglia (Fig. 5*A*). In contrast, preOLs remained the same as controls (Fig. 5*A*). In agreement with this observation, quantification with dichlorohydrofluorescin (23) also demonstrated that LPS-activated microglia generated abundant ROS and/or peroxynitrite in contrast to preOLs (Fig. 5*B*). Because both ROS and peroxynitrite can oxidize dichlorohydrofluorescin, we next tested the effect of inhibiting NO generation and thus peroxynitrite formation on the fluorescence signal. Blockade of NO production with L-NMMA efficiently inhibited LPS-induced oxidation of dichlorohydrofluorescin, indicating that peroxynitrite was indeed the major reactive species in LPS-activated microglia (Fig. 5*B*).

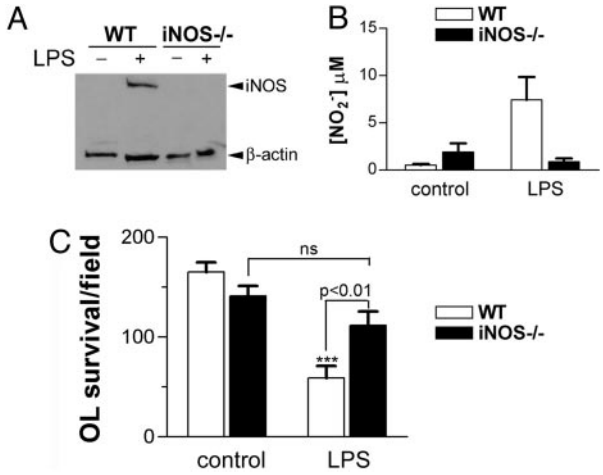

Fig. 4. Microglia isolated from iNOS-deficient mice failed to produce NO and induce toxicity to preOLs. Microglia were isolated from iNOS-deficient and wild-type mice and cocultured with preOLs. The cocultures were treated with and without 5 μg/ml LPS for 24 h. (*A* and *B*) Expression of iNOS and generation of NO were demonstrated by Western blot (*A*) and the Greiss reaction (*B*), respectively. (*C*) Surviving preOLs were counted based on immunostaining for O4. ***, $P < 0.0001$ wild-type cultures with LPS treatment compared with wild type without LPS treatment; ns, not significant. Results are representative of three independent experiments.

Activation of the Superoxide-Generating NADPH Oxidase in Microglia. Phagocytic cells, such as macrophages, neutrophils, and monocytes, are known to undergo respiratory burst when encountering invading microorganisms (24). During this process, they produce large amounts of superoxide by an NADPH oxidase. Activation of the oxidase represents an essential mechanism of host defense against various pathogens. It has been recently demonstrated that microglia also possess a similar NADPH oxidase (25). Because microglia are thought to be derived from monocytes during early brain development, and because microglia have the full capacity to be induced and develop into macrophages in the CNS, we next asked whether NADPH oxidase was required for the cytotoxic effect of activated microglia.

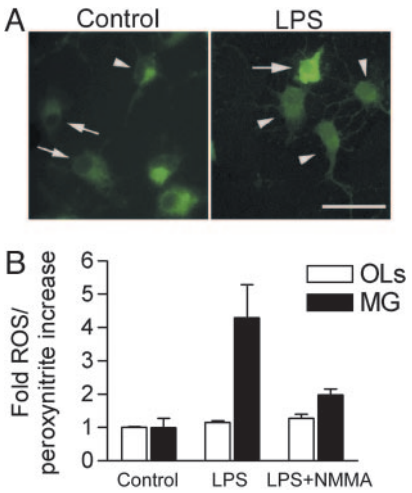

Fig. 5. Microglia, but not preOLs, challenged with LPS generated ROS. (*A*) preOL plus microglia cocultures were treated with or without 0.1 μg/ml LPS. After 24 h, dihydrorhodamine 123 was added at 8 μM for 20 min, and images were taken with a fluorescence microscope to evaluate oxidative stress in microglia and preOLs. Microglia became brightly fluorescent with LPS treatment. Microglia (arrows) and preOLs (arrowheads) were distinguishable by their characteristic morphology. (Scale bar, 20 μm.) (*B*) Quantification of ROS/peroxynitrite generation was carried out with dichlorohydrofluorescin by using a fluorescence plate reader (mean ± SD, $n = 3$). L-NMMA, which blocked formation of peroxynitrite by inhibiting NO production, significantly decreased the fluorescence intensity. MG, microglia.

