Tumor Necrosis Factor α Mediates Lipopolysaccharide-Induced Microglial Toxicity to Developing Oligodendrocytes When Astrocytes Are Present

Jianrong Li,¹ E. Radhika Ramenaden,¹ Jie Peng,² Hisami Koito,² Joseph J. Volpe,¹ and Paul A. Rosenberg¹

¹Department of Neurology and the F. M. Kirby Neurobiology Center, Children's Hospital Boston, and the Program in Neuroscience, Harvard Medical School, Boston, Massachusetts 02115, and ²Department of Veterinary Integrative Biosciences, Texas A & M University, College Station, Texas 77843

Reactive microglia and astrocytes are present in lesions of white matter disorders, such as periventricular leukomalacia and multiple sclerosis. However, it is not clear whether they are actively involved in the pathogenesis of these disorders. Previous studies demonstrated that microglia, but not astrocytes, are required for lipopolysaccharide (LPS)-induced selective killing of developing oligodendrocytes (preOLs) and that the toxicity is mediated by microglia-derived peroxynitrite. Here we report that, when astrocytes are present, the LPS-induced, microglia-dependent toxicity to preOLs is no longer mediated by peroxynitrite but instead by a mechanism dependent on tumor necrosis factor- α (TNF α) signaling. Blocking peroxynitrite formation with nitric oxide synthase (NOS) inhibitors or a decomposition catalyst did not prevent LPS-induced loss of preOLs in mixed glial cultures. PreOLs were highly vulnerable to peroxynitrite; however, the presence of astrocytes prevented the toxicity. Whereas LPS failed to kill preOLs in cocultures of microglia and preOLs deficient in inducible NOS (iNOS) or gp91^{phox}, the catalytic subunit of the superoxide-generating NADPH oxidase, LPS caused a similar degree of preOL death in mixed glial cultures of wild-type, iNOS^{-/-}, and gp91^{phox}^{-/-} mice. TNF α neutralizing antibody inhibited LPS toxicity, and addition of TNF α induced selective preOL injury in mixed glial cultures. Furthermore, disrupting the genes encoding TNF α or its receptors TNFR1/2 completely abolished the deleterious effect of LPS. Our results reveal that TNF α signaling, rather than peroxynitrite, is essential in LPS-triggered preOL death in an environment containing all major glial cell types and underscore the importance of intercellular communication in determining the mechanism underlying inflammatory preOL death.

Key words: oligodendrocyte precursors; cell death; white matter injury; cerebral palsy; glia; nitric oxide

Introduction

Glial cells are essential for the development and function of the CNS (Volterra and Meldolesi, 2005). Microglia are the resident macrophage-like cells in the CNS and extremely responsive to environmental stress and immunological challenges (Kreutzberg, 1996). Activation of microglia has been implicated in a number of neurological disorders, including the white matter disorders periventricular leukomalacia (PVL) (Haynes et al., 2005) and multiple sclerosis (Trapp et al., 1999). Reactive astrocytes are frequently present in various lesions of the nervous system. A localized activation of microglia and astrocytes may have both beneficial and detrimental effects on neighboring cells (John et al., 2003; Minghetti, 2005). Both cell types are prime

immune regulators in the CNS, exhibit great plasticity toward injury, and are capable of producing many inflammatory mediators, including cytokines, such as tumor necrosis factor α (TNF α), and chemokines, as well as reactive oxygen/nitrogen species and trophic factors (Ridet et al., 1997; Dong and Benveniste, 2001; Hanisch, 2002; Pekny and Nilsson, 2005).

Brain injury in premature infants is a common perinatal disorder and a major cause of life-long neurological disability that accounts for enormous personal and societal burden. PVL is the major form of cerebral white matter injury that underlies most of the neurological sequelae, including cognitive deficits associated with prematurity (Volpe, 2001b; Haynes et al., 2005). Preoligodendrocytes (preOLs) are the major cell type selectively injured in PVL. Hypoxia/ischemia and immune responses in the CNS to maternal/fetal infection are two primary components of the pathogenesis of PVL (Volpe, 2001a; Riddle et al., 2006), which is characterized by both focal necrosis with loss of all cellular elements and diffuse white matter injury leading to subsequent myelination deficits (Volpe, 2003).

Considerable clinical, *in vivo*, and *in vitro* evidence points to a strong link between bacterial endotoxin lipopolysaccharide (LPS) and PVL (Gilles et al., 1976; Grether and Nelson, 1997; Lehnardt et al., 2002; Pang et al., 2003; Wang et al., 2006). Many studies have demonstrated selective white matter lesions in fetal

Received Aug. 31, 2007; revised April 2, 2008; accepted April 4, 2008.

This work was supported in part by National Institutes of Health Grants P01NS38475 (J.J.V., P.A.R.) and NS060017 (J.L.); by grants from the National Multiple Sclerosis Society, the Hearst Foundation, the United Cerebral Palsy Foundation, and the Priscilla and Richard Hunt Fellowship (J.L.); by the startup fund from Texas A & M University (J.L.); and by a National Institutes of Health Developmental Disability Research Center Grant HD18655 (to Children's Hospital). We thank Ling Dong and Leon Massillon for technical assistance.

Correspondence should be addressed to either of the following: Dr. Paul A. Rosenberg, Department of Neurology, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, E-mail: paul.rosenberg@childrens.harvard.edu; or Dr. Jianrong Li at her present address, Department of Veterinary Integrative Biosciences, Texas A & M University, Mail Stop 4458, College Station, TX 77843, E-mail: jrli@cvm.tamu.edu.

DOI:10.1523/JNEUROSCI.3995-07.2008

Copyright © 2008 Society for Neuroscience 0270-6474/08/285321-10\$15.00/0

and neonatal animals after local, systemic, or intrauterine administration of LPS (Hagberg et al., 2002). However, the mechanisms underlying this inflammatory injury to preOLs remain elusive. Microglia and astrocytes are profoundly activated in the diffuse white matter lesions of PVL (Haynes et al., 2003), suggesting a role in mediating preOL injury. *In vitro*, LPS toxicity to OLs is not cell autonomous and only occurs in cultures containing microglia through activation of the microglial Toll-like receptor 4 (TLR4) signaling pathway (Lehnardt et al., 2002). We recently demonstrated that peroxynitrite, a short-lived potent oxidant and the reaction product of nitric oxide (NO) and superoxide, is the toxic microglial factor responsible for LPS-induced death of preOLs (Li et al., 2005). Unexpectedly, we found that, although astrocytes do not contribute directly to LPS toxicity to preOLs, their presence completely changed LPS-induced cell death mechanisms. In this study, we demonstrate that, when astrocytes are present together with microglia and preOLs, LPS-induced, microglia-dependent toxicity to preOLs is mediated instead by a mechanism dependent on TNF α signaling.

