Published in final edited form as:
J Neurochem. 2011 January ; 116(1): 53–66. doi:10.1111/j.1471-4159.2010.07084.x.

Astrocytes Promote TNF-Mediated Toxicity to Oligodendrocyte Precursors

SunJa Kim, **Andrew J. Steelman**, **Hisami Koito**, and **Jianrong Li**‡
Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, Texas, USA 77843

Abstract

Neuroinflammation and increased production of tumor necrosis factor (TNF) in the central nervous system have been implicated in many neurological diseases including white matter disorders periventricular leukomalacia and multiple sclerosis. However, the exact role of TNF in these diseases and how it mediates oligodendrocyte injury remain unclear. Previously we demonstrated that lipopolysaccharide (LPS) selectively kills oligodendrocyte precursors (preOLs) in a non-cell autonomous fashion through the induction of TNF in mixed glial cultures. Here we report that activation of oligodendroglial, but not astroglial and microglial, TNFR1 is required for LPS toxicity, and that astrocytes promote TNF-mediated preOL death through a cell contact-dependent mechanism. Microglia were the sole source for TNF production in LPS-treated mixed glial cultures. Ablation of TNFR1 in mixed glia completely prevented LPS-induced death of preOLs. TNFR1-expressing preOLs were similarly susceptible to LPS treatment when seeded into wildtype and TNFR1$^{-/-}$ mixed glial cultures, demonstrating a requirement for oligodendroglial TNFR1 in the cell death. Although exogenous TNF failed to cause significant cell death in enriched preOL cultures, it became cytotoxic when preOLs were in contact with astrocytes. Collectively, our results demonstrate oligodendroglial TNFR1 in mediating inflammatory destruction of preOLs and suggest a previously unrecognized role for astrocytes in promoting TNF toxicity to preOLs.

Keywords

oligodendrocyte precursors; cell death; microglia; white matter injury; inflammation; periventricular leukomalacia

Introduction

Inflammatory responses associated with activation of microglia within the central nervous system (CNS) have been implicated in many pathological conditions including white matter disorder periventricular leukomalacia (PVL). PVL is the major form of cerebral white matter injury and the most important determinant of the neurologic morbidity in premature infants (Volpe 2003). Pathologically, it is characterized by focal necrotic lesions deep in the cerebral white matter that correlates with the development of cerebral palsy, and by diffuse white matter lesions leading to subsequent myelination abnormalities that likely contribute to cognitive and behavioral deficits frequently observed in survivors of prematurity (Volpe 2003). Diffuse PVL lesions are now recognized as the predominant form of brain injury of

‡ Corresponding author: Jianrong Li, Ph.D., Department of Veterinary Integrative Biosciences, Texas A&M University, Mail Stop 4458, College Station, TX 77843, Phone: 979-862-7155, Fax: 979-847-8981, jrli@cvm.tamu.edu.

The authors have no conflict of interest to declare.

prematurity with hallmarks of prominently activated astrocytes and microglia and dying premyelinating oligodendrocytes (preOLs) (Haynes *et al.* 2003). Although the role of these reactive astrocytes and microglia remains unclear, they are likely involved in preOL injury by releasing highly reactive oxygen/nitrogen species and proinflammatory cytokines, such as tumor necrosis factor (TNF) (Haynes *et al.* 2005, Back 2006, Rezaie & Dean 2002). In fact, preOLs, the primary OL lineage cells in human cerebral white matter during the peak risk period for PVL (Back *et al.* 2001), are intrinsically highly sensitive to oxidative/nitrative (Back *et al.* 1998, Back *et al.* 2002) and excitotoxic insults (McDonald *et al.* 1998, Follett *et al.* 2000).

Hypoxia/ischemia and maternal/fetal infection are the two primary triggers for PVL (Volpe 2001). Accumulating experimental and clinical evidence suggests a strong link between the gram negative bacterial component lipopolysaccharide (LPS) and PVL. Many animal studies employing systemic, local, or intrauterine LPS administration and hypoxia/ischemia have demonstrated selective injury to developing cerebral white matter, leading to hypomyelination (reviewed by (Hagberg *et al.* 2002)). Proinflammatory cytokine TNF is a central player in cerebral ischemia and neuroinflammation, and may contribute to the pathogenesis of PVL. TNF is known to exert most of its biological functions by signaling through cognate receptors TNF receptor 1 (TNFR1, p55) and/or receptor 2 (TNFR2, p75). Increased TNF production and TNFR1/2 expression have been reported for human PVL (Deguchi *et al.* 1996, Kadhim *et al.* 2006). Furthermore, transgenic animal studies have demonstrated that overexpression of TNF in astrocytes results in OL apoptosis and demyelination (Akassoglou *et al.* 1998), implying a pathogenic effect of local TNF signaling in the CNS.

Using an *in vitro* model for inflammatory injury to preOLs, we demonstrated that LPS induces selective preOL death indirectly by activating microglia (Li *et al.* 2008, Li *et al.* 2005). We further identified that peroxynitrite underlies the potent direct killing capability of activated microglia to preOLs and that astrocytes can shift the killing mechanism to one dependent on TNF signaling (Li *et al.* 2008). However, neither the cellular source for TNF production nor the TNF receptor mediating preOL death was identified. Since primary glial cells all express TNF receptors (Dopp *et al.* 1997), they can all engage in TNF signaling. Here, we systematically dissected the cellular source for TNF production, determined the TNF receptor required for LPS toxicity, and further identified an essential role for oligodendroglial TNFR1 in TNF-mediated killing of preOLs in mixed glial cultures. Most importantly, we provided the first evidence demonstrating that astrocytes sensitize preOLs to TNF toxicity in a contact-dependent manner.

Materials and Methods

Animals and reagents

Wildtype B6.129SF2/J and C57BL/6J mice, transgenic eGFP mice (003291, background C57BL/6J), and TNF (background B6.129SF2/J) and TNFR1 (background C57BL/6J) knockout mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Recombinant TNF was obtained from R&D Systems (Minneapolis, MN). PDGF and basic FGF were from PeproTech (Rocky Hill, NJ). Recombinant GFP adenovirus was from Gene Transfer Vector Core, University of Iowa. Rabbit polyclonal antibodies against Iba1 were purchased from Wako Chemicals (Richmond, VA). Rat antimouse TNF (clone MP6-XT22) was obtained from eBioscience (San Diego, CA). Olig2 antibody was a generous gift from Dr. Richard Lu (University of Texas Southwestern Medical Center). Lactate dehydrogenase (LDH) cytotoxicity kit was from Roche Applied Science (Indianapolis, IN). Unless specified otherwise, all other reagents were from Sigma (St. Louis, MO).

Primary cell cultures

Primary preOLs, microglia, astrocytes and mixed glial cultures were prepared from the forebrains of 1 to 2-d-old Sprage-Dowley rats or mice using a differential detachment method as detailed previously (Li *et al.* 2008). Briefly, mixed glial cultures were grown in poly-D-lysine coated culture plates or in flasks for individual cell type isolation. Microglia were isolated by shaking mixed glia-containing flasks for 1 h at 200 rpm. The purity of microglia at this stage was consistently >95%. To obtain highly purified microglial monocultures (>99%), cells were further plated into regular petri dishes for 1 h and then detached from the plates with ice cold EBSS. PreOLs isolated from mixed glial cultures were maintained in growth medium, i.e., the serum-free Basal Defined Medium (BDM) (DMEM, 0.1% bovine serum albumin, 50 μg/ml human apo-transferrin, 50 μg/ml insulin, 30 nM sodium selenite, 10 nM D-biotin and 10 nM hydrocortisone) supplemented with PDGF (10 ng/ml) and bFGF (10 ng/ml) for 5–9 days. The OL mono-cultures consisted of OL precursors [O_4^+, O_1^-] and were of at least 95% pure. Mature OLs were obtained by culturing preOLs in differentiation medium (BDM supplemented with 10 ng/ml CNTF and 15 nM T3) for 7–10 days.

Astrocytes were purified from the astrocyte layer in the flask that has been exposed to the specific microglia toxin L-leucine methyl ester (1 mM) for 1 h, followed by 1~2 cycles of subculture and repeated exposure to L-leucine methyl ester. The enriched astrocytes were consistently more than 95% pure with preOLs being the major contaminating cells. For astrocytes and preOLs co-cultures, astrocytes (2.4×10^5 cells per well) were plated into 24-well culture plates 1~2 days before seeding preOLs (6×10^4 cells per well), and the co-cultures were maintained in growth medium for 1–2 days before use. For bridged no cell contact co-cultures, preOLs grown on PO-coated coverslips with mini PDMS columns attached at four corners were transferred to 24-wells containing astrocytes. The distance between the astrocyte layer and the preOL layer was 1mm. Unless stated otherwise, all cell treatments were carried out in the growth medium. Mouse mixed glial cultures and various glia monocultures were prepared with the same methods as described above from wildtype (WT) and TNF and TNFR1 knockout (KO) mice. Mosaic mouse mixed cultures using cells from WT and KO mice were prepared by seeding isolated astrocytes (4×10^4/well) in poly-D-lysine coated 8 well chamber slide (Nunc, Rochester, NY), followed by microglia seeding (2×10^4/well) on the next day, and preOLs plating (0.8×10^4/well) plating the following day. Five to seven days after preOLs plating, these mixed glial cultures were subjected to vehicle or LPS treatment for 48 h and preOL survival was determined by counting the number of $O4^+$ cells.

Cell viability determination

Cell death was induced by exposure to LPS (*Escherichia coli* O111:B4, Sigma) or TNF as specified in the figure legend. Survival of preOLs was determined by counting O4 positive 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× magnification with a total of >1000 cells counted in the control conditions. Cell survival is expressed as mean ± SD. When monocultures of preOLs were used, cell viability was evaluated by Alamar blue, a tetrazolium dye that is reduced by living cells to a colored product, as described previously (Li *et al.* 2003). Cell toxicity was also assessed by lactate dehydrogenase (LDH) release assay according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN).

Infection of preOLs with recombinant adenovirus

Purified rat preOLs were infected with adenovirus containing green fluorescent protein (GFP) cDNA (AdGFP) as described previously (Baud *et al.* 2004). Briefly, cells were exposed to 1×10^8 pfu/ml of AdGFP overnight in BDM followed by a complete medium change the next day. Cells were allowed to recover for 48 h. The infection rate was consistently more than 90% with minimum toxicity. Cells were then trypsinized off the culture dish and seeded at density of $1–2 \times 10^4$ per well into established mixed glial cultures. The next day, the cultures were treated with either vehicle or LPS for 24 h, and immunostained for Iba1 and GFP.

