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Poly(ADP-Ribose) Polymerase-1 Promotes Microglial Activation, Proliferation, and Matrix Metalloproteinase-9-Mediated Neuron Death¹

Tiina M. Kauppinen and Raymond A. Swanson²

Activated microglia contribute to cell death in ischemic and neurodegenerative disorders of the CNS. Microglial activation is regulated in part by NF- κ B, and the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) enhances NF- κ B binding to DNA. In this study, the role of PARP-1 in microglia-mediated neurotoxicity was assessed using microglia from wild-type (wt) and PARP-1^{-/-} mice. Cultured microglia were incubated with TNF- α , a cytokine that is up-regulated in many neurological disorders. When stimulated with TNF- α , wt microglia proliferated, underwent morphological changes characteristic of activation, and killed neurons placed in coculture. The effects of TNF- α were markedly attenuated both in PARP-1^{-/-} microglia and in wt microglia treated with the PARP enzymatic inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2h)-isoquinolinone. These effects were also blocked by (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile, which inhibits translocation of NF- κ B to the nucleus. TNF- α also up-regulated microglial release of matrix metalloproteinase-9 (MMP-9), an enzyme with potential neurotoxic properties that is transcriptionally regulated by NF- κ B. This up-regulation was blocked in PARP-1^{-/-} microglia and in wt microglia by the PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2h)-isoquinolinone. Microglia from MMP-9^{-/-} mice were used to evaluate the contribution of MMP-9 to microglial neurotoxicity. MMP-9^{-/-} microglia treated with TNF- α showed substantially reduced neurotoxicity relative to the wt microglia. TNF- α -stimulated wt microglia treated with the MMP inhibitor ilomastat also showed reduced neurotoxicity. These findings suggest that PARP-1 activation is required for both TNF- α -induced microglial activation and the neurotoxicity resulting from TNF- α -induced MMP-9 release. *The Journal of Immunology*, 2005, 174: 2288–2296.

Microglial activation involves morphological changes, proliferation, and increased release of proinflammatory agents (1). These agents, which include cytokines, reactive oxygen species, and proteases, have been shown to exacerbate brain injury in stroke, multiple sclerosis, and other conditions in which inflammation contributes to CNS injury (2–4). Microglial activation is regulated in part by the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1)³ (5, 6). PARP-1 is one of several poly(ADP-ribose) polymerases that form ADP-ribose polymers at protein acceptor sites. PARP-1 accounts for >90% of total cell PARP activity, and it has been extensively characterized with respect to its functions in DNA surveillance and repair (7, 8). Widespread activation of PARP-1 triggers a cell death pathway involving NAD depletion and mitochondrial release of apoptosis-inducing factor (9, 10), and PARP inhibitors have been shown to markedly reduce brain injury in a variety of disease conditions (11, 12).

PARP-1 is also known to interact with transcription factors, including Oct-1, AP-1, SP-1, Stat-1, YY-1, and NF- κ B (13). With NF- κ B, PARP-1 acts as a coactivator by facilitating DNA binding and promoter activation (14, 15). PARP-1 interactions with NF- κ B have been shown to influence microglial migration and the release of NO and cytokines (5, 6, 13). These interactions suggest that the salutary effects of PARP-1 inhibition in many disease conditions may result not only from interruption of the PARP-1 cell death pathway, but also from blocking the microglial inflammatory response. Whether PARP-1 enzymatic activity is needed for its action as a coactivator of NF- κ B remains unsettled (6, 16).

Matrix metalloproteinases (MMPs) are among the factors released by microglia that contribute to neural injury. In particular, MMP-9 release and activation cause opening of the blood-brain barrier (17) and direct neurotoxicity (18). Microglia express and release MMP-2, -3, and -9 in vitro and in animal models of stroke (19, 20), but the factors regulating MMP release are not well established. The aim of the present study was to determine whether microglial neurotoxicity and MMP-9 release are regulated by PARP-1, and if so, whether PARP-1 enzymatic activity is required. Our findings indicate that microglia stimulated by TNF- α in vitro exhibit increased PARP-1 activity. Inhibition of PARP-1 activity prevented TNF- α -induced microglia proliferation, MMP-9 release, and microglia-induced neurotoxicity. The findings identify MMP-9 as a novel mediator of microglia-mediated neurotoxicity and establish PARP-1 activation as a key factor regulating microglial MMP-9 release.