Materials and Methods

Materials. LPS (*Escherichia coli* O111:B4) was obtained from Sigma (St. Louis, MO). Wild-type, mutant, or knock-out mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Various cytokines were obtained from R & D Systems (Minneapolis, MN). PDGF and basic FGF were from PeproTech (Rocky Hill, NJ). 3-Morpholino-sydnonimine (SIN-1), N^G -monomethyl-L-arginine (L-NMMA), iron (III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (FeTMPyP), and peroxynitrite were purchased from Cayman Chemical (Ann Arbor, MI). Recombinant reporter adenovirus was from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). Antibodies against CD68 and GFAP were from Millipore Bioscience Research Reagents (Temecula, CA), and inducible nitric oxide synthase (iNOS) was from BD Transduction Laboratory (San Jose, CA). Caspase inhibitors were from Axora (San Diego, CA). Unless specified otherwise, all other reagents were from Sigma.

Primary cell cultures. Primary preOLs, microglia, astrocytes, and mixed glial cultures were prepared from the forebrains of 1- to 2-d-old rats or mice using a differential detachment method (McCarthy and de Vellis, 1980; Li et al., 2005; Chen et al., 2007). Briefly, forebrains free of meninges were digested with HBSS containing 0.01% trypsin and 10 μ g/ml DNase and triturated with DMEM containing 20% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin. Dissociated cells were plated onto poly-D-lysine-coated 75 cm² flasks or directly into 24-well plates for experiments using mixed glia and fed every other day for 7–10 d. Microglia were isolated by shaking the mixed glia-containing flasks for 1 h at 200 rpm. The purity of microglia was consistently >95%. After removing microglia, the flasks were subjected to shaking at 200 rpm overnight to separate preOLs from the astrocyte layer (Li et al., 2005). The suspension was plated onto uncoated Petri dishes for 1 h to further remove residual contaminating microglia/astrocytes. PreOLs were plated either by themselves or onto 24-well plates containing microglia or astrocytes for cocultures. PreOLs were maintained in a serum-free basal defined medium (BDM) (DMEM, 0.1% bovine serum albumin, 50 μ g/ml human apo-transferrin, 50 μ g/ml insulin, 30 nM sodium selenite, 10 nM D-biotin, and 10 nM hydrocortisone) containing 10 ng/ml PDGF and 10 ng/ml basic FGF for 5–9 d. The OL cultures were primarily progenitors and precursors (A₂B₅⁺, O₄⁺, O₁-negative (O₁[−]), myelin basic protein[−]) and are therefore referred to as preOLs. Contamination by astrocytes and microglia was <2% in preOL monocultures. Astrocytes were purified from the astrocyte layer in the flask after being exposed to a specific microglia toxin L-leucine methyl ester (1 mM) for 1 h. The enriched astrocytes were consistently >95% pure, with preOLs being the major contaminating cells. For cocultures, fixed cell numbers of microglia (2–3 $\times$ 10⁴ cells per well), astrocytes (1 $\times$ 10⁴ cells per well), and preOLs (4–5 $\times$ 10⁴ cells per well) were plated into 24-well culture plates and used within 2–4 d. Mouse

mixed glial cultures were prepared with the same methods as described above from iNOS, gp91^{phox}, TNF α , TNF receptors 1/2 (TNFR1/2), or interferon γ (IFN γ) knock-out mice.

Cell treatment and cell survival determination. Cell death was induced by exposure to LPS (*E. coli* O111:B4; Sigma) or cytokines in the presence of various reagents as specified in the figure legends. Accumulation of nitrite in the medium was determined by the Griess reaction as described previously (Li et al., 2005). SIN-1 and peroxynitrite treatment was performed as described previously (Zhang et al., 2006). Cells were washed twice and then placed in Earle's balanced salt solution. Increasing concentrations of SIN-1 or authentic peroxynitrite were then added to cells. After 1 h incubation with SIN-1 or peroxynitrite, the cells were switched back to BDM medium, and cell survival was analyzed 20–48 h later. Survival of preOLs was determined by counting O₄-positive (O₄⁺) cells with normal nuclei. Briefly, cells were treated in triplicate as specified for 24–48 h. After washing with PBS and fixation with 4% paraformaldehyde, cells were immunostained with O₄ antibody (1:500). Total number of cells was revealed by staining all nuclei with Hoechst 33258. Five random consecutive fields were counted in each coverslip under 200 $\times$ magnification with a total of >1000 cells counted in the control conditions. Cell survival is expressed as mean $\pm$ SD.

Infection of preOLs with recombinant adenovirus. Purified preOLs were infected with adenovirus containing green fluorescent protein (GFP) cDNA (AdGFP) as we described previously (Baud et al., 2004a). Briefly, cells were exposed to 1 $\times$ 10⁸ pfu/ml AdGFP overnight in regular culture medium followed by a complete medium change the next day. Cells were allowed to recover and express GFP for 48 h. The infection rate was consistently >90% with minimum toxicity. Cells were then trypsinized off the culture dish and seeded at density of 1–2 $\times$ 10⁴ per well into regular mixed glial cultures. The next day, cell cultures were challenged with LPS or TNF for 24–48 h, and morphology and the extent of loss of GFP⁺ cells were analyzed.

Analysis of TNF α production. The concentrations of TNF α in the culture media of cells treated as specified were measured using commercially available ELISA kits according to the instructions of the manufacturer (eBioScience, San Diego, CA). Absorption at 450 nm was determined in a microplate reader (Fluostar Optima; BMG Labtech, Offenburg, Germany). The detection limit of the ELISA was 8 pg/ml for TNF α .

Immunocytochemistry, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, and immunofluorescence microscopy. After treatments, cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and blocked with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100) or TBS (for O₄ immunostaining) containing 5% goat serum. The coverslips were incubated with antibody O₄ (1:500) or antibodies against CD68 (1:100), GFAP (1:1000), or iNOS (1:1000) overnight at 4°C. After washes, secondary antibody conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (1:1000 dilution; Invitrogen, Carlsbad, CA) was incubated with the coverslips for 1 h at room temperature. In the case of labeling fragmented DNA, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using an In Situ Cell Death detection kit according to the protocol of the manufacturer (Roche, Indianapolis, IN). After more washes, nuclei were stained with Hoechst 33258 at a final concentration of 2 μ g/ml for 1 min. The coverslips were then washed two to three times and mounted onto glass slides with FluoroMount and kept in the dark at 4°C. Cell images were captured with a fluorescence microscope (model IX71; Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera.