Analysis of TNF production

The levels of TNF in the culture media of cells treated as specified were measured using a commercially available ELISA kit according to the manufacturer's instruction (eBioScience, San Diego, CA). Absorption at 450 nm was determined in a microplate reader (Fluostar Optima, BMG Labtech). The detection limit of the ELISA was 8 pg/ml.

RT-PCR

Microglia and astrocyte monocultures were plated onto poly-D-lysine coated 6-well plates at a density of 1.0×10^6 cells per well in DMEM containing 10% fetal bovine serum. After 24 h, cells were rinsed twice with BDM media, and stimulated with LPS (0–1.0 μg/ml) or vehicle for 0–24 h. RNA was extracted using an RNeasy kit according to the manufacturers' instructions (Qiagen, Valencia, CA). Residual DNA was removed by incubating RNA samples with DNase I for 15 min at room temperature followed by DNase inactivation at 65°C for 10 min according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The samples were reverse transcribed to cDNA using the reverse transcription system kit (Promega, Madison, WI) and random primers in the presence or absence of reverse transcriptase for 10 min at 25°C followed by 15 min at 45°C, 5 min at 95°C, and 5 min at 4°C. RT-PCR was used to test for the presence of TNF, IL-1β, IL-10, TLR2, TLR3, TLR4, GFAP, Iba1 and β-actin cDNA using the following specific primers: TNF, forward-GCCCACGTCGTAGCAAAC, reverse-GCAGCCTTGTCCCTTGAA; IL-1β, forward-TGACCCATGTGAGCTGAAAG, reverse-AGGGATTTTGTCGTTGCTTG; IL-10, forward-GAGTGAAGACCAGCAAAGGC, reverse-TTGTCCAGCTGGTCCTTCTT; TLR2, forward-AGCTGGAGAACTCTGACCCA, reverse-CAAAGAGCCTGAAGTGGGAG; TLR3, forward-TGCGATTGGCAAGTTATTCG, reverse-GCGCAGGCTGTTGTAGGAAA; TLR4, forward-TGCTCAGACATGGCAGTTTC, reverse-TCAAGGCTTTTCCATCCAAC; GFAP, forward-CAGCTT CGAGCCAAGGAG, reverse-TGTCCCTCTCCACCTCCA; Iba1, forward-CTTTTGGACTGCTGAAAGCC, reverse-GTT TCTCCAGCATTCGCTTC; and β-actin, forward- AGACTTCGAGCAGGAGATGG, reverse-CCATCATGAAGTGTGACGTTG. Briefly, after an initial denature step at 95°C for 10 min, 100 ng cDNA per reaction was subjected to 30 cycles of PCR (95°C 15 sec, 56°C 15 sec, 72°C 30 sec) followed by a final elongation step at 72°C for 5 min. Products were electrophorezed on 2% agarose gels and visualized under UV light using a Bio-Rad Chemidoc XRS gel documentation system and Quantity-one software.

Immunocytochemistry and fluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 10 min and blocked with TBST (50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% Triton X-100) or TBS (for O4 immunostaining) containing 5% goat serum. The coverslips were then incubated with antibody O4 (1:500) or antibodies against Iba1 (1:1000) or GFAP (1:1000) overnight at 4°C. After washes, secondary antibody conjugated with either Alexa Fluor 488 or Alexa Fluor 594 (1:1000

dilution, Molecular Probes, Eugene, OR) was incubated with the coverslips for 1 h at room temperature. Following more washes, nuclei were stained with Hoechst 33258 at a final concentration of 2 μg/ml for 1 min. The coverslips were then washed 2–3 times and mounted onto glass slides with FluoroMount and kept in the dark at 4°C. Cell images were captured with a fluorescence microscope equipped with an Olympus DP70 digital camera (Olympus IX71).

For intracellular TNF staining, mixed glial cultures from C57BL/6 mice were washed twice with media and then treated with LPS at 0, 0.1 or 1.0 μg/ml for 8 or 24 h. During the last 5 h of stimulation, protein secretion was blocked using the protein transport inhibitor Brefeldin A (eBioscience, San Diego, CA) diluted 1:1000 in BDM alone or in BDM containing LPS. Cells were then fixed, permeabilized, blocked, and immunostained as described above.

Annexin-V detection

Cell surface phosphatidylserine (PS) was detected with an annexin-V-FITC apoptosis detection kit (Sigma) according to the manufacturer's instructions with slight modification. Cells were treated with LPS (1.0μg/ml) or media for 2, 6, 8, 12, 24 and 48h, at which time an aliquot of the supernatants was removed and stored at −80°C for subsequent TNF ELISA and LDH analyses. The cells were washed twice with sterile filtered TBS, and 500μl of binding buffer containing 5μl annexin-V-FITC was added to each well for exactly 10 min. The cells were then fixed with 2% PFA and stained for O4.

Western blotting analysis

Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by electrotransferring of separated proteins to PVDF membrane. Nonspecific binding was blocked with TBS-T (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk for 1 h at room temperature. Anti TNFR1 (1: 500 dilution, Rabbit IgG, Santa Cruz Biotech, Santa Cruz, CA.), and β-actin (1:10,000 dilution) antibodies were diluted in TBS-T containing 5% non-fat milk and incubated overnight with the membrane at 4°C. After washing 3–5 times with TBS-T, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1:2000) for 1 h and was visualized by chemiluminescence using the SuperSignal detection kit (Thermo Scientific, Rockford, IL).

Statistical Analysis

All cell culture treatments were performed in triplicate. Unless specified otherwise, data were presented as mean ± SD. Results were analyzed by one-way ANOVA followed by Bonferroni's post-hoc 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-activated microglia kill preOLs and phagocytose dying cells in mixed glial cultures

As mentioned above, multiple lines of evidence have revealed a strong relationship between maternal/fetal infection, LPS, and pathogenesis of PVL. To elucidate the mechanism underlying endotoxin-induced white matter injury, an *in vitro* model was developed. Using co-cultures of preOLs with other cells such as microglia or astrocytes, we and others identified an indispensible role of microglia in mediating preOL cell death (Lehnardt *et al.* 2002, Li *et al.* 2005, Li *et al.* 2008). Consistent with previous findings, in mixed glial cultures prepared from neonatal rat forebrains, addition of LPS induced robust microglia activation and proliferation with concomitantly significant loss of preOLs (Fig. 1A–C). LPS

activated microglia exhibit increased immunoreactivity to Iba1 and larger cell bodies and processes. A significant portion of activated microglia appeared to be amoeboid (Fig. 1C).

It is well accepted that apoptotic cells are rapidly cleared by phagocytes *in vivo* without intracellular content release (Lauber *et al.* 2004). We found that GFP^+ preOLs, when seeded on top of established mixed glial cultures, were susceptible to LPS and their cell corpses were engulfed by microglia (Fig. 1D). Consistent with this finding, we found increased cell surface PS on preOLs in mixed glial cultures treated with LPS, as determined by Annexin V staining (Fig. 1E, H). Translocation of PS from the inner leaflet of plasma membrane to the outer leaflet represents a "eat-me" signal for phagocytes (Lauber *et al.* 2004). This LPS-induced PS translocation in preOLs correlated with TNF production and proceeded cell death (Fig. 1F, G). These results show that dying preOLs were efficiently removed by microglia via phagocytosis, and are in agreement with our observation that despite significant loss of preOLs in LPS-treated mixed glial cultures, there was minimal cell debris.

Microglia, but not astrocytes, produce TNF in LPS-stimulated mixed glial cultures

We previously demonstrated that production of TNF is required for LPS toxicity to preOLs in mixed glial cultures (Li *et al.* 2008). However, the cellular source for TNF production was not identified. Since both microglia and astrocytes are the main responders in the CNS to inflammation and capable of producing various chemokines and cytokines *in vivo* and *in vitro* (Hanisch 2002, Farina *et al.* 2007), we examined which cell types or both of them produce TNF in response to LPS in cultures. Using enriched rat microglia and astrocyte monocultures, we found that LPS induced robust TNF production in microglia, but not astrocytes, in a concentration-dependent manner (Fig. 2A, B).

Previous studies suggest that astrocytes constitutively express low level of the LPS receptor, toll-like receptor 4 (TLR4) (Carpentier *et al.* 2005, Bowman *et al.* 2003). To further answer the question whether astrocytes produce TNF in response to LPS, we prepared highly purified monocultures (>99%), exposed them to LPS for 6 and 24 h, and examined various transcripts by RT-PCR (Fig. 2C). Whereas both astrocytes and microglia express TLR3, TLR4 was only detected in microglia, a finding consistent with a previous study (Lehnardt *et al.* 2002). LPS exposure resulted in a transient minor upregulation of TNF and IL-1β mRNA in astrocytes at 6 h, but not at 24 h (Fig. 2C). In contrast, LPS-stimulated microglia continuously expressed TNF and IL-1β. Additionally, the anti-inflammatory cytokine IL-10 was also detected in LPS-activated microglia, especially at the later time point, suggesting an autoregulatory mechanism for microglial activation, potentially mediated through phagocytosis of dying preOLs (Fig. 1).

To investigate whether astrocytes are capable of producing TNF in response to factors produced by LPS-activated microglia, we immunostained for TNF in mixed glia treated with LPS for 8 and 24h. Whereas no TNF immunoreactivity was detected in the absence of LPS stimulation, LPS at concentrations of 0.1–1 μg/ml induced robust TNF immunoreactivity in cells with activated microglial morphology (Fig. 2D). We did not detect a single TNF-positive cell that was also GFAP positive at both time points (>1000 TNF^+ cells examined). Instead, TNF immunoreactivity was completely restricted to microglia at various activation stages, with the most active amoeboid microglia producing the strongest TNF signal (Fig. 2E, arrow vs. arrowhead). Taken together, our data demonstrate that microglia but not astrocytes produce TNF upon LPS stimulation.