Materials and Methods

Materials

Cell culture reagents were obtained from Mediatech, unless otherwise stated. Culture plates (24-well plates) and 75-cm² polystyrene culture

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³ Abbreviations used in this paper: PARP, poly(ADP-ribose) polymerase; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2h)-isoquinolinone; GFAP, glial fibrillary acidic protein; ILO, ilomastat; MAP, microtubule-associated protein; MMP, matrix metalloproteinase; PAR, poly(ADP-ribose); TIMP, tissue inhibitor of metalloproteinase; wt, wild type.

flasks were from Falcon/BD Biosciences. The 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1-(2h)-isoquinolinone (DPQ) and *N*-[(2R)-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (ilomastat (ILO)) were obtained from Chemicon International. The (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082) was obtained from Alexis Biochemicals. TNF- α and LPS (from *Escherichia coli*) were obtained from Sigma-Aldrich. Primary Abs used were: rat polyclonal anti-poly(ADP-ribose) (Trevigen), rat monoclonal anti-mouse F4/80 (Serotec), rat polyclonal anti-gial fibrillar acid protein (GFAP; Chemicon International), rabbit polyclonal anti-microtubule-associated protein 2 (MAP2; Chemicon International), and polyclonal rabbit anti-rat MMP-9 (Chemicon International). Secondary Abs used were: anti-rabbit and anti-rat IgG conjugated with Texas Red or Alexa Fluor 594 (Molecular Probes) and peroxidase-conjugated anti-rabbit IgG (Vector Laboratories).

Neuron cultures

The cell culture protocols used in this study were approved by the San Francisco Veterans Affairs Medical Center animal studies committee and follow the National Institutes of Health guidelines for humane care of animals. Neuron cultures were prepared from wild-type (wt) Swiss-Webster mice (Simonsen), as described previously (21). In brief, cortices were removed from embryonic day 16 wt mice, dissociated into MEM containing 10 mM glucose, and supplemented with 10% FBS (HyClone) and 2 mM glutamine, and plated on poly(D-lysine)-coated 24-well plates at a density of 7×10^5 cells/well. After 2 days in vitro, 22 μ M cytosine β -D-arabinofuranoside (Sigma-Aldrich) was added to inhibit the growth of non-neuron cells. After 24 h, the medium was removed and replaced with a 1:1 mixture of glial condition medium and MEM. This medium was 50% exchanged with fresh medium after 5 days. The cultures contained ~97% neurons and 3% astrocytes, as assessed by immunostaining for the neuron marker MAP2 and the astrocyte marker GFAP.

Microglia and microglia-neuron cocultures

Microglial cultures were prepared from wt, PARP-1^{-/-}, and MMP-9^{-/-} mice. Wt mice were Swiss-Webster strain. The PARP-1^{-/-} mice were the 29S-Adprt1^{tm1Zq^w} strain, originally developed by Wang et al. (22), and obtained from The Jackson Laboratory. The MMP-9^{-/-} mice were originally developed by Vu et al. (23) and kindly provided by Dr. L. Noble (University of California, San Francisco, CA) (24). These mice were bred on the FVBn background. Separate microglia preparations were generated from each of the 1-day-old progeny of heterozygote crosses, with genotyping subsequently performed on the tail samples. The wt and targeted alleles were separately amplified with specific primer combinations for the MMP-9 wt allele (5'-GCATACTTGTTACCGCTATGG-3' and 5'-TAACCGGAGGTGCAAACTGG-3') and the MMP-9-NEO-targeted allele (5'-CTCAGAAGAAGCTCGTCAAGA-3' and 5'-GGATTGCAC CAGTTCTCC-3').

Microglial cultures were prepared from mixed glial cultures, as described previously (25), with minor modifications. In brief, cortices were dissected from 1-day-old mice. Cells were dissociated by mincing, followed by incubation in papain and DNase for 10 min at 37°C. After centrifugation for 5 min at 500 \times g, the cells were resuspended and triturated with a fire-polished Pasteur pipette into MEM containing 5 mM glucose and supplemented with 10% FBS (HyClone), 2 mM glutamine, 100 nM sodium selenate, and 200 nM α -tocopherol. Cells were plated on 24-well plates at a density of 2×10^5 cells/well or 75-cm² flasks at a density of 5×10^6 cells per flask and maintained in a 37°C, 5% CO₂ incubator. The medium was changed at 3 days in vitro and once per week thereafter. This procedure results in cultures consisting of astrocytes and microglial cells. After 2 wk in vitro, microglia were harvested by mildly shaking the cultures and collecting the floating cells. These cells were replated at a density of 5×10^5 cells/well in 24-well plates for pure microglial cultures, or at the density of 5×10^4 cells/well on top of 6-day in vitro neuron cultures in 24-well plates for microglia-neuron cocultures. The purity of the replated microglial cultures was >99%, and the microglia-neuron cocultures contained ~7% microglia, 90% neurons, and 3% astrocytes.