Statistical analysis. All cell culture treatments were performed in triplicate. Results were analyzed by one-way ANOVA, followed by Bonferroni's *post hoc t* test to determine statistical significance. Comparison between two experimental groups was based on two-tailed *t* test. *p* < 0.05 was considered statistically significant.

Results

LPS-induced killing of oligodendrocyte precursors is mediated through activated microglia

Previous studies showed that LPS causes significant toxicity to OLs in culture and *in vivo* (Merrill et al., 1993; Pang et al., 2000;

Figure 1. LPS is cytotoxic to preOLs by activating microglial cells. Mixed glial cultures consisting of astrocytes, preOLs, and microglia, highly enriched preOLs, and various cocultures were treated with or without LPS (1 μ g/ml) for 48 h. Survival of preOLs was evaluated by immunocytochemistry and by counting O_4^+ cells. **A**, Representative images of mixed glial cultures treated as indicated and immunostained with antibody O_4 (red). Nuclei were stained with bisbenzamide (blue). Scale bar, 50 μ m. **B**, LPS-induced toxicity was not cell autonomous and required microglial activation. PreOL viability was unaffected by LPS in preOL monocultures lacking microglia and astrocytes. LPS had minimal effect in cocultures of preOLs and astrocytes. Data are representative of at least three independent experiments. ** $p < 0.001$ compared with corresponding controls.

Lehnardt et al., 2002; Li et al., 2005; Wang et al., 2006). Subsequent studies demonstrated that the effect of LPS on developing OLs is not cell autonomous but rather dependent on microglia activation through TLR4 (Lehnardt et al., 2002). Using mixed glial cultures containing all major CNS glial cell types (microglia, astrocytes, and OLs), we found that LPS selectively killed preOLs (O_4^+ , O_1^-) (Fig. 1A). To determine which cell type was responsible for the LPS toxicity, we tested the effect of LPS on preOLs in various monocultures and cocultures. Consistent with previous findings (Lehnardt et al., 2002; Li et al., 2005), LPS had no effect on pure preOLs or preOLs cocultured with astrocytes but was highly toxic to preOLs when preOLs were cocultured with microglia (Fig. 1B). These results confirmed a pivotal role for activated microglia in LPS-mediated toxicity. Using this preOL plus microglia coculture model, we determined that peroxynitrite generated by LPS-activated microglia is the toxin responsible for LPS-induced death of preOLs (Li et al., 2005).

To visualize morphological features of dying preOLs and to validate the methodology used to quantify preOL survival in mixed glial cultures, we infected purified preOLs with adenovirus containing GFP cDNA and then seeded them into established mixed glial cultures that contained regular preOLs. Expression of GFP in infected preOLs allowed us to clearly visualize the morphology of the entire preOL, including their fine processes (Fig. 2A). Exposure of mixed glial cultures containing added GFP $^+$ preOLs to LPS resulted in significant degeneration and loss of GFP $^+$ cells when observed at 24 h after LPS treatment (Fig. 2B,C). It appeared that dying preOLs had multiple abnormal

Figure 2. LPS causes degeneration of GFP-expressing preOLs in mixed glial cultures. Purified preOLs were infected with adenovirus containing GFP and seeded onto established mixed glial cultures that contained uninfected preOLs. **A**, Left, Representative image of the morphology of a GFP-expressing preOL 1 d after seeding into mixed glial cultures; right, colocalization of O_4 antigen with GFP in an infected preOL (arrow). Arrowhead indicates an endogenous preOL. Scale bars, 10 μ m. **B**, The above cell cultures were treated with LPS for 24 h, and degenerating GFP $^+$ preOLs cells were visualized with a fluorescence microscope. Arrows indicate degenerating cells, and arrowheads indicate dying cells with morphological features of apoptotic bodies. Scale bars, 20 μ m. **C**, Survival of GFP $^+$ preOLs was determined by counting live GFP $^+$ cells 48 h after specified treatments. Data are from one representative experiment of three that were performed. * $p < 0.01$; ** $p < 0.001$ when compared with control.

morphologies. Some appeared to have beaded processes and bulb formation (Fig. 2B, top and bottom left), whereas others appeared to form apoptotic bodies (Fig. 2B, bottom right). No degenerating GFP $^+$ cells were observed in controls. The extent of preOL survival as determined by counting live GFP $^+$ cells was similar to that determined by counting live O_4^+ preOLs. These data suggest that LPS-induced death of preOLs is not synchronous and may occur by multiple pathways. Our data also demonstrate that the method used to determine preOL survival by counting O_4^+ preOLs in this study is reliable and accurate.

Nitric oxide is not required for LPS toxicity in mixed glial cultures in which all CNS glial cell types are present

Peroxynitrite is a short-lived potent oxidant formed when NO reacts with superoxide anion, a reaction that occurs at a diffusion-limited rate (Pacher et al., 2007). We found that iNOS was rapidly upregulated in microglia in mixed glial cultures exposed to LPS (Fig. 3*A,B*). iNOS upregulation and thus NO production appeared to correlate with preOL process retraction and cell death (Fig. 3*A*). However, to our surprise and in striking contrast to the fact that blocking NO production effectively prevents LPS toxicity in cocultures of preOLs and microglia (Li et al., 2005), the NOS inhibitor L-NMMA had no effect on LPS toxicity in the mixed glial cultures despite the fact that it completely blocked NO production (Fig. 3*C,D*). The iNOS-specific inhibitor 1400W also did not abolish LPS toxicity when mixed glial cultures were used (Fig. 3*E*). Furthermore, the peroxynitrite decomposition catalyst and superoxide scavenger FeTMPyP (Misko et al., 1998), which blocks LPS toxicity in cocultures (Li et al., 2005), did not prevent preOL death in mixed glial cultures. These results indicate that, although astrocytes are not required for LPS toxicity (Fig. 1*B*), their presence changed the death mechanism from an NO-dependent mechanism to a mechanism independent of NO and peroxynitrite production.