Microglia-derived TNF is necessary for LPS-induced preOL death in mixed glial cultures

Ablation of *tnf* in mixed glia completely prevented LPS toxicity (Li *et al.* 2008). If microglia are indeed responsible for TNF generation, then selective ablation of *tnf* in microglia, but

not in astrocytes and OLs, should prevent LPS toxicity. To test this hypothesis, we isolated each individual cell type from WT and TNF KO mice, and reconstituted them in different combinations to create mosaic mixed glial cultures with a composition similar to that of regular mixed glial cultures. We then subjected these cultures to vehicle or LPS treatment for 48 h and examined preOL survival by counting the number of $O4^+$ cells.

Consistent with our above finding in regular mixed glial cultures (Fig. 1A), LPS also induced significant loss of preOLs in reconstituted mixed cultures containing WT astrocytes, microglia and OLs (Fig. 3A). However, in mixed glia containing TNF KO instead of WT microglia, the LPS toxicity was abrogated (Fig. 3B). In contrast, TNF deficiency in astrocytes or OLs did not render any protection (Fig. 3C–E). These results clearly demonstrate an indispensible role for microglial TNF in mediating LPS toxicity to preOLs. Consistent with the survival data, analyses of secreted TNF in these mosaic mixed glial culture revealed that when LPS failed to cause preOL death in cultures containing TNF KO microglia (Fig. 3B), LPS-induced TNF production was also significantly diminished (Fig. 3G vs. F). It should be noted that LPS-induced TNF production was not completely abrogated in cultures containing TNF KO microglia, most likely due to contaminating WT microglia in WT astrocytes and OLs preparations.

Oligodendroglial TNFR1 is necessary for LPS toxicity in mixed glial cultures

We next questioned whether microglia-derived TNF exerts its toxic effects on preOLs in an autocrine or a paracrine fashion. Since ligation of TNFR1 has been shown to be primarily related to cell death and activation of TNFR2 to cell proliferation and protection (Arnett *et al.* 2001, Probert *et al.* 2000), we tested whether TNFR1 is responsible for the LPS toxicity. Indeed, ablation of TNFR1 in all glial cells completely prevented LPS-induced LDH release and loss of preOLs, even though TNFR1 KO cultures produced similar levels of TNF as the WT (Fig. 4A–D). These data demonstrate that TNFR1 is necessary for LPS-induced killing of preOLs.

PreOLs, microglia and astrocytes all express TNFR1 (Dopp *et al.* 1997). As activation of the TNFR1 pathway in either microglia or astrocytes could potentially induce OL death via secreted factors, it was important to determine which cell was responsible for the TNFR1-mediated effect. Therefore, we isolated GFP-expressing preOLs, which also expressed TNFR1, and seeded them onto either WT or TNFR1 KO mixed glial cultures (Fig. 4E). If oligodendroglial TNFR1, but not microglial and astroglial TNFR1, is indeed essential for LPS-induced preOL death, these exogenously added $GFP^+/TNFR1^+$ preOLs should demonstrate similar vulnerability toward LPS regardless being in WT or TNFR1 KO mixed glia. On the other hand, if activation of TNFR1 on microglia or astrocytes is responsible for LPS-induced preOL death, then these $GFP^+/TNFR1^+$ preOLs should be less vulnerable in TNFR1 KO mixed glial cultures. We found that upon LPS treatment, similar number of $GFP^+/TNFR1^+$ preOLs underwent degeneration regardless the TNFR1 status of the recipient mixed glial cultures (Fig. 4E, F). As an internal control, the endogenous $TNFR1^{-/-}$ preOLs in the $TNFR1^{-/-}$ mixed glial cultures were completely resistant to LPS (Fig. 4B). These data strongly suggest that ligation of oligodendroglial TNFR1 by TNF generated from microglia accounts for LPS-induced death of preOLs in an environment where all types of glia co-exist.

TNF does not trigger cell death in pure preOLs

Our results demonstrated that activation of oligodendroglial TNFR1 by LPS-induced production of TNF is required for selective killing of preOLs in mixed glial cultures. Interestingly, when preOLs were exposed to increasing concentrations of recombinant TNF, no significant cell loss was observed (Fig. 5A). Similarly, TNF also had limited effect on

mature OLs (Fig. S1A). Conditioned media from mixed glia previously treated with LPS were not toxic when added to pure preOLs (Fig. 5B, C). This is in sharp contrast to selective preOL death in LPS-treated mixed glial cultures. Thus, the above data indicate that a non-cell autonomous pathway is operative together with oligodendroglial TNFR1 for TNF-mediated toxicity in mixed glial cultures.

Astrocytes promote TNF toxicity to preOLs in a contact-dependent manner

We previously showed that LPS-activated microglia kill preOLs via peroxynitrite in co-cultures, but via TNF in mixed glial cultures (Li *et al.* 2005, Li *et al.* 2008). Since TNF is not toxic to pure preOLs as demonstrated above, we hypothesized that astrocytes directly contribute to TNF-mediated toxicity. Whereas preOLs themselves were not sensitive to TNF, they became so when cultured together with astrocytes (Fig. 6A), demonstrating an essential role for astrocytes in TNF-induced loss of preOLs. In contrast, mature MBP-expressing OLs were not significantly affected by TNF (Fig. S1B). This observation is in agreement with earlier studies demonstrating maturation-dependent vulnerability of preOLs to oxidative stress, excitotoxicity and cytokines (Haynes et al. 2005, Butts *et al.* 2008, Andrews *et al.* 1998, Horiuchi *et al.* 2006). Interestingly, when preOLs were co-cultured with astrocytes in the absence of cell-cell contact, as illustrated in Fig. 6B, they were no longer vulnerable to TNF (Fig. 6C). Similarly, conditioned medium from TNF-treated astrocytes was not toxic to preOLs. These results suggest that direct cell contact between preOLs and astrocytes is a prerequisite for TNF toxicity.

One possibility is that astrocytes promote TNF-mediated preOL death by upregulating TNFR1 in preOLs since oligodendroglial TNFR1 is required for the toxicity (Fig. 4). However, LPS neither increased the level of total TNFR1 of mixed glia (Fig 7A, top panel), nor did it induce TNFR1 specifically in preOLs that were plated into $TNFR1^{-/-}$ mixed glial cultures (Fig. 7A, lower panel), a condition where these exogenously added preOLs were susceptible to LPS treatment. Moreover, although astrocytes are required for TNF-induced toxicity to preOLs, activation of astrocytic TNFR1 is not (Fig. 7B). This is in line with our above finding (Fig. 4) and demonstrates that TNFR1 on preOLs is necessary but not sufficient for TNF-induced cell death.

Discussion

In this study we demonstrated that oligodendroglial TNFR1 is indispensible for LPS-triggered death of preOLs, and revealed a non-cell autonomous death pathway that involves astrocytes and production of TNF from activated microglia. We further demonstrated that microglia not only are responsible for LPS toxicity to preOLs but also actively phagocytose dying preOLs in culture. We showed that conditioned media derived from LPS-challenged mixed glia is not toxic to preOLs, and that recombinant TNF does not cause significant cell loss in pure preOLs, suggesting that activation of TNFR1 alone is not sufficient to cause cell death. In contrast, astrocytes render preOLs vulnerable to TNF in a contact-dependent manner (Fig. 8). Our results suggest, for the first time, that astrocytes contribute positively to TNF-mediated toxicity to preOLs, and may reconcile previous contradictory observations from different laboratories on the effect of TNF on OLs. Our data clearly indicate that intercellular interactions among various glial cells are important and necessary for preOL destruction under inflammatory conditions. Complete understanding of these communications and death of oligodendroglial cells would enable development of strategies that selectively target deleterious aspects of proinflammatory cytokines such as TNF while preserve their beneficial effects.

Multiple lines of evidence have indicated a deleterious role for TNF in OL injury *in vivo* (Probert et al. 2000, Akassoglou et al. 1998, Probert *et al.* 1995, Stalder *et al.* 1998), in

organotypic cultures (Selmaj & Raine 1988), and *in vitro* (Jurewicz *et al.* 2005, Andrews *et al.* 1998, Li *et al.* 2008). Production of TNF and expression of TNF receptor R1 and R2 are found to be locally up-regulated in human PVL (Kadhim *et al.* 2001, Deguchi *et al.* 1996) as well as in multiple sclerosis (MS) lesions (Selmaj *et al.* 1991a, Hofman *et al.* 1989, Raine *et al.* 1998). Blockade of TNF prevents initiation of pathology and ameliorates progression of demyelination in animal models of MS (Selmaj *et al.* 1991b, Korner *et al.* 1997, Baker *et al.* 1994, Ruddle *et al.* 1990). Moreover, localized over-expression of TNF in the CNS selectively induces OL death and demyelination in the context of an intact blood brain barrier and absence of immune cell infiltration into the CNS parenchyma (Akassoglou *et al.* 1998, Kassiotis & Kollias 2001, Probert *et al.* 1995). This TNF pathology appears to be mediated through TNF/TNFR1 since both cytotoxic and inflammatory effects of TNF are abrogated in mice deficient in TNFR1 (Akassoglou *et al.* 1998). Other animal studies also revealed critical roles for TNF/TNFR signaling in triggering CNS inflammation and demyelination (Probert et al. 2000, Akassoglou *et al.* 1997, Stalder et al. 1998), and demonstrated that macrophage/microglial cells, but not lymphocytes play a central role in mediating injury (Stalder et al. 1998). Unexpectedly, anti-TNF therapies in human MS was of little efficacy or even resulted in worsened symptoms in some patients (TNF neutralization in MS 1999), clearly indicating the complexity of TNF signaling and call upon further understanding and investigation into these pathways. On the other hand, several studies have demonstrated a protective role for TNF. Using genetically modified mice, TNF was found to be required for preOLs proliferation and remyelination in a toxin-induced demyelination model, and this beneficial effect of TNF appears to be mediated by TNFR2 (Arnett *et al.* 2001). Although TNF is elevated in PVL and MS as well as in many other neurological disorders including ischemia, neurodegenerative diseases and traumatic brain injury, whether TNF signaling actively contributes to pathogenesis, prevents cell injury, and/or participates in repair process in these disorders has yet to be established due to the complex actions of TNF on neurons and glia (reviewed by (McCoy & Tansey 2008, Chadwick *et al.* 2008). The contrasting protective and deleterious effects of TNF in models of CNS demyelination suggest that selectively targeted, rather than global blockade of TNF signaling events, is required for achieving any meaningful therapeutic outcome. Our current study indicates that selective inhibition of oligodendroglial TNFR1 may offer therapeutic advantages and avoid the adverse effect of TNF-targeted therapies in the CNS. Future animal studies employing conditional knockout of TNFR1 in OL lineage cells is thus needed to explore this revenue.