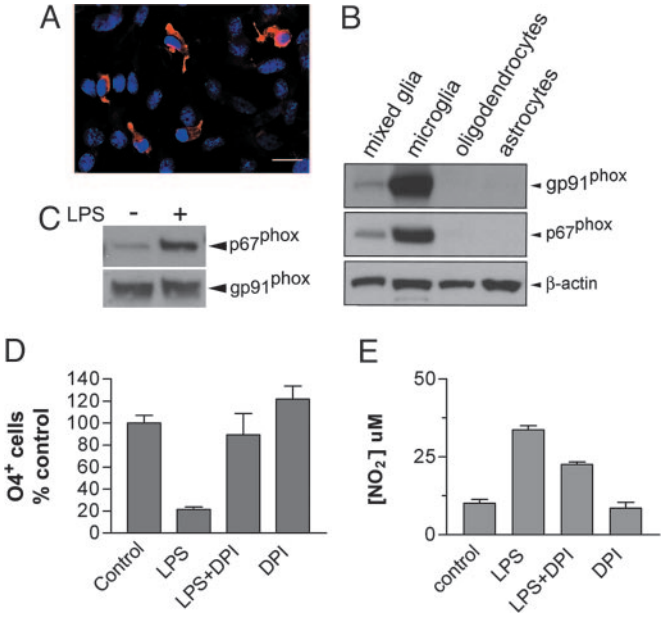

Fig. 6. The microglial superoxide-generating NADPH oxidase contributed to LPS-induced toxicity to preOLs. (*A* and *B*) Microglia expressed the phagocytic NADPH oxidase. (*A*) Immunostaining of mixed glial cultures for gp91^{phox} showed that only microglia stained strongly for gp91^{phox}. (Scale bar, 25 μm.) (*B*) Western blot of different cultures assaying for components of NADPH oxidase. (*C*) Activation of NADPH oxidase by LPS. Western blot analysis showed translocation of p67^{phox} to plasma membrane upon 1 μg/ml LPS challenge for 18 h. The same membrane was then reprobed for gp91^{phox}. (*D* and *E*) NADPH oxidase inhibitor DPI (10 nM) prevented LPS-induced toxicity in preOL plus microglia cocultures. Data are representative of three separate experiments.

The phagocytic NADPH oxidase is a dormant enzyme in resting cells consisting of two membrane-bound components, gp91^{phox} and p22^{phox}, and several cytosolic components, including p47^{phox}, p40^{phox}, p67^{phox}, and rac1/2 (24). Gp91^{phox} is a flavocytochrome and the catalytic core of the enzyme. Upon activation, the cytosolic components translocate to the membrane and associate with membrane components to form an assembled, activated, and superoxide-producing enzyme complex. We first determined which cell types express the NADPH oxidase. Immunolocalization of gp91^{phox} revealed that only cells with the characteristic morphology of microglia in mixed glial cultures expressed this protein (Fig. 6*A*). By using highly purified primary cultures we confirmed that microglia were the only cell type expressing gp91^{phox} and the cytosolic component p67^{phox} (Fig. 6*B*).

Upon LPS challenge, the microglial NADPH oxidase became activated, as demonstrated by the translocation of p67^{phox} to the plasma membrane (Fig. 6*C*). The level of membrane-bound gp91^{phox} remained the same (Fig. 6*C*). A selective NADPH oxidase inhibitor, diphenyleneiodonium (DPI), at 10 nM completely prevented LPS-induced killing, suggesting a role for this enzyme in producing the cytotoxic effect (Fig. 6*D*). DPI also partially inhibited the NO production by activated microglia (Fig. 6*E*). This inhibition was likely due to a partial blockade of the enzymatic activity of iNOS, which is also a flavoprotein. Because of this potential pitfall regarding the specificity of DPI, we next used gp91^{phox}-deficient microglial cultures to prove the