To determine the mechanism underlying LPS-induced preOL death in mixed glial cultures, we next tested whether excitotoxicity and oxidative injury were involved because astrocytes are capable of releasing glutamate under inflammatory conditions and preOLs are known to be highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity as well as oxidative stress (Oka et al., 1993; Back et al., 1998; Fern and Moller, 2000; Follett et al., 2000; Deng et al., 2003; Rosenberg et al., 2003; Baud et al., 2004b). Neither the AMPA/kainate receptor antagonist NBQX nor the antioxidants vitamin E and vitamin K₂, shown previously to be effective in preventing oxidative injury to preOLs (Li et al., 2003), had any protective effect (Fig. 3*E*). To determine whether the cells died through a caspase-dependent apoptotic pathway, we next tested the effect of caspase inhibitors. The pan caspase inhibitor zVAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) and the caspase-3 inhibitor z-DEVD-fmk (*z*-Asp-Glu-Val-fluoromethyl ketone) did not prevent the loss of preOLs (data not shown), indicating that a caspase-independent cell death pathway was operative. Consistent with this observation, there was minimal caspase-3 activation during LPS challenge in mixed glial cultures (data not shown). Because we have observed apparent apoptotic body formation in LPS-treated

Figure 3. Nitric oxide production is not necessary for LPS-induced preOL death in mixed glial cultures. **A–C**, LPS induced robust iNOS expression and NO production in mixed glial cultures. Mixed glia were treated with or without LPS (1 μg/ml) for 24 h and analyzed by immunocytochemistry (**A**) and Western blot (**B**) for iNOS expression (arrowheads). Dying preOLs with retracted processes were evident during LPS challenge (arrows). Double immunostaining for iNOS and activated microglial marker CD68 showed that iNOS was upregulated in activated microglia (bottom). **C, D**, The NOS inhibitor L-NMMA (1 mM) blocked NO production but had no effect on preOL survival. ***p* < 0.001 when compared with controls; ns, not significant. **E**, LPS-induced death of preOLs in mixed glial cultures was not dependent on production of NO and peroxynitrite, oxidative stress, or activation of the AMPA/kainate receptor. Mixed glial cultures were treated as indicated with or without 1 μg/ml LPS for 48 h in the presence of NOS inhibitor (1 mM NMMA), iNOS-specific inhibitor (10 μM 1400W), peroxynitrite decomposition catalyst (5 μM FeTMPyP), AMPA/kainate receptor antagonist (100 μM NBQX), or antioxidants (10 μM vitamin E or 10 μM vitamin K₂). PreOL survival was quantified by counting O₄⁺ cells. Data are representative of three separate experiments. **F**, Representative photomicrograph of double labeling of dying preOLs with O₄ and TUNEL in preOLs plus microglia cocultures exposed to LPS for 24 h. Arrows indicate TUNEL⁺ preOLs.

mixed glial cultures containing GFP⁺ preOLs (Fig. 2*B*), we asked whether dying preOLs contain fragmented chromatin, a common feature of apoptotic cell death. Unexpectedly, we did not observe TUNEL⁺ O₄⁺ preOLs (data not shown). In contrast with this observation made in the mixed glial cultures, TUNEL⁺ preOLs were evident in cocultures of microglia and preOLs treated with LPS (Fig. 3*F*), in which we demonstrated previously that LPS-induced preOL toxicity is mediated by generation of peroxynitrite (Li et al., 2005). These results provide additional evidence for a different cell death mechanism induced by LPS when astrocytes are present. It should be noted that programmed cell death can take many forms, ranging from necrosis-like, apoptotic-like programmed cell death to classical apoptosis (Leist and Jäätelä, 2001).

Figure 4. Functional iNOS and NADPH oxidase are not required for LPS- or cytokine-induced preOL death in mixed glial cultures. **A–C**, Combinations of proinflammatory cytokines induce preOL death independent of NO production. Mixed glial cultures were treated as indicated for 48 h. PreOL survival was quantified by counting O₄⁺ cells, and NO production was measured by the Griess reaction. Combinations of TNFα (100 ng/ml), IFNγ (100 U/ml), and/or IL-1β (100 ng/ml) triggered significant death of preOLs (**A**, **B**) and upregulated iNOS in both microglia and astrocytes identified by their distinct morphology (**C**; arrows, astrocytes; arrowheads, microglia). In contrast to preOLs plus microglia cocultures, death of preOLs in mixed glial cultures was not blocked by the NOS inhibitor L-NMMA. Red, O₄; blue, Hoechst 33258; green, iNOS. Data are representative of three separate experiments. **D**, Mixed glial cultures with disrupted iNOS and gp91^{phox} genes, therefore deficient functional iNOS and NADPH oxidase, remained sensitive to LPS- and cytokine-induced toxicity to preOLs. Mixed glial cultures were isolated from mutant mice and treated as indicated with LPS (1 μg/ml) or TNFα (100 ng/ml) plus IFNγ (100 U/ml) for 48 h. **p* < 0.01, ***p* < 0.001 compared with corresponding controls. ns, Not significant.

Inflammatory cytokines are highly toxic to oligodendrocyte precursors in mixed glial cultures regardless of their ability to induce high levels of nitric oxide

Besides reactive nitrogen/oxygen species, activated microglia are also capable of producing a number of proinflammatory cytokines, including TNFα and interleukin-1β (IL-1β) (Hanisch, 2002). Because it has been shown previously that preOLs are highly sensitive to TNFα and interferon γ (IFNγ) (Merrill et al., 1993; Andrews et al., 1998) and combinations of these cytokines are powerful inducers of iNOS (Possel et al., 2000; Saha and Pahan, 2006), we asked whether these proinflammatory cytokines are toxic to preOLs in the mixed glial culture and whether blockade of inducible NO production abolishes cell death. Combinations of cytokines, such as TNFα, IFNγ, and IL-1β, induced profound preOL death as well as iNOS expression and NO production (Fig. 4A–C). However, although L-NMMA completely blocked NO production, it had no protective effect against this cytokine-induced toxicity, indicating that increased NO production in response to cytokines does not account for the toxicity.

More importantly, this result also demonstrates that NO, even at high concentrations, was not toxic to preOLs in mixed glial cultures. To confirm our above findings, we prepared mixed glial cultures from mice deficient in iNOS or gp91^{phox}, the catalytic subunit of the superoxide-generating NADPH oxidase, and subjected the cells to LPS or cytokine mixture treatment. We showed previously that microglia deficient in iNOS or gp91^{phox} failed to kill preOLs during LPS activation. However, when astrocytes were present together with preOLs and microglia, LPS as well as TNFα plus IFNγ caused similar levels of preOL death in wild-type, iNOS^{-/-}, and gp91^{phox}^{-/-} cells (Fig. 4D). Together, these data indicate that NO or peroxynitrite is not essential to LPS- or cytokine-induced preOL death in mixed glial cultures that include astrocytes.