Our data support a prominent role for activated microglia in inflammatory OL injury. Primary microglia express an array of TLRs including TLR4 that transduces LPS signaling, respond robustly to LPS stimulation, produce proinflammatory cytokines such as TNF and IL-1β, and cause selective preOL death via a mechanism involving oligodendroglial TNFR1. Interestingly, we also observed efficient engulfment of dying/dead preOLs by reactive microglia. It is not clear whether these phagocytozing microglia are of different populations or activation stages from microglia that are producing TNF and kill preOLs. When removing dying cells or myelin debris, phagocytozing microglia were shown to release anti-inflammatory factors (Magnus *et al.* 2001, Liu *et al.* 2006), consistent with our observation on microglial production of the anti-inflammatory cytokine IL-10 over time (Fig. 2C). Recent evidence suggests that when challenged, microglia undergo a sequence of activated states with different phenotypic responses and functional diversity, being either protective or toxic, but how these are regulated remain unclear (Hanisch & Kettenmann 2007). Indeed, LPS activated microglia in mixed glial cultures exhibit an array of morphology with amoeboid microglia, the likely fully activated state, producing the highest amount and other activation stages producing lower amount of TNF (Fig. 2E). It is possible that it is these amoeboid microglia that are cytotoxic to preOLs while some less activated microglia may be beneficial either by removing dying cells or by secreting trophic factors. Further studies will

be required to investigate the underpinning mechanisms. On the other hand, this microglial functional diversity also highlights the need for specific blockade of only deleterious signaling events.

To date, the mechanism by which TNF causes a toxic effect to OL lineage cells remains unresolved. Conflicting data on the effect of TNF on OL lineage cells have been reported from various laboratories. While some found toxicity of TNF on cultured OLs, others did not; and some observed developmental-dependent toxicity (Merrill & Scolding 1999, Andrews *et al.* 1998, Horiuchi *et al.* 2006, Scurlock & Dawson 1999, Cammer 2000, Jurewicz *et al.* 2005, Pang *et al.* 2005, Taylor *et al.* 2010). In light of our finding of an essential role for astrocytes in TNF-mediated toxicity to preOLs, some discrepancies may in part due to different culture compositions and the presence of astrocytes. How exactly astrocytes promote TNF toxicity to preOLs is currently unknown, but direct cell-cell contact appears to be essential. TNF is a pleiotropic cytokine that exerts multiple cellular responses including apoptosis, cell survival and proliferation in a receptor- and cell context-dependent manner. Both OLs and astrocytes express receptors for TNF, therefore, it is possible that astrocytes respond to TNF (through receptor other than TNFR1, Fig. 7B) and express factors that, through direct interaction with preOLs, enhance TNF/TNFR1 death signaling in preOLs or suppress survival signals. One possibility for contact-dependent cell killing is through gap junctions since gap junctions are known to couple astrocytes and OLs (Nagy & Rash 2000, Orthmann-Murphy *et al.* 2008) and have been previously suggested in propagation of cell injury (Lin *et al.* 1998, Farahani *et al.* 2005, Froger *et al.* 2010). Further study is needed to dissect, at molecular level, the interplay between OLs and astrocytes under inflammatory conditions. It should be noted that despite astrocytes are generally considered protective by releasing trophic factors such as CNTF, LIF and IL-11, a pathological role for reactive astrocytes has also been implicated in white matter diseases (Williams *et al.* 2007, Haynes et al. 2005). It is also noteworthy that neurons or axons could directly or indirectly affect preOL injury, or vise versa, in the cerebral white matter, as axonal injury was recently reported in PVL diffuse lesions (Haynes *et al.* 2008). Since reciprocal axon-glial interactions are essential for myelinogenesis of the developing brain (Barres & Raff 1999, Simons & Trajkovic 2006), it would be interesting to examine whether activated microglia or TNF differentially affect various cell population in the white matter.

In summary, our study reveals a detrimental role for engaging oligodendroglial TNFR1 in LPS-triggered death of preOLs in an environment where all glial cells coexist, identifies an essential role for astrocytes in TNF toxicity to preOLs, and offers new insights into the mechanisms of TNF-mediated injury to preOLs. This study also suggests that selected inhibition of TNFR1 signaling in preOLs may be beneficial in white matter diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MS	multiple sclerosis
OLs	oligodendrocytes
PDMS	polydimethylsiloxane

preOLs	oligodendrocyte precursors
PVL	periventricular leukomalacia
TLR	Toll-like receptor
TNF	tumor necrosis factor alpha
TNFR1	TNF receptor 1 (p55)

Acknowledgments

This work was supported by NIH grant R01NS060017, by the research grant RG3975 from the National Multiple Sclerosis Society, and by the start-up funds from Texas A&M University. We thank Jie Peng and Sarah Dobrovolny for technical assistance.

References

Akassoglou K, Bauer J, Kassiotis G, Pasparakis M, Lassmann H, Kollias G, Probert L. Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. Am J Pathol 1998;153:801–813. [PubMed: 9736029]

Akassoglou K, Probert L, Kontogeorgos G, Kollias G. Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. J Immunol 1997;158:438–445. [PubMed: 8977220]

Andrews T, Zhang P, Bhat NR. TNFalpha potentiates IFNgamma-induced cell death in oligodendrocyte progenitors. J Neurosci Res 1998;54:574–583. [PubMed: 9843148]

Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JPY. TNF[alpha] promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci 2001;4:1116–1122. [PubMed: 11600888]

Back SA. Perinatal white matter injury: the changing spectrum of pathology and emerging insights into pathogenetic mechanisms. Ment Retard Dev Disabil Res Rev 2006;12:129–140. [PubMed: 16807910]

Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. J Neurosci 1998;18:6241–6253. [PubMed: 9698317]

Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, Arvin KL, Holtzman DM. Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. J Neurosci 2002;22:455–463. [PubMed: 11784790]

Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. J Neurosci 2001;21:1302–1312. [PubMed: 11160401]

Baker D, Butler D, Scallon BJ, O'Neill JK, Turk JL, Feldmann M. Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins. Eur J Immunol 1994;24:2040–2048. [PubMed: 8088324]

Barres BA, Raff MC. Axonal Control of Oligodendrocyte Development. J. Cell Biol 1999;147:1123–1128. [PubMed: 10601327]

Baud O, Li J, Zhang Y, Neve RL, Volpe JJ, Rosenberg PA. Nitric oxide-induced cell death in developing oligodendrocytes is associated with mitochondrial dysfunction and apoptosis-inducing factor translocation. Eur J Neurosci 2004;20:1713–1726. [PubMed: 15379992]

Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express toll-like receptors for bacterial products. Glia 2003;43:281–291. [PubMed: 12898707]

Butts BD, Houde C, Mehmet H. Maturation-dependent sensitivity of oligodendrocyte lineage cells to apoptosis: implications for normal development and disease. Cell Death Differ 2008;15:1178–1186. [PubMed: 18483490]

Cammer W. Effects of TNF[alpha] on immature and mature oligodendrocytes and their progenitors in vitro. Brain Research 2000;864:213–219. [PubMed: 10802028]

Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ, Miller SD. Differential activation of astrocytes by innate and adaptive immune stimuli. Glia 2005;49:360–374. [PubMed: 15538753]

Chadwick W, Magnus T, Martin B, Keselman A, Mattson MP, Maudsley S. Targeting TNF-[alpha] receptors for neurotherapeutics. Trends in Neurosciences 2008;31:504–511. [PubMed: 18774186]

Deguchi K, Mizuguchi M, Takashima S. Immunohistochemical expression of tumor necrosis factor alpha in neonatal leukomalacia. Pediatr Neurol 1996;14:13–16. [PubMed: 8652010]

Dopp JM, Mackenzie-Graham A, Otero GC, Merrill JE. Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. J Neuroimmunol 1997;75:104–112. [PubMed: 9143243]

Farahani R, Pina-Benabou MH, Kyrozis A, et al. Alterations in metabolism and gap junction expression may determine the role of astrocytes as “good samaritans” or executioners. Glia 2005;50:351–361. [PubMed: 15846800]

Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol 2007;28:138–145. [PubMed: 17276138]

Follett PL, Rosenberg PA, Volpe JJ, Jensen FE. NBQX attenuates excitotoxic injury in developing white matter. J Neurosci 2000;20:9235–9241. [PubMed: 11125001]

Froger N, Orellana JA, Calvo C-F, Amigou E, Kozoriz MG, Naus CC, Sáez JC, Giaume C. Inhibition of cytokine-induced connexin43 hemichannel activity in astrocytes is neuroprotective. Molecular and Cellular Neuroscience 2010;45:37–46. [PubMed: 20684043]

Hagberg H, Peebles D, Mallard C. Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. Ment Retard Dev Disabil Res Rev 2002;8:30–38. [PubMed: 11921384]

Hanisch U-K, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 2007;10:1387–1394. [PubMed: 17965659]

Hanisch UK. Microglia as a source and target of cytokines. Glia 2002;40:140–155. [PubMed: 12379902]

Haynes RL, Baud O, Li J, Kinney HC, Volpe JJ, Folkerth DR. Oxidative and nitrative injury in periventricular leukomalacia: a review. Brain Pathol 2005;15:225–233. [PubMed: 16196389]

Haynes RL, Billiards SS, Borenstein NS, Volpe JJ, Kinney HC. Diffuse axonal injury in periventricular leukomalacia as determined by apoptotic marker fractin. Pediatr Res 2008;63:656–661. [PubMed: 18520330]

Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, Volpe JJ, Kinney HC. Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. J Neuropathol Exp Neurol 2003;62:441–450. [PubMed: 12769184]

Hofman FM, Hinton DR, Johnson K, Merrill JE. Tumor necrosis factor identified in multiple sclerosis brain. J. Exp. Med 1989;170:607–612. [PubMed: 2754393]

Horiuchi M, Itoh A, Pleasure D, Itoh T. MEK-ERK signaling is involved in interferon-gamma - induced death of oligodendroglial progenitor cells. J. Biol. Chem 2006;281:20095–20106. [PubMed: 16728393]

Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K. Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. Brain 2005;128:2675–2688. [PubMed: 16219674]

Kadhim H, Khalifa M, Deltenre P, Casimir G, Sebire G. Molecular mechanisms of cell death in periventricular leukomalacia. Neurology 2006;67:293–299. [PubMed: 16864823]

Kadhim H, Tabarki B, Verellen G, De Prez C, Rona AM, Sebire G. Inflammatory cytokines in the pathogenesis of periventricular leukomalacia. Neurology 2001;56:1278–1284. [PubMed: 11376173]

Kassiotis G, Kollias G. Uncoupling the Proinflammatory from the Immunosuppressive Properties of Tumor Necrosis Factor (TNF) at the p55 TNF Receptor Level: Implications for Pathogenesis and Therapy of Autoimmune Demyelination. J. Exp. Med 2001;193:427–434. [PubMed: 11181695]

Korner H, Lemckert FA, Chaudhri G, Etteldorf S, Sedgwick JD. Tumor necrosis factor blockade in actively induced experimental autoimmune encephalomyelitis prevents clinical disease despite activated T cell infiltration to the central nervous system. Eur J Immunol 1997;27:1973–1981. [PubMed: 9295034]

Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. Mol Cell 2004;14:277–287. [PubMed: 15125832]

Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, Jensen FE, Rosenberg PA, Volpe JJ, Vartanian T. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. J Neurosci 2002;22:2478–2486. [PubMed: 11923412]

Li J, Baud O, Vartanian T, Volpe JJ, Rosenberg PA. Peroxynitrite generated by inducible nitric oxide synthase and NADPH oxidase mediates microglial toxicity to oligodendrocytes. Proc Natl Acad Sci U S A 2005;102:9936–9941. [PubMed: 15998743]

Li J, Lin JC, Wang H, Peterson JW, Furie BC, Furie B, Booth SL, Volpe JJ, Rosenberg PA. Novel role of vitamin K in preventing oxidative injury to developing oligodendrocytes and neurons. J Neurosci 2003;23:5816–5826. [PubMed: 12843286]

Li J, Ramenaden ER, Peng J, Koito H, Volpe JJ, Rosenberg PA. Tumor necrosis factor {alpha} mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present. J. Neurosci 2008;28:5321–5330. [PubMed: 18480288]

Lin JH, Weigel H, Cotrina ML, Liu S, Bueno E, Hansen AJ, Hansen TW, Goldman S, Nedergaard M. Gap-junction-mediated propagation and amplification of cell injury. Nat Neurosci 1998;1:494–500. [PubMed: 10196547]

Liu Y, Hao W, Letiembre M, Walter S, Kulanga M, Neumann H, Fassbender K. Suppression of microglial inflammatory activity by myelin phagocytosis: role of p47-PHOX-mediated generation of reactive oxygen species. J Neurosci 2006;26:12904–12913. [PubMed: 17167081]

Magnus T, Chan A, Grauer O, Toyka KV, Gold R. Microglial phagocytosis of apoptotic inflammatory T cells leads to down-regulation of microglial immune activation. J Immunol 2001;167:5004–5010. [PubMed: 11673508]

McCoy M, Tansey M. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. Journal of Neuroinflammation 2008;5:45. [PubMed: 18925972]

McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP. Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat Med 1998;4:291–297. [PubMed: 9500601]

Merrill JE, Scolding NJ. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. Neuropathol Appl Neurobiol 1999;25:435–458. [PubMed: 10632895]

Nagy JI, Rash JE. Connexins and gap junctions of astrocytes and oligodendrocytes in the CNS. Brain Res Brain Res Rev 2000;32:29–44. [PubMed: 10751655]

Orthmann-Murphy JL, Abrams CK, Scherer SS. Gap junctions couple astrocytes and oligodendrocytes. Journal of Molecular Neuroscience 2008;35:101–116. [PubMed: 18236012]

Pang Y, Cai Z, Rhodes PG. Effect of tumor necrosis factor-alpha on developing optic nerve oligodendrocytes in culture. J Neurosci Res 2005;80:226–234. [PubMed: 15765524]

Probert L, Akassoglou K, Pasparakis M, Kontogeorgos G, Kollias G. Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. Proc Natl Acad Sci U S A 1995;92:11294–11298. [PubMed: 7479982]

Probert L, Eugster H-P, Akassoglou K, Bauer J, Frei K, Lassmann H, Fontana A. TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease. Brain 2000;123:2005–2019. [PubMed: 11004118]

Raine CS, Bonetti B, Cannella B. Multiple sclerosis: expression of molecules of the tumor necrosis factor ligand and receptor families in relationship to the demyelinated plaque. Rev Neurol (Paris) 1998;154:577–585. [PubMed: 9809372]

Rezaie P, Dean A. Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. Neuropathology 2002;22:106–132. [PubMed: 12416551]

Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, Clark RB. An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. J Exp Med 1990;172:1193–1200. [PubMed: 2212948]

Scurlock K, Dawson G. Differential responses of oligodendrocytes to tumor necrosis factor and other pro-apoptotic agents: Role of ceramide in apoptosis. Journal of Neuroscience Research 1999;55:514–522. [PubMed: 10723061]

Selmaj K, Raine CS. Tumor necrosis factor mediates myelin damage in organotypic cultures of nervous tissue. Ann N Y Acad Sci 1988;540:568–570. [PubMed: 2462832]

Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. J Clin Invest 1991;87:949–954. [PubMed: 1999503]

Selmaj K, Raine CS, Cross AH. Anti-tumor necrosis factor therapy abrogates autoimmune demyelination. Ann Neurol 1991;30:694–700. [PubMed: 1722388]

Simons M, Trajkovic K. Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. J Cell Sci 2006;119:4381–4389. [PubMed: 17074832]

Stalder AK, Carson MJ, Pagenstecher A, Asensio VC, Kincaid C, Benedict M, Powell HC, Masliah E, Campbell IL. Late-Onset Chronic Inflammatory Encephalopathy in Immune-Competent and Severe Combined Immune-Deficient (SCID) Mice with Astrocyte-Targeted Expression of Tumor Necrosis Factor. Am J Pathol 1998;153:767–783. [PubMed: 9736027]

Taylor DL, Pirianov G, Holland S, et al. Attenuation of proliferation in oligodendrocyte precursor cells by activated microglia. J Neurosci Res 2010;88:1632–1644. [PubMed: 20091773]

TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. Neurology 1999;53:457–465. [PubMed: 10449104]

Volpe JJ. Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res 2001;50:553–562. [PubMed: 11641446]

Volpe JJ. Cerebral white matter injury of the premature infant-more common than you think. Pediatrics 2003;112:176–180. [PubMed: 12837883]

Williams A, Piaton G, Lubetzki C. Astrocytes - Friends or foes in multiple sclerosis? Glia 2007;55:1300–1312. [PubMed: 17626262]

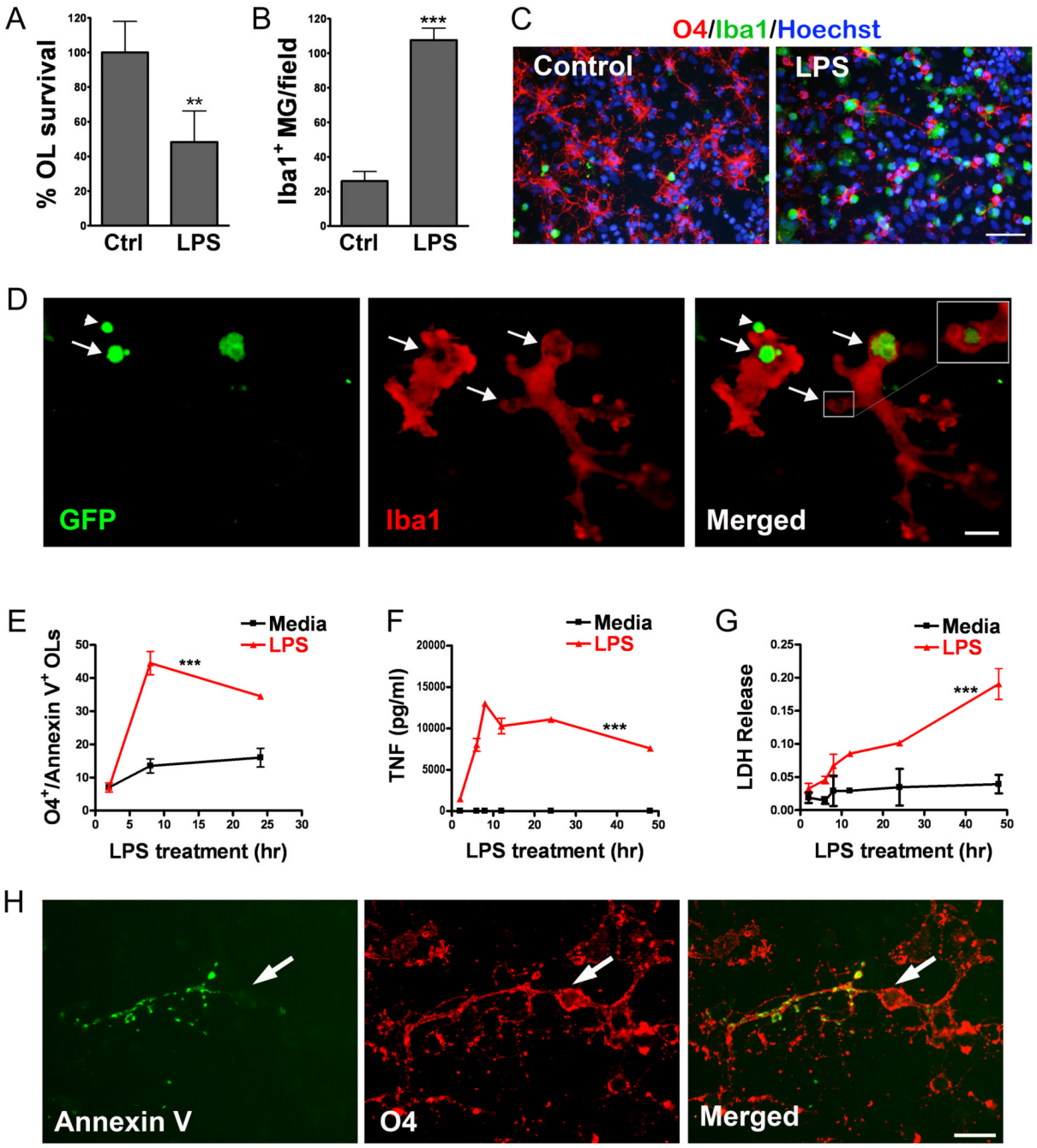

Fig. 1. LPS potently activates microglia and induces death of preOLs in mixed glial cultures