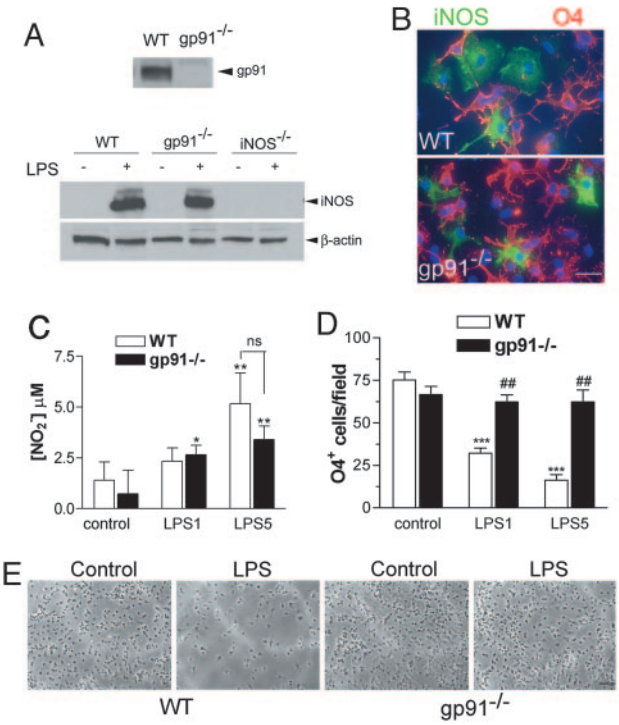

Fig. 7. Microglia isolated from gp91^{phox}-deficient mice failed to induce toxicity to preOLs. (*A–C*) Microglia deficient in gp91^{phox} produced a similar level of NO when challenged with LPS. preOL plus microglia cocultures were treated with and without 1 μg/ml LPS for 24 h. (*A*) Western blot analysis. (*B*) Immunostaining of iNOS. (Scale bar, 10 μm.) (*C*) Nitrite levels (mean $\pm$ SD, $n = 3$). *, $P < 0.05$; **, $P < 0.01$; when compared with control; ns, not significant. (*D* and *E*) Microglia were isolated from gp91^{phox}-deficient and wild-type mice and cocultured with preOLs. Cultures were treated with and without 1 or 5 μg/ml LPS for 24 h. (*D*) preOL survival. ***, $P < 0.0001$ compared with wild-type control; ##, $P < 0.0001$ compared with corresponding treatment for wild type. (*E*) Phase contrast image of cells treated with and without 5 μg/ml LPS. (Scale bar, 40 μm.) Results are representative of three independent experiments.

role of the NADPH oxidase in LPS-induced toxicity. Mice deficient in gp91^{phox} do not have a functional NADPH oxidase and therefore are not able to produce superoxide via activation of this enzyme.

Gp91^{phox}-Deficient Microglia Fail to Kill preOLs. Microglia isolated from gp91^{phox} knockout mice did not express any gp91^{phox} but were capable of expressing the same level of iNOS protein as wild type upon LPS induction (Fig. 7*A*). Immunostaining of iNOS also revealed that microglia from wild type or knockout mice expressed iNOS when treated with LPS (Fig. 7*B*). Again, as described earlier, preOLs did not contain iNOS (Fig. 7*B*). Consistent with the levels of iNOS expression, there were no significant differences in NO production between microglia isolated from the wild type and the gp91^{phox} knockouts (Fig. 7*C*). Although the gp91^{phox}-deficient microglia were capable of producing NO as well as the wild-type cells, they did not cause significant death of preOLs when challenged with LPS (Fig. 7 *D* and *E*). This result clearly demonstrated that superoxide generated from the microglial NADPH oxidase played an essential role in producing LPS-induced cell death.

Discussion

In this study, we investigated the mechanism by which activation of microglia leads to cytotoxicity to preOLs. We report here that peroxynitrite produced by activated microglia is the primary cytotoxic factor to these preOLs. Most importantly, we further identified the cellular and molecular sources for NO and superoxide generation, and, thereby, peroxynitrite production. Our results suggest that iNOS and NADPH oxidase contribute to the killing of preOLs by activated microglia.

As the resident macrophage-like cells of the CNS, microglia not only represent the first line of the innate immunity defense system against a variety of pathogens but also regulate many aspects of the adaptive immune response (1, 26). Several lines of clinical, neuropathological, and experimental studies suggest that infection/inflammation play important roles in the pathogenesis of PVL (10). Neuropathological analysis of PVL brains revealed the presence of an increased number of reactive microglia throughout the diffuse white matter lesion (5), suggesting that these immune cells may contribute to injury to developing OLs and hypomyelination. A relationship between infection/inflammation, circulating LPS, and the pathogenesis of PVL was suggested initially by Gilles *et al.* (12), who showed that systemic administration of LPS to neonatal kittens resulted in white matter injury similar to human PVL, and later by others (14, 27, 28). Furthermore, selective white matter injury was shown in the neonatal rat brain after intracerebral injection of LPS (13). CNS inflammation is often associated with ischemia and infection, and evidence for prenatal inflammation significantly increases the incidence of cerebral palsy (29). Our report showing that activation of microglia through Toll-like receptor 4 is required for LPS-induced cytotoxicity to OLs and white matter injury provides a mechanistic link between innate immunity and white matter injury (11). NO (30); glutamate; proinflammatory cytokines, such as TNF-α and INF-γ (31–33); and disialoganglioside GD3 (34) have all been suggested as soluble factors toxic to OLs produced by activated microglia *in vitro*. By using highly enriched primary cultures of microglia and preOLs, we show here that peroxynitrite is the primary factor released from LPS-activated microglia that is toxic to preOLs. This conclusion is based on the evidence that (*i*) removal of NO or superoxide with selective inhibitors/scavengers abolished LPS-induced death of preOLs; (*ii*) a specific peroxynitrite decomposition catalyst, FeTMPyP, effectively prevented the cell death; (*iii*) inhibition of NO production with L-NMMA significantly abolished LPS-initiated oxidation of dichlorohydrofluorescin in microglia, indicating that peroxynitrite was the major oxidant generated by LPS-activated microglia; and (*iv*) microglia isolated from mice genetically deficient in iNOS or gp91^{phox} failed to induce preOL death when activated by LPS. Our results are in agreement with recent neuropathological findings that tyrosine nitration, indicative of peroxynitrite formation, is evident in preOLs in the diffuse white matter lesion of PVL (5).