TNFα production is necessary for LPS-induced death of oligodendrocyte precursors

Next, we investigated whether LPS-induced cytokine production accounts for LPS toxicity. Mixed glia exposed to LPS for 24 h produced significant amounts of extracellular TNFα (1299 ± 62 pg/ml; *n* = 3, mean ± SD) compared with controls (22.2 ± 27 pg/ml; *n* = 3, mean ± SD). Antibodies neutralizing soluble TNFα, but not control IgG, significantly prevented LPS-induced toxicity (Fig. 5A), indicating that LPS-induced TNFα production mediates, at least in part, preOL death in mixed glial cultures. In agreement with this observation, exogenous TNFα caused significant preOL death when applied to mixed glia (Fig. 5B). A similar level of toxicity was found when TNFα was added to mixed glial cultures containing GFP⁺ preOLs (data not shown). It should be noted

that the level of toxicity induced by exogenous TNFα was often less than that induced by LPS, although TNFα was used at a concentration approximately two orders of magnitude higher than the level induced by LPS, indicating that endogenous TNFα is much more efficient in killing preOLs. Interestingly, the effect of TNFα appears to be not cell autonomous because TNFα by itself was not toxic to pure preOLs but was injurious to preOLs when other glial cells were simultaneously present (Fig. 5B), suggesting the importance of glial cell–cell communication in causing preOL death. Conditioned media collected from LPS-treated mixed glia were not toxic to pure preOLs (data not shown). These results suggest that cell–cell contact and/or other local factors are required for efficient LPS-triggered killing of preOLs.

TNFα signaling through TNF receptors mediates LPS-induced toxicity to preOLs

To verify that TNFα signaling accounts for LPS-induced preOL death in mixed glial cultures, we isolated mixed glia from wild-type mice and mice deficient in TNFα or TNFR1/2. Cells defi-

Figure 5. TNF α is produced in response to LPS and mediates LPS-induced toxicity in mixed glial cultures. **A**, Neutralizing TNF α partially prevented LPS toxicity. Mixed glial cultures were treated as indicated with or without LPS (1 μ g/ml) in the presence of neutralizing antibody against TNF α (10 μ g/ml) or control IgG for 48 h. PreOL survival was analyzed by counting O₄⁺ cells. **B**, Exogenous TNF α was cytotoxic to preOLs in mixed glial cultures but not in pure preOL cultures. Purified preOLs and mixed glial cultures were treated as indicated for 48 h. Data are representative of three independent experiments. * p < 0.05, ** p < 0.001. ns, Not significant.

Figure 6. TNF α signaling is essential for LPS-induced preOL death in mixed glial cultures. **A**, LPS caused TNF α release in wild-type and TNFR1/2^{-/-} but not TNF α ^{-/-} mixed glial cultures treated with LPS for 24 h. **B**, TNF α signaling is essential to LPS toxicity. Mixed glial cultures were prepared from wild-type mice or mice deficient in TNF α , TNFR1/2, or IFN γ . Cells were treated with or without LPS (1 μ g/ml) and were fixed 48 h later. PreOLs were visualized by immunocytochemistry with O₄ antibody, and their survival was quantified. Data are presented as mean $\pm$ SD and representative of three separate experiments. ** p < 0.001 compared with corresponding controls. WT, Wild type; KO, knock-out.

cient in TNF α were unable to produce TNF α during LPS stimulation (Fig. 6A) and were resistant to LPS-induced killing of preOLs (Fig. 6B). Because TNF α signals through receptors TNFR1 and TNFR2, disruption of both receptors should silence TNF α -mediated signaling. Indeed, when mixed glia from TNFR1/2 knock-outs were subjected to LPS, TNF α was still produced, but preOL death was completely abolished (Fig. 6). In contrast, LPS exposure of wild-type cells resulted in marked killing of preOLs.

It is known that IFN γ potently activates astrocytes and that transgenic overexpression of IFN γ in mature OLs results in demyelination (Corbin et al., 1996; Horwitz et al., 1997). IFN γ was found to be upregulated in reactive astrocytes in the diffuse white matter lesions of PVL (Folkerth et al., 2004). Because TNF α and IFN γ act synergistically in causing injury to preOLs and their toxicity is also independent of NO production (Fig. 4), we asked whether IFN γ may act together with TNF α in causing preOL death in mixed glial cultures treated with LPS, a possibility that also explains why exogenous TNF α appears less toxic than LPS. We found that, in IFN γ ^{-/-} mixed glial cultures, LPS was as effective in killing preOLs as in wild-type cultures (Fig. 6B). Therefore, we conclude that TNF α -mediated signaling is essential for LPS-induced toxicity to preOLs in mixed glial cultures and that this toxicity is independent of IFN γ .

Astrocytes prevent peroxynitrite-induced death of oligodendrocyte precursors

As we showed above and previously (Li et al., 2005), LPS is toxic to preOLs but only in the presence of microglia (Fig. 1B). We identified peroxynitrite as the microglial toxin responsible for LPS-induced death in cocultures of preOLs and microglia. In contrast, LPS-treated astrocytes had minimal effect on preOL viability, consistent with previous findings that microglia but not astrocytes and OLs express functional TLR4 *in vitro* (Lehnardt et al., 2002). However, in the present study, we found that, in an environment in which all three CNS glial cell types are present, the mechanism underlying LPS-induced toxicity is no longer mediated through peroxynitrite but rather by a different mechanism involving TNF α signaling. This apparent dilemma could be resolved if astrocytes protect against peroxynitrite-induced preOL death, thereby allowing other mechanisms such as those mediated by TNF α to become dominant. This hypothesis is in agreement with evidence that astrocytes have high antioxidative capacities (Peuchen et al., 1997).

To test this hypothesis, we first treated preOLs with SIN-1, a widely used peroxynitrite generator (Zhang et al., 2006), and found that preOLs are indeed highly vulnerable to peroxynitrite (Fig. 7). Even lower concentrations of SIN-1 (200 μ M) caused nearly complete cell death when preOLs were evaluated 24 h later. We then tested the effect of SIN-1 on preOL viability in mixed glial cultures in which OLs, microglia, and astrocytes coexist. To our surprise, SIN-1 did not kill preOLs in these cultures, even at concentrations of 1 mM and when evaluated 48 h later. To determine the major cell type responsible for this protection of preOLs against the toxic effect of SIN-1, we examined the effect of SIN-1 on preOL viability when preOLs were cocultured with microglia or astrocytes. As predicted, preOLs were still sensitive to SIN-1 when cocultured with microglia, in agreement with our previous identification of peroxynitrite as the microglial toxin that mediates LPS toxicity. In contrast, SIN-1 had minimal effect when preOLs were cocultured with astrocytes (Fig. 7). Similarly, authentic peroxynitrite was also highly toxic to preOLs. At concentrations of 100 μ M, peroxynitrite, but not vehicle controls, caused massive preOL death (Fig. 8). However, the presence of astrocytes significantly prevented the peroxynitrite-induced preOL death (Fig. 8). High concentrations of peroxynitrite were not specifically toxic to preOLs but were toxic to astrocytes as well. In summary, our data demonstrate that, although astrocytes prevent LPS-activated, microglial peroxynitrite-mediated toxicity, they do not change preOL cell fate but rather shift the death mechanism toward a TNF α -dependent mechanism.