A–C: Exposure of rat mixed glia to LPS resulted in preOLs death and activation and proliferation of microglia. Mixed glial cultures were treated with or without LPS (1 μg/ml) for 48 hr and then double immunostained for OLs (O4, red) and microglia (Iba1, green). Nuclei of all cells were visualized with Hoechst 33258 (blue). Survival of preOLs was determined by counting $O4^+$ cells and proliferation of microglia was evaluated by counting $Iba1^+$ cells. Data represent at least three independent experiments. **, $p<0.01$; ***, $p<0.001$ when compared to controls. Scale bar, 50 μm. ***D:*** Representative photomicrographs showing LPS-activated microglia engulfing dying/dead preOLs. Purified preOLs were prelabeled with GFP via adenoviral infection and subsequently seeded into established mixed glial

cultures. The cells were then treated with LPS (0.1 μg/ml) for 24 hr and fixed and immunostained for Iba1 (red) and GFP (green). Microglia engulfing OL debris were frequently observed in LPS-treated but not control cultures. Arrows indicate formation of phagosomes encircling preOL corpses and arrowhead indicates a microglia cell in contact with a GFP^+ body. Insert is an enhanced and enlarged image of the boxed area showing a microglial process engulfing a GPF^+ cell corpse. Scale bar, 5 μm. ***E–F:*** Treatment of mixed glia with LPS increased the number of annexin-V^+ $O4^+$ cells in a time-dependent fashion that correlated with TNF secretion and preceded cell death . ***H:*** Representative photomicrograph of annexin-V (green) colocalizing with an $O4^+$ (red) OL. Scale bar, 10 μm.

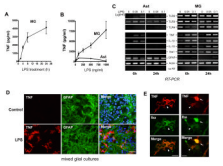

Fig. 2. Microglia but not astrocytes are the primary source for LPS-stimulated TNF production

A, B: Microglia (MG), but not astrocytes (Ast), responded robustly to LPS and secreted TNF in a dose-dependent manner. Enriched rat microglia or astrocytes were exposed to LPS (0.5 μg/ml) for indicated duration or to increasing concentrations of LPS (0.01–1 μg/ml) for 24 h. TNF was measured by ELISA. Data are representative of 2–4 independent experiments. ***C:*** RT-PCR analyses of TLRs and cytokine transcripts in LPS-treated cells. Highly purified microglia and astrocyte monocultures were subjected to LPS stimulation (0, 0.05 and 0.1 μg/ml) for 6 and 24 h and subjected to RT-PCR. ***D, E:*** Activated microglia, but not astrocytes, produced TNF in mixed glial cultures treated with LPS. Mixed glia were treated as indicated with LPS (0.1 μg/ml) for 8 and 24 h. TNF immunoreactivity was only detected in $Iba1^+$ cells in LPS-treated cultures at both time points. Arrow indicates amoeboid microglia and arrowheads activated microglia. Scale bars, 50 μm (***D***); 20 μm (***E***).

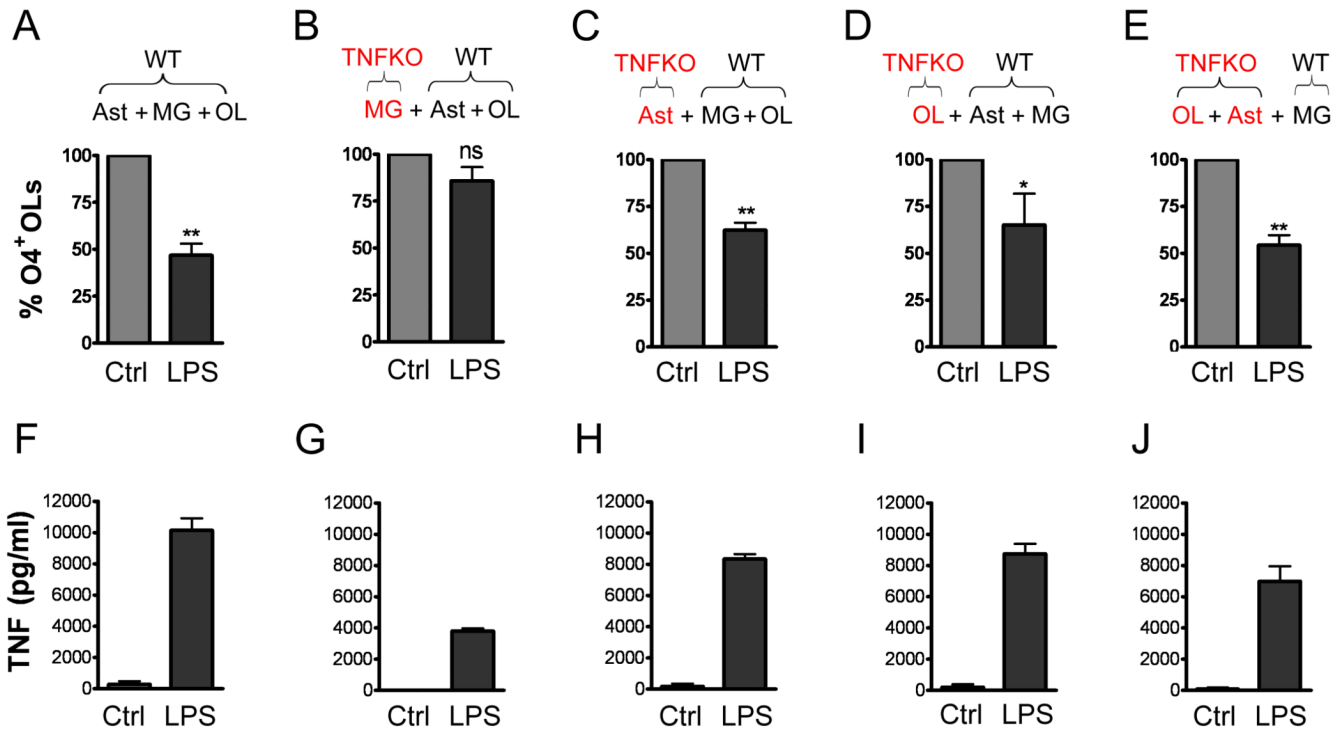

Fig. 3. TNF secreted from LPS-activated microglia is required for preOLs death in mixed glial cultures

A–E: Reconstituted mosaic mixed glial cultures demonstrated the requirement for microglial TNF in LPS-induced preOL death. Enriched microglia, astrocytes and preOLs were isolated from TNF WT and KO mice, and mixed in various combinations as indicated. Cells were treated with or without LPS (1 μg/ml) for 48 h. PreOLs survival was determined by counting $O4^+$ cells. Deletion of *tnf* gene in microglia (***B***), but not in astrocytes (***C***) or OLs (***D***), efficiently abrogated LPS-induced death of preOLs. Data represent mean ± SEM of 3–4 independent experiments. *, $p<0.01$; **$p<0.001$; ns, not significant when compare with corresponding controls without LPS treatment. ***F–J:*** Production of TNF in the above experiments was determined in parallel in culture media by ELISA.

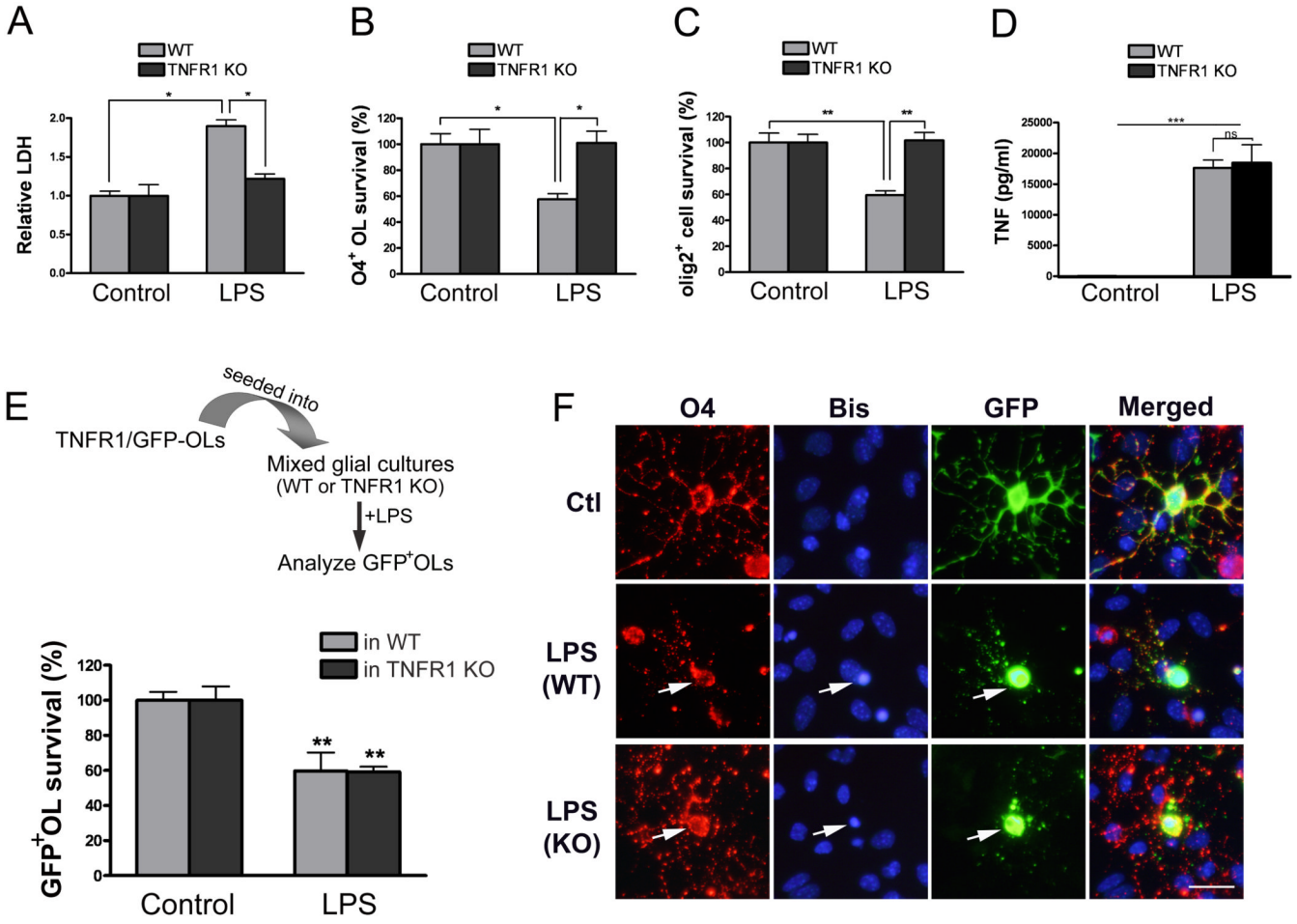

Fig. 4. Oligodendroglial TNFR1 is required for LPS-induced killing of preOLs in mixed glial cultures