Immunostaining

To determine the proportions of different cell types present, representative cultures were taken at the time experiments were performed and fixed with 1:1 methanol:acetone at 4°C for 10 min. Cells were permeabilized, and nonspecific protein binding was blocked with 30-min incubations in blocking buffer (1% BSA and 0.3% Triton X-100 in 1 mM PBS). The cultures were incubated at 4°C for 24 h with primary Abs (diluted in blocking buffer) specific for neurons (rabbit anti-MAP2, 1/1000 dilution), astrocytes (rabbit anti-GFAP, 1/1000 dilution), or microglia (rat anti-mouse F4/80, 1/100 dilution). After washing in 0.3% Triton X-100/1 mM PBS, the cul-

tures were incubated for 2 h with rabbit or rat anti-IgG conjugated with Texas Red or Alexa Fluor 594 diluted 1/300 in blocking buffer. The number of immunopositive and unstained cells was counted in five arbitrary areas of three to four wells stained with each Ab to provide an estimate of the relative proportions of neurons, astrocytes, and microglia in the cultures.

Immunostaining for poly(ADP-ribose) (PAR), the enzymatic product of PARP-1, was used to provide a qualitative measure of PARP-1 activity in microglia. Microglial cultures were fixed and immunostaining was performed, as described above, using rabbit polyclonal anti-PAR Ab (1/1000 dilution) as a primary Ab and anti-rabbit IgG conjugated with Alexa Fluor 594 as the secondary Ab. Secondary Abs used alone without prior incubation with primary Abs showed no staining.

Experimental procedures

Microglial cultures were used at day 2–3 after replating (in vitro day 16–17). The cultures were incubated with 10–100 ng/ml TNF- α or 50 ng/ml LPS. In some experiments, TNF- α or LPS was coincubated with a PARP inhibitor, DPQ (25 μ M), or with an inhibitor of NF- κ B nuclear translocation, BAY 11-7082 (BAY, 5 μ M). All compounds were dissolved in the MEM that was used alone as a control. The cultures were incubated with test compounds for 24 h in a 37°C, 5% CO₂ incubator. None of the experimental conditions reduced viability of microglial cells, as analyzed by the lactate dehydrogenase assay (26) and propidium iodide staining (27) at 24 and 48 h (data not shown).

Neuron cultures and microglia-neuron cocultures were used at neuron day 7 in vitro. Cultures were exposed to 10–100 ng/ml TNF- α alone or combined with 25 μ M DPQ, 5 μ M BAY 11-7082, or a broad spectrum MMP inhibitor, ILO (10 μ M) for 36–42 h in a 37°C, 5% CO₂ incubator. ILO is also known as GM6001 and galardin (28–30). All compounds were dissolved in the mixture of glial condition medium and MEM (1:4) that was used alone as a control condition.

Immunoblotting

Aliquots of cell culture medium were concentrated by lyophilizing and redissolving into 22 times lower volume. The samples were mixed with loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 4% SDS, 0.01% bromophenol blue, and 10% 2-ME, and incubated for 15 min at 65°C. The samples were then loaded onto 12% SDS-PAGE gels, with the loading volumes adjusted in proportion to protein content in the culture well from which sample was taken. Proteins were separated by electrophoresis and transferred onto Hybond membrane (Amersham Biosciences). After a 1-h incubation in blocking buffer (5% fat-free milk solution in 1 mM PBS/0.1% Tween), membranes were incubated overnight at 4°C with a 1/2000 dilution of rabbit anti-rat MMP-9 Ab in blocking buffer, followed by a 2-h incubation with peroxidase-conjugated anti-rabbit IgG (1/5000 dilution). Protein bands were visualized using the ECL WB Detection kit (Amersham Biosciences) and Kodak X-OMAT AR film.

Microglial proliferation and neuron survival

TNF- α - and LPS-induced microglial proliferation and neuron survival were quantified by cell counting after the designated treatments. Cell counting was done in blinded fashion on five randomly selected $\times 40$ microscopic fields in each well. Values were normalized to counts in control (medium-only) wells from the same 24-well plate.