Figure 7. Astrocytes prevent SIN-1-mediated preOL death. Mixed glial cultures, purified preOLs, or their cocultures with microglia (MG) or astrocytes (Ast) were subjected to increasing concentrations of SIN-1 for 24–48 h. PreOL survival under various conditions was determined by counting immunostained O_4^+ cells. Data represent one of three separate experiments with similar results. **A**, Representative immunofluorescence images of O_4 staining 48 h after treatment. **B**, Quantification of preOL survival after SIN-1 challenge in various cultures. Presence of astrocytes clearly prevented SIN-1-induced preOL death. * $p < 0.05$; ** $p < 0.001$ when compared with corresponding controls.

Discussion

This study has investigated the cellular mechanism by which LPS induces injury to oligodendrocyte precursors, the cell type predominantly damaged in the diffuse white matter lesion of PVL. To investigate the mechanism underlying LPS-induced selective loss of preOLs in primary mixed glial cultures, various single and cocultures were prepared. Consistent with previous reports (Lehnardt et al., 2002; Li et al., 2005), we found that activation of microglia, but not astrocytes, is absolutely required for LPS toxicity. In preOL-microglia cocultures, a diffusible potent oxidant,

peroxynitrite, was identified as the primary underlying toxic factor killing pre-OLs (Li et al., 2005). Blocking peroxynitrite formation by preventing either NO production or superoxide production or enhancing peroxynitrite decomposition abolished preOL death in these cocultures. However, when the mechanism of LPS-induced toxicity to preOLs was reexamined in mixed glial cultures in which astrocytes were also present, paradoxically, LPS-induced toxicity was independent of peroxynitrite but instead relied on TNF α signaling, although copious amount of NO was produced. This apparent paradox was reconciled by the fact that astrocytes block peroxynitrite-mediated toxicity to preOLs.

The determination of a peroxynitrite-independent cell death pathway when astrocytes are present is based on the following evidence: (1) blocking NO production with the NOS inhibitors L-NMMA or 1400W had minimal effect on LPS toxicity in mixed glial cultures; (2) a peroxynitrite decomposition catalyst and superoxide scavenger, FeTMPyP, as well as other antioxidants did not protect preOLs; (3) disruption of genes encoding iNOS or the gp91^{phox} NADPH oxidase, two enzymes that we identified previously to be indispensable for LPS-induced microglial killing of preOLs in cocultures with microglia (Li et al., 2005), did not prevent LPS-induced toxicity in mixed glial cultures; and (4) astrocytes blocked peroxynitrite toxicity to preOLs. These data suggest that astrocytes respond to activated microglia and unmask a cell death mechanism mediated by TNF α signaling.

Microglia activated by LPS have been shown to release proinflammatory cytokines such as TNF α and IL-1 β (Hanisch, 2002). As a potent source of immunologically relevant cytokines, including TNF α , astrocytes also play a pivotal role in the type and extent of CNS immune and inflammatory responses. A previous study showed that induction of iNOS in astrocytes by IFN γ and IL-1 β potentiates NMDA-receptor mediated excitotoxicity (Hewett et al., 1994). Activated microglia also enhance TNF α production and glutamate release from astrocytes, resulting in amplified neurotoxicity (Bezzi et al.,

2001). Neuropathological studies have revealed that, in human PVL cases, but not age-matched controls, abundant hypertrophic reactive astrocytes and activated microglia populate diffuse white matter lesions (Haynes et al., 2003). Therefore, it is most likely that intercellular communication among these activated glia may play an important role in the pathogenesis of PVL. Our data demonstrate that, with the peroxynitrite cell death pathway inhibited by astrocytes, a cell death pathway orchestrated by TNF α /TNFR signaling becomes dominant for LPS-triggered injury to preOLs in culture.

Figure 8. Astrocytes protect against peroxynitrite-induced preOL death. PreOLs alone and in coculture with astrocytes (Ast) were exposed to increasing concentrations of authentic peroxynitrite for 1 h. PreOL survival was determined 20–24 h later. At 200 μ M peroxynitrite, preOLs were killed when cultured alone but were protected in the presence of astrocytes. Data are representative of three separate experiments. ** $p < 0.001$ when compared with corresponding controls. ns, Not significant.

Multiple lines of evidence suggest that several proinflammatory cytokines, including IFN γ (Folkerth et al., 2004), TNF α (Deguchi et al., 1996; Kadhimi et al., 2001), and IL-6 (Yoon et al., 1997) and IL-2 (Kadhimi et al., 2002) are elevated in PVL and may play pivotal roles in perinatal white matter injury (Dammann and Leviton, 1997; Rezaie and Dean, 2002; Pang et al., 2006; Smith et al., 2007). Proinflammatory cytokines such as TNF α are also potent regulators of glial activation and iNOS induction (John et al., 2003). TNF α is a prototypical proinflammatory cytokine that plays a central role in initiating inflammatory reactions of the innate immune system, in part through the induction of expression and release of other cytokines (Wajant et al., 2003). TNF α exerts its biologic functions by binding to and signaling through TNFR1 and TNFR2 in an autocrine and/or paracrine manner. *In situ* immunohistochemical studies revealed locally increased TNFR1/2 expression and TNF α production in both reactive astrocytes and microglia in human PVL lesions (Deguchi et al., 1996; Yoon et al., 1997; Kadhimi et al., 2001). TNF α /TNFR1 was also responsible for optical nerve OL death and subsequent retinal ganglion cell loss in a mouse model of glaucoma (Nakazawa et al., 2006). Transgenic overexpression of TNF α in astrocytes, but not neurons, results in demyelination and selective OL apoptosis (Akassoglou et al., 1998). It is not clear whether preOLs are damaged in these transgenic mice during early development. Other *in vivo* studies also revealed critical roles of the TNF/TNFR signaling pathway in CNS inflammation and white matter degeneration (Akassoglou et al., 1997; Probert et al., 2000). Our data demonstrate that TNF α /TNFR signaling is necessary for LPS-initiated death of preOLs in mixed glial cultures. The cellular source for LPS-induced TNF α production in mixed glia was not defined in the current study, but activated microglia are most likely responsible for the LPS-induced initial TNF α production given our observation that microglia respond robustly to LPS and produce NO and TNF α in culture, whereas astrocytes respond poorly (J. Li and J. Peng, unpublished observation). However, astrocytes, microglia, and preOLs all express TNF α receptors (Dopp et al., 1997) and thus all are capable of engaging in TNF α /TNFR signaling. Therefore, TNF α released from activated microglia may activate astroglial TNF α receptors, resulting in additional production of this cytokine and/or other toxic factors and amplification of microglial responses to LPS. Interestingly, TNF α itself had minimal effect on preOL viability in preOL monocul-

tures, suggesting a non-cell-autonomous cell death pathway. The actual mediator(s) regulated by TNF α signaling and responsible for preOL death in mixed glial cultures remains to be identified.