A–D: TNFR1 is required for LPS toxicity in mixed glial cultures. Mixed glial cultures were prepared from WT and TNFR1 KO mice, and were treated with or without LPS (1 μg/ml) for 48 h. Cytotoxicity was evaluated by measuring released LDH in culture media (***A***). PreOLs were determined by counting $O4^+$ or OL lineage specific transcription factor $olig2^+$ cells (***B, C***). TNF was determined by ELISA (***D***). Data represent mean ± SEM of 3 independent experiments. *, $p<0.01$; **, $p<0.001$ compared with corresponding controls. ***E–F:*** Activation of TNFR1 on preOLs is essential for LPS toxicity. Mixed glial cultures were prepared from WT or TNFR1 KO. Enriched $GFP^+/TNFR1^+$ preOLs were prepared from GFP mice and were plated onto WT or TNFR1 KO mixed glial cultures. The cells were then stimulated with or without LPS (1μg/ml) for 48 h. Survival of GFP^+ OLs was determined by counting the number of GFP^+ OLs. Data demonstrate that while LPS toxicity to preOLs was completely abrogated in TNFR1 KO mixed glia cultures (***B, C***), exogenously added $GFP^+/TNFR1^+$ OLs were equally vulnerable to LPS in the WT or KO mixed glia (***E***). **, $p<0.001$ when compared with corresponding controls. ***F:*** Representative photomicrographs of GFP^+ OLs in WT and TNFR1KO mixed glial cultures treated with or without LPS. Arrows indicate degenerating GFP^+ OLs upon LPS treatment of the indicated mixed glial cultures. Scale bar, 20 μm.

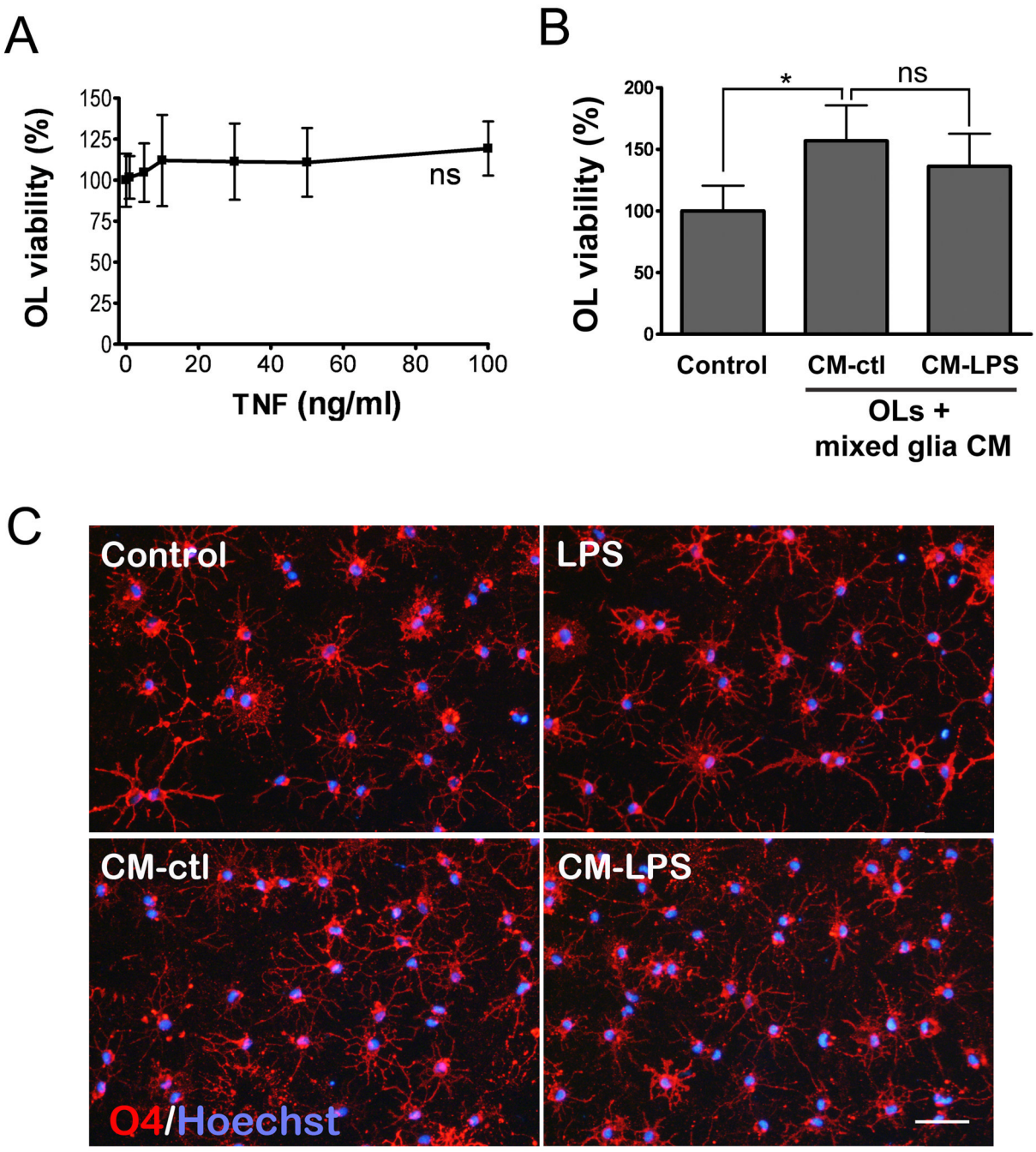

Fig. 5. TNF does not induce significant preOLs death in highly enriched preOLs

A: exogenous TNF did not affect OL viability. Highly enriched rat preOLs were exposed to increasing concentrations of TNF for 48 hr. Cell viability was evaluated by Alamar Blue viability assay and confirmed by visual inspection under phase contrast microscopy. Data represent at least 3–5 independent experiments. Ns, not significant. ***B:*** Conditioned media (CM) from control or LPS-treated mixed glial cultures were not toxic to pure preOLs. CM were obtained from mixed glia cultures treated with or without LPS (1 μg/ml) for 48 h and were added to pure preOLs for another 48 h. Cell viability was measured as the above. Results are a representative of at least 3 independent experiments. *, $p<0.05$; ns, not

significant. ***C:*** Representative photomicrographs of immunostained preOLs treated as indicated for 48h. Scale bar, 50μm.