Because peroxynitrite is a highly reactive oxidant capable of inducing injury to a number of cell types, the identification of peroxynitrite as a toxic factor released from LPS-activated microglia may have broader implications. This finding may represent a common mechanism by which activated microglia cause bystander injury to surrounding cells, including neurons and OLs. In fact, peroxynitrite appears to be responsible for amyloid β- and LPS-induced toxicity to immature cortical neurons in culture (35). Although the molecular origin of peroxynitrite was not identified in that study, it is likely to be the same as we report here. It should be noted that our study does not necessarily exclude the possibility that other factors, such as proinflammatory cytokines released from activated microglia, may affect the survival of OLs, especially *in vivo*.

Production of intracellular superoxide results from normal processes of cellular function and metabolism (36). Potential sources include the electron transport chain, xanthine oxidase, lipoxygenase, cycloxygenase, and peroxisomes. Activation of NADPH oxidase is a central mechanism for ROS generation in phagocytes (24). This superoxide-generating enzyme is dormant

in resting cells and produces superoxide only upon activation. Unlike the regulation of iNOS, the principal regulation of NADPH oxidase is posttranslational and depends on assembly of several membrane-bound and cytosolic components to form an active enzyme complex. Although the kinetics and magnitude of superoxide generation appear to be different in microglia as compared with, for example, neutrophils, microglia appear to express all of the components of the oxidase and generate superoxide upon activation (25). Our results demonstrate that microglia are the primary cells expressing the oxidase, and LPS challenge did not increase $gp91^{phox}$ expression but, instead, activated the enzyme. In contrast, astrocytes and preOLs did not express detectable NADPH oxidase. These results do not, however, exclude the possibility that the oxidase can be transcriptionally up-regulated in these cells under certain pathophysiological conditions (37). In fact, exposure to zinc results in up-regulation of NADPH oxidase in cultured cortical neurons and astrocytes, and this up-regulation may contribute to zinc-induced neuronal death (38). We found that LPS-activated, microglia-dependent toxicity to preOLs was completely blocked by the NAPDH oxidase inhibitor DPI. Most importantly, although LPS-activated $gp91^{phox}$-deficient microglia generated a similar amount of NO as the wild-type cells, they failed to induce preOL death, strongly suggesting that the microglial NADPH oxidase-derived superoxide reacts with NO to form peroxynitrite that is toxic. How LPS activates NADPH oxidase is currently unknown and requires further investigation. A recent study suggests a direct interaction between Toll-like receptor 4 and NAD(P)H oxidase isozyme 4 (39). NADPH oxidase appears also to be critical in mediating injury under other pathological conditions. For example, the oxidase has been implicated in nerve growth factor deprivation- (40), fibrillar amyloid β- (41) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity *in vitro* (42), in death of dopaminergic neurons in an animal model of Parkinson's disease (43), and in neurotoxicity in a stroke model (44). All of the emerging evidence suggests that NADPH oxidase may play crucial roles in multiple diseases and disorders of the CNS, including neurodegenerative diseases, hypoxic/ischemic injury, and white matter injury.

In summary, this study demonstrates the cellular mechanism underlying how bacterial endotoxin-activated microglia kill preOLs. In this paradigm, peroxynitrite is the primary cytotoxic factor for preOLs. Superoxide-generating NADPH oxidase and NO-generating iNOS are indispensable for death of preOLs caused by activated microglia. A similar mechanism appears to be operative for mature, myelin basic protein-expressing OLs. It would therefore be interesting to test whether NADPH oxidase and iNOS play a causal role in animal models of white matter injury. Based on current knowledge concerning the pathogenesis of PVL, our study provides insights into the mechanism by which activated microglia kill OLs and could lead to development of strategies of prevention and treatment for cerebral palsy.

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