MMP activity

Zymography assessment of MMP-2 and MMP-9 activity was performed, as described previously (31, 32), with minor modifications. Cell-free aliquots of culture medium were mixed with loading buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, and 0.01% bromophenol blue, and loaded onto 10% SDS-PAGE gels containing 1 mg/ml gelatin. The loading volume of each sample was adjusted in proportion to the protein content in the culture well from which sample was taken, and ranged from 8 to 45 μ l. Samples were electrophoresed at 100 V for 95 min on a minigel apparatus. The gel was rinsed with 2.5% Triton X-100 to remove SDS and restore enzyme activity, then incubated 16–20 h at 37°C in substrate buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 0.02% dodecylpoly(ethyleneglycoether)). The gels were stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol/7% acetic acid for 1 h, then destained with 40% methanol/7% acetic acid until bands were apparent. MMP-9 activity was assessed by comparing the integrated ODs of the MMP-9 bands (both pro and active forms) on each gel.

A biochemical assay using the colorimetric MMP substrate, thiopeptolide, was used to provide a more quantitative assessment of MMP activity. The assay was performed with an assay kit (BIOMOL), according to the

manufacturer's instructions. In brief, 50- μ l aliquots of medium from microglial cultures were mixed with 50 μ l of assay buffer containing 50 mM HEPES, pH 7.5, 10 mM CaCl_2 , 0.05% Brij-35, 100 μ M thiopeptolide, and 1 mM Ellmans'reagent, on a 96-well plate. These conditions optimize MMP-9 activity, but do not exclude contribution from other MMPs. The colorimetric reaction was followed by measuring absorbance at 412 nm over a 20-min interval at 37°C. The time interval showing linear absorbance changes was used for quantitation. Results were expressed as change of absorbance in arbitrary units (Vmax) per μ g of protein in the source culture wells.

Statistics

Each data point is calculated as the mean value from three or four independent experiments, with each experiment using three to four culture wells per condition. Results are presented as means \pm SE. Statistical significance was evaluated by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test. Values of $p < 0.05$ were considered significant.

Results

PARP-1 activation facilitates microglial activation

TNF- α is up-regulated in several brain disorders, such as stroke, multiple sclerosis, and Alzheimer's disease, in which CNS inflammation may contribute to pathology (33–38). Wt microglia and PARP-1^{-/-} microglia were incubated with 10–100 ng/ml TNF- α for 24 h to assess the effects of PARP-1 gene expression on TNF- α -induced microglial activation. Incubation of wt microglia with 10 ng/ml TNF- α for 24 h led to cell body enlargement and reduced peripheral branching, resulting in the ameboid morphology characteristic of activated microglia (Fig. 1a). TNF- α also dose dependently increased proliferation of wt microglia (Fig. 1b). In contrast, microglia cultured from PARP-1^{-/-} mice showed negligible morphological or proliferative response to TNF- α (Fig. 1). Wt microglia treated with the PARP inhibitor DPQ (25 μ M) similarly showed no increase in proliferation and only minimal changes in morphology at TNF- α concentrations that were 10-fold higher (100 ng/ml) than those that induced significant changes in wt microglia (10 ng/ml) (Fig. 1 and data not shown). Although DPQ inhibits PARP species other than PARP-1 (39), the very similar results of PARP-1 gene disruption and DPQ suggest that the morphological and proliferative effects of TNF- α require PARP-1 enzymatic activity. To confirm that TNF- α induces PARP activation in microglia, we studied formation of PAR by immunocytochemistry. PAR formation was markedly increased in wt microglia incubated with TNF- α , and this increase was blocked by coincubation with the PARP inhibitor DPQ (Fig. 2).

A subset of these studies was performed using 50 ng/ml LPS in place of TNF- α to stimulate microglia. The morphological changes induced by LPS were somewhat different from those induced by TNF- α (Fig. 1), but as with TNF- α these changes were suppressed in PARP-1^{-/-} microglia and in wt microglia coincubated with DPQ (Fig. 1a). Unlike TNF- α , LPS did not induce microglial proliferation, consistent with prior reports (40, 41). Because TNF- α is an endogenous cytokine relevant to several neurodegenerative diseases and LPS is more relevant to infectious diseases, all subsequent experiments were performed using only TNF- α .