Our data do not support a role for iNOS in LPS-induced preOL death in mixed glial cultures. However, this does not necessarily mean that reactive nitrogen species, in particular peroxynitrite, play no role in white matter injury *in vivo*. Immunoreactive nitrotyrosine, a footprint of peroxynitrite formation, is found in astrocytes and preOLs in the diffuse component of human PVL lesions (Haynes et al., 2003). Interestingly, morphologically identified microglia/macrophages within the subacute necrotic foci, but not in the diffuse lesions, are immunostained positively for nitrotyrosine (Haynes et al., 2003), indicating that a robust and significant burst of oxidative/nitrative stress may be present in the necrotic foci. In culture, peroxynitrite is highly toxic to preOLs and is indeed the molecule directly responsible for the death of preOLs triggered by acutely activated microglia (Li et al., 2005). However, the presence of astrocytes switches the death mechanism from a peroxynitrite-dependent to a TNF α -dependent pathway. These results have several important implications. First, peroxynitrite generated by activated microglia/macrophages within the necrotic foci in PVL may play a role there in direct killing of preOLs. Conversely, in the diffuse white matter lesion, which is populated by abundant reactive astrocytes as well as microglia, a different mechanism of toxicity, such as that mediated by TNF α signaling, may be more important. Second, understanding how astrocytes communicate with activated microglia and influence the mechanism of toxicity and identifying TNF α -regulated mediators of preOL injury should provide us with mechanistic insights that can be exploited to augment protective signals while suppressing deleterious signals. It should be noted that the role of reactive nitrogen species in neonatal white matter injury has yet to be established in animal models. Blocking iNOS may have only a limited beneficial effect. In fact, in inflammatory demyelination models of multiple sclerosis, ablation of the iNOS gene actually exacerbates OL injury and clinical symptoms (Fenyk-Melody et al., 1998; Sahrbacher et al., 1998). The role of iNOS and its product NO in neonatal white matter injury *in vivo* therefore remains unknown. Multiple pathogenic mechanisms of preOL destruction are likely to exist. Combinatorial approaches such as blocking TNF α signaling and NO production may prove beneficial in preventing white matter injury.

In summary, this study highlights the importance of both astrocytes and microglia in mediating and regulating injury to oligodendrocyte precursors and identifies a distinct cellular mechanism by which endotoxin-activated microglia kill preOLs in an environment in which all three types of CNS glial cells interact. Although peroxynitrite is upregulated, its toxicity to preOL is blunted by astrocytes. Instead, activation of TNF α /TNFR signaling results in preOL death when astrocytes are present. Our study provides new mechanistic insights into inflammatory injury to preOLs and underscores the necessity to consider cell–cell interactions when developing new strategies for the prevention and treatment of white matter injury.

References

- Akassoglou K, Probert L, Kontogeorgos G, Kollias G (1997) Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J Immunol* 158:438–445.
- Akassoglou K, Bauer J, Kassiotis G, Pasparakis M, Lassmann H, Kollias G, Probert L (1998) Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central ner-