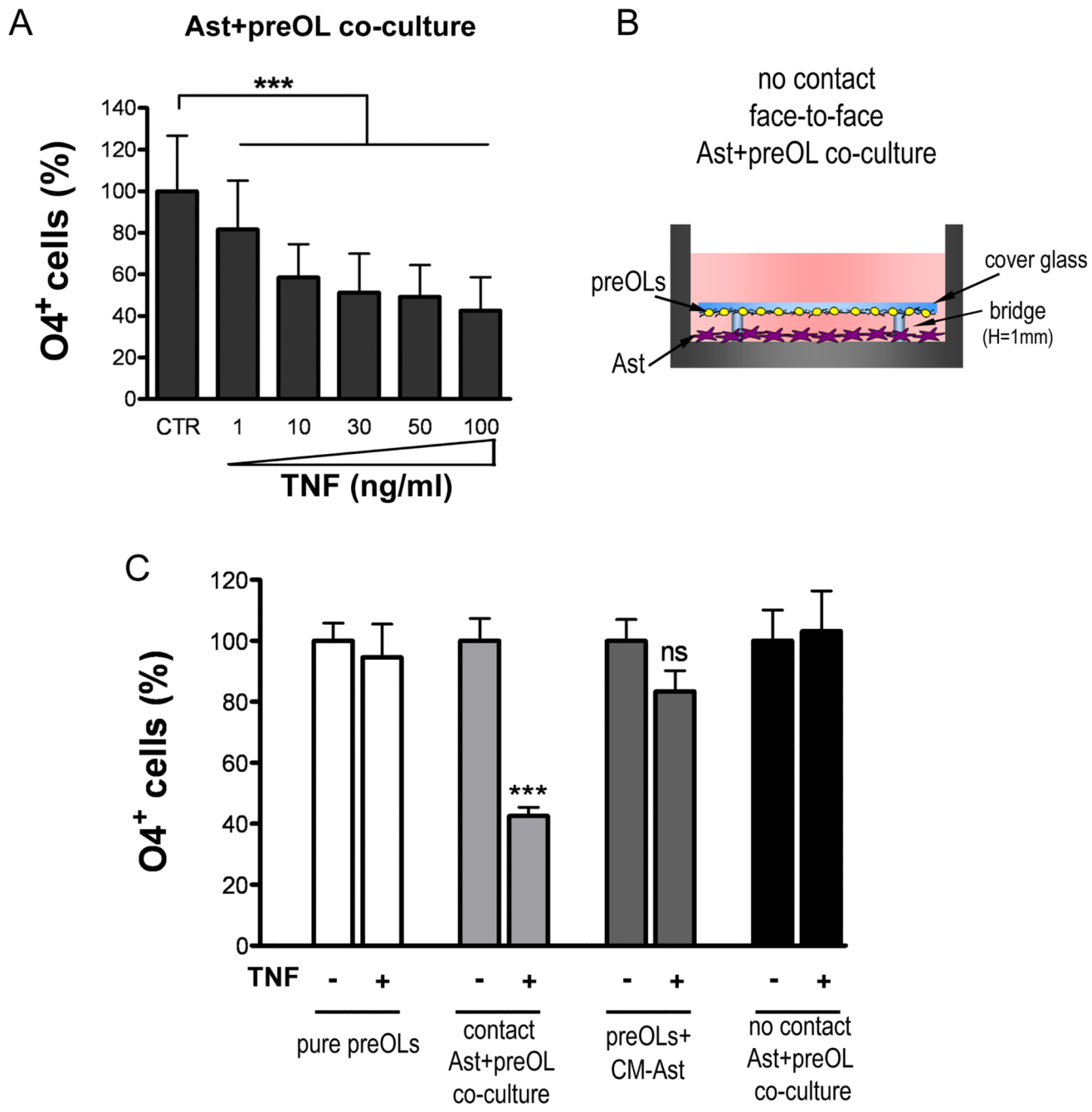

Fig. 6. Astrocytes promote TNF toxicity to preOLs in a contact-dependent manner

A, TNF was toxic to preOLs in astrocytes/preOLs co-cultures. Cells were isolated from neonatal rats and prepared as described. Astrocytes/preOL co-cultures were treated with increasing concentrations of TNF for 48 h. PreOL survival was determined by counting $O4^+$ cells. Data represent 3 independent experiments. ***, $p<0.001$. ***B:*** schematic illustration of "no contact" co-cultures of astrocytes and preOLs. PreOLs grown on coverslip were suspended in astrocyte cultures by four pre-mounted PDMS mini-bridges. The space between the two cell layers was 1mm. ***C:*** PreOLs were sensitive to TNF treatment only when they were in direct cell-cell contact with astrocytes. Specified cell cultures were treated with TNF (100 ng/ml) for 48 h. PreOL survival was evaluated by counting $O4^+$ cells.

Whereas TNF induced preOL cell death in contact co-cultures, it did not cause significant cell death in pure or no-contact co-cultures. Conditioned medium (CM-Ast) was obtained from astrocytes treated with or without TNF for 24h. Data represent three independent experiments. ***, $p<0.001$.

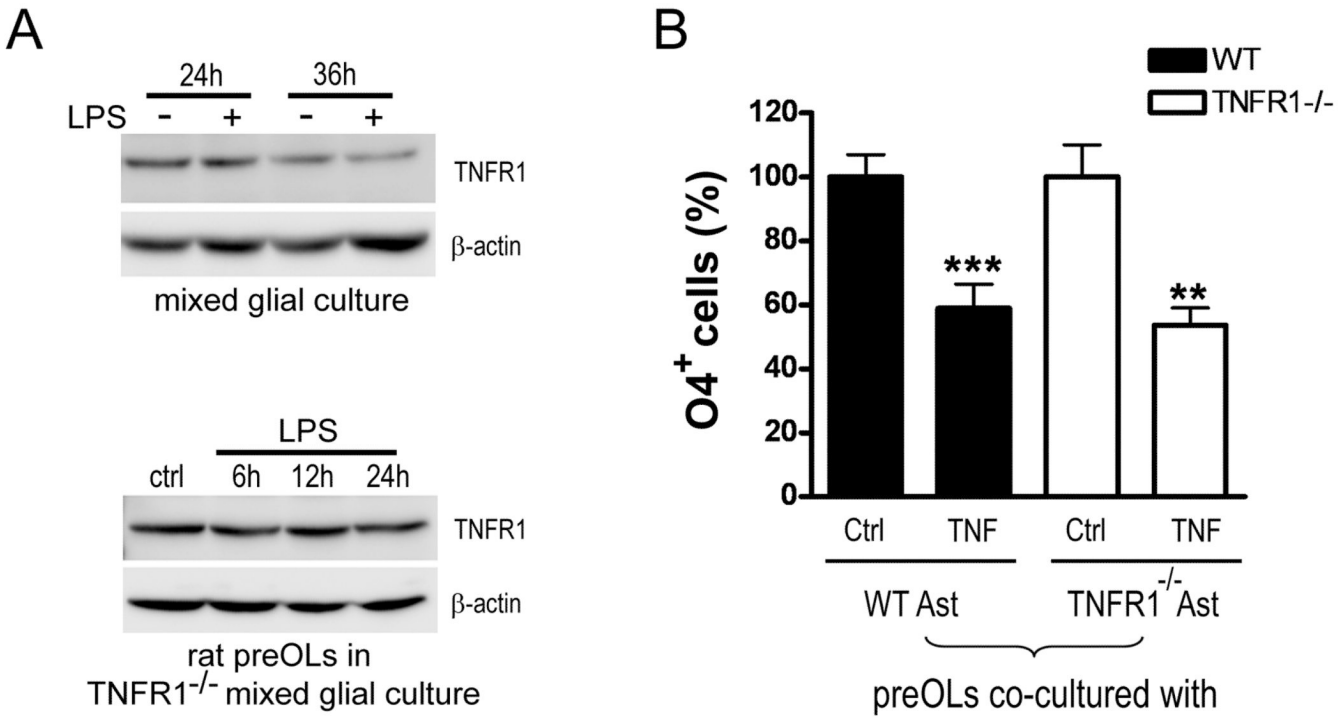

Fig. 7. Astrocytes do not increase TNFR1 expression in preOLs

A: Western blotting analyses on TNFR1 expression. *Top panel*, rat mixed glial cultures were treated as indicated and total cell lysates were subjected to western blot analysis. *Low panel*, since all glia express TNFR1, to specifically analyze oligodendroglial TNFR1 changes, rat preOLs were plated into established TNFR1$^{-/-}$ mixed glial cultures and treated with LPS. LPS treatment did not affect TNFR1 expression in preOLs even though rat preOLs were sensitive to LPS treatment under this condition. ***B:*** TNFR1-deficient astrocytes promote TNF toxicity to preOLs as efficiently as WT astrocytes. Rat preOLs were co-cultured with WT or TNFR1$^{-/-}$ astrocytes. Addition of TNF triggered the same level of preOL death in both co-cultures. **, $p<0.01$; ***, $p<0.001$ when compared with corresponding controls. Data represent three independent experiments.

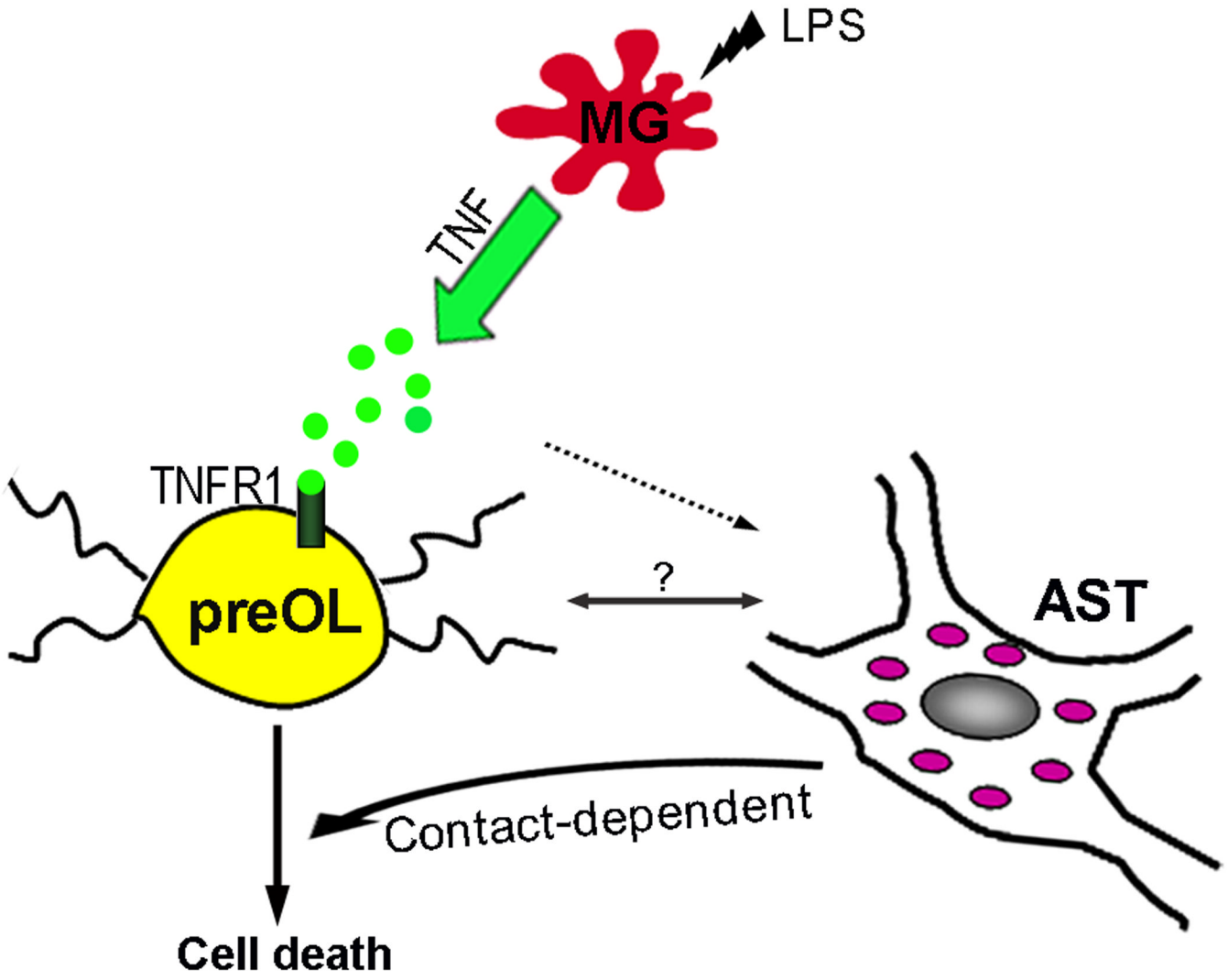

Fig. 8. Simplified schematic of how LPS triggers TNF-dependent death of preOLs

Upon LPS stimulation, microglia become activated and produce TNF. When preOLs are in contact with astrocytes, TNF triggers cell death via the TNFR1 on preOLs. TNF may also act on astrocytes, which in turn affect TNF signaling in preOLs through a cell-cell contact mechanism.