- vous system of transgenic mice: models for multiple sclerosis with primary oligodendroglialopathy. *Am J Pathol* 153:801–813.
- Andrews T, Zhang P, Bhat NR (1998) TNF α potentiates IFN γ -induced cell death in oligodendrocyte progenitors. *J Neurosci Res* 54:574–583.
- Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ (1998) Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J Neurosci* 18:6241–6253.
- Baud O, Li J, Zhang Y, Neve RL, Volpe JJ, Rosenberg PA (2004a) Nitric oxide-induced cell death in developing oligodendrocytes is associated with mitochondrial dysfunction and apoptosis-inducing factor translocation. *Eur J Neurosci* 20:1713–1726.
- Baud O, Greene AE, Li J, Wang H, Volpe JJ, Rosenberg PA (2004b) Glutathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J Neurosci* 24:1531–1540.
- Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, Vescovi A, Bagetta G, Kollias G, Meldolesi J, Volterra A (2001) CXCR4-activated astrocyte glutamate release via TNF α : amplification by microglia triggers neurotoxicity. *Nat Neurosci* 4:702–710.
- Chen Y, Balasubramanian V, Peng J, Hurlock EC, Tallquist M, Li J, Lu QR (2007) Isolation and culture of rat and mouse oligodendrocyte precursor cells. *Nat Protoc* 2:1044–1051.
- Corbin JG, Kelly D, Rath EM, Baerwald KD, Suzuki K, Popko B (1996) Targeted CNS expression of interferon- γ in transgenic mice leads to hypomyelination, reactive gliosis, and abnormal cerebellar development. *Mol Cell Neurosci* 7:354–370.
- Dammann O, Leviton A (1997) Maternal intrauterine infection, cytokines, and brain damage in the preterm newborn. *Pediatr Res* 42:1–8.
- Deguchi K, Mizuguchi M, Takashima S (1996) Immunohistochemical expression of tumor necrosis factor α in neonatal leukomalacia. *Pediatr Neurol* 14:13–16.
- Deng W, Rosenberg PA, Volpe JJ, Jensen FE (2003) Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. *Proc Natl Acad Sci USA* 100:6801–6806.
- Dong Y, Benveniste EN (2001) Immune function of astrocytes. *Glia* 36:180–190.
- Dopp JM, Mackenzie-Graham A, Otero GC, Merrill JE (1997) Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. *J Neuroimmunol* 75:104–112.
- Fenyk-Melody JE, Garrison AE, Brunnert SR, Weidner JR, Shen F, Shelton BA, Mudgett JS (1998) Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J Immunol* 160:2940–2946.
- Fern R, Moller T (2000) Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. *J Neurosci* 20:34–42.
- Folkerth RD, Keefe RJ, Haynes RL, Trachtenberg FL, Volpe JJ, Kinney HC (2004) Interferon- γ expression in periventricular leukomalacia in the human brain. *Brain Pathol* 14:265–274.
- Follett PL, Rosenberg PA, Volpe JJ, Jensen FE (2000) NBQX attenuates excitotoxic injury in developing white matter. *J Neurosci* 20:9235–9241.
- Gilles FH, Leviton A, Kerr CS (1976) Endotoxin leucoencephalopathy in the telencephalon of the newborn kitten. *J Neurol Sci* 27:183–191.
- Grether JK, Nelson KB (1997) Maternal infection and cerebral palsy in infants of normal birth weight. *JAMA* 278:207–211.
- Hagberg H, Peebles D, Mallard C (2002) Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment Retard Dev Disabil Res Rev* 8:30–38.
- Hanisch UK (2002) Microglia as a source and target of cytokines. *Glia* 40:140–155.
- Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, Volpe JJ, Kinney HC (2003) Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. *J Neuropathol Exp Neurol* 62:441–450.
- Haynes RL, Baud O, Li J, Kinney HC, Volpe JJ, Folkerth DR (2005) Oxidative and nitrate injury in periventricular leukomalacia: a review. *Brain Pathol* 15:225–233.
- Hewett SJ, Csernansky CA, Choi DW (1994) Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron* 13:487–494.
- Horwitz MS, Evans CF, McGavern DB, Rodriguez M, Oldstone MB (1997) Primary demyelination in transgenic mice expressing interferon- γ . *Nat Med* 3:1037–1041.
- John GR, Lee SC, Brosnan CF (2003) Cytokines: powerful regulators of glial cell activation. *The Neuroscientist* 9:10–22.
- Kadhimi H, Tabarki B, Verellen G, De Prez C, Rona AM, Sebire G (2001) Inflammatory cytokines in the pathogenesis of periventricular leukomalacia. *Neurology* 56:1278–1284.
- Kadhimi H, Tabarki B, De Prez C, Rona AM, Sebire G (2002) Interleukin-2 in the pathogenesis of perinatal white matter damage. *Neurology* 58:1125–1128.
- Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 19:312–318.
- Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T (2002) The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J Neurosci* 22:2478–2486.
- Leist M, Jüttelä M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2:589–598.
- Li J, Lin JC, Wang H, Peterson JW, Furie BC, Furie B, Booth SL, Volpe JJ, Rosenberg PA (2003) Novel role of vitamin K in preventing oxidative injury to developing oligodendrocytes and neurons. *J Neurosci* 23:5816–5826.
- Li J, Baud O, Vartanian T, Volpe JJ, Rosenberg PA (2005) Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. *Proc Natl Acad Sci USA* 102:9936–9941.
- McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85:890–902.
- Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE (1993) Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J Immunol* 151:2132–2141.
- Minghetti L (2005) Role of inflammation in neurodegenerative diseases. *Curr Opin Neurol* 18:315–321.
- Misko TP, Highkin MK, Veenhuizen AW, Manning PT, Stern MK, Currie MG, Salvemini D (1998) Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J Biol Chem* 273:15646–15653.
- Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI (2006) Tumor necrosis factor- α mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. *J Neurosci* 26:12633–12641.
- Oka A, Belliveau MJ, Rosenberg PA, Volpe JJ (1993) Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. *J Neurosci* 13:1441–1453.
- Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87:315–424.
- Pang Y, Cai Z, Rhodes PG (2000) Effects of lipopolysaccharide on oligodendrocyte progenitor cells are mediated by astrocytes and microglia. *J Neurosci Res* 62:510–520.
- Pang Y, Cai Z, Rhodes PG (2003) Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. *Brain Res Dev Brain Res* 140:205–214.
- Pang Y, Fan LW, Zheng B, Cai Z, Rhodes PG (2006) Role of interleukin-6 in lipopolysaccharide-induced brain injury and behavioral dysfunction in neonatal rats. *Neuroscience* 141:745–755.
- Pekny M, Nilsson M (2005) Astrocyte activation and reactive gliosis. *Glia* 50:427–434.
- Peuchen S, Bolanos JP, Heales SJ, Almeida A, Duchon MR, Clark JB (1997) Interrelationships between astrocyte function, oxidative stress and antioxidant status within the central nervous system. *Prog Neurobiol* 52:261–281.
- Posselt H, Noack H, Putzke J, Wolf G, Sies H (2000) Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies. *Glia* 32:51–59.
- Probert L, Eugster H-P, Akassoglou K, Bauer J, Frei K, Lassmann H, Fontana A (2000) TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease. *Brain* 123:2005–2019.
- Rezaie P, Dean A (2002) Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. *Neuropathology* 22:106–132.

- Riddle A, Ling Luo N, Manese M, Beardsley DJ, Green L, Rorvik DA, Kelly KA, Barlow CH, Kelly JJ, Hohimer AR, Back SA (2006) Spatial heterogeneity in oligodendrocyte lineage maturation and not cerebral blood flow predicts fetal ovine periventricular white matter injury. *J Neurosci* 26:3045–3055.
- Ridet JL, Privat A, Malhotra SK, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* 20:570–577.
- Rosenberg PA, Dai W, Gan XD, Ali S, Fu J, Back SA, Sanchez RM, Segal MM, Follett PL, Jensen FE, Volpe JJ (2003) Mature myelin basic protein-expressing oligodendrocytes are insensitive to kainate toxicity. *J Neurosci Res* 71:237–245.
- Saha RN, Pahan K (2006) Regulation of inducible nitric oxide synthase gene in glial cells. *Antioxid Redox Signal* 8:929–947.
- Sahrbacher UC, Lechner F, Eugster HP, Frei K, Lassmann H, Fontana A (1998) Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur J Immunol* 28:1332–1338.
- Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. *J Neurosci* 27:10695–10702.
- Trapp BD, Bo L, Mork S, Chang A (1999) Pathogenesis of tissue injury in MS lesions. *J Neuroimmunol* 98:49–56.
- Volpe JJ (2001a) Neurobiology of periventricular leukomalacia in the premature infant. *Pediatr Res* 50:553–562.
- Volpe JJ (2001b) *Neurology of the newborn*, Ed 4. Philadelphia: Saunders.
- Volpe JJ (2003) Cerebral white matter injury of the premature infant—more common than you think. *Pediatrics* 112:176–180.
- Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci* 6:626–640.
- Wajant H, Pfizenmaier K, Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10:45–65.
- Wang X, Rousset CI, Hagberg H, Mallard C (2006) Lipopolysaccharide-induced inflammation and perinatal brain injury. *Semin Fetal Neonatal Med* 11:343–353.
- Yoon BH, Romero R, Kim CJ, Koo JN, Choe G, Syn HC, Chi JG (1997) High expression of tumor necrosis factor- α and interleukin-6 in periventricular leukomalacia. *Am J Obstet Gynecol* 177:406–411.
- Zhang Y, Wang H, Li J, Dong L, Xu P, Chen W, Neve RL, Volpe JJ, Rosenberg PA (2006) Intracellular zinc release and ERK phosphorylation are required upstream of 12-lipoxygenase activation in peroxynitrite toxicity to mature rat oligodendrocytes. *J Biol Chem* 281:9460–9470.