TNF- α -induced release of MMP-9 from microglia requires PARP-1 activation

Gelatin zymography was used to evaluate MMP-2 and MMP-9 activity in medium collected from the microglia cultures. As shown in Fig. 3a, medium from unstimulated wt microglia generated a band on zymography corresponding to the low activity, 92-kDa proform of MMP-9. Medium collected from TNF- α -stimulated wt microglia showed the additional presence of the 88-kDa, activated MMP-9 species (Fig. 3a). Western blots showed that this increase in activity was associated with a large increase in MMP-9

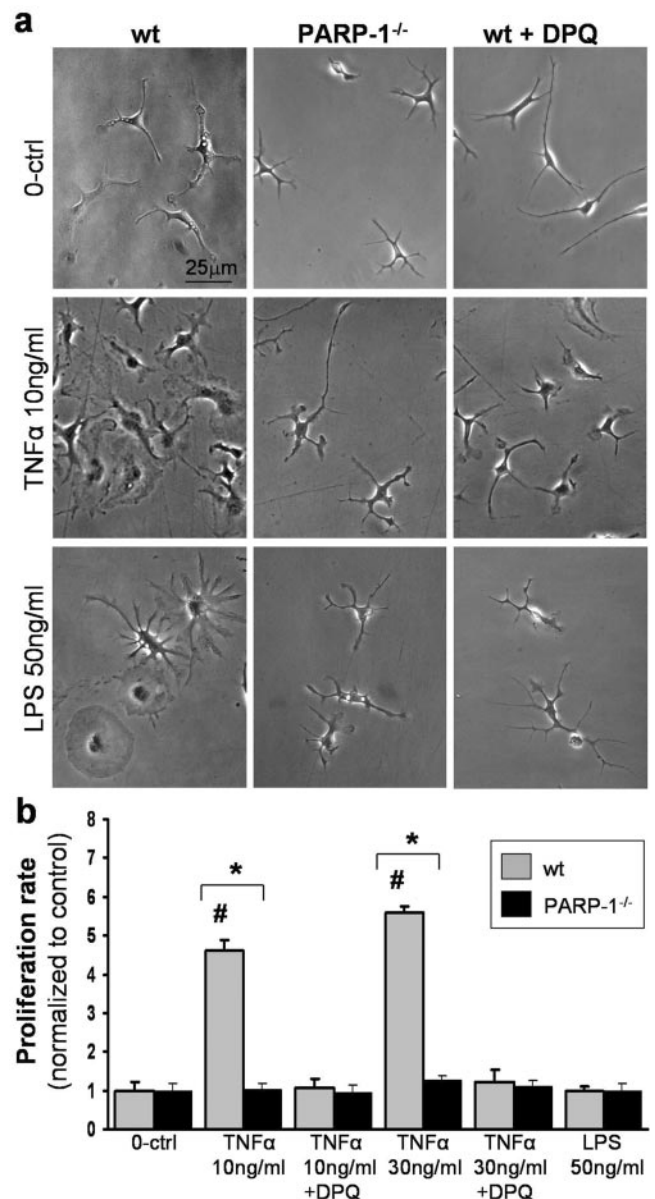


FIGURE 1. Microglia activation is regulated by PARP-1. *a*, Representative phase contrast photomicrographs show morphological changes in microglia after 24-h incubations with TNF- α (10 ng/ml) or LPS (50 ng/ml). The retraction of processes and enlargement of cell soma induced by these agents were blocked in PARP^{-/-} microglia and in wt microglia treated with the PARP inhibitor DPQ (25 μ M). *b*, Microglia proliferation was induced by TNF- α , but not by LPS. The TNF- α -induced proliferation was blocked in PARP^{-/-} microglia and in wt microglia treated with the PARP inhibitor DPQ (25 μ M). The basal (control) proliferation rate was 10 \pm 3% over 24 h in both wt and PARP-1^{-/-} microglia. Data are combined from three independent experiments ($n = 3$), each with three to four culture wells per treatment condition. #, $p < 0.001$ compared with control (0-ctrl). *, $p < 0.001$ wt vs PARP-1^{-/-}.

protein in the culture medium (Fig. 3b). An additional activity band corresponding to MMP-2 was evident at roughly equal intensity in zymographs prepared from both untreated and TNF- α -stimulated wt microglia. Whether this MMP-2 band is the 72-kDa proform or the 68-kDa active form could not be determined. Compared with wt microglia, medium from PARP-1^{-/-} microglia showed lower basal activity of both MMP-2 and MMP-9. The PARP-1^{-/-} microglia also failed to generate the 88-kDa activated MMP-9 species in response to TNF- α (Fig. 3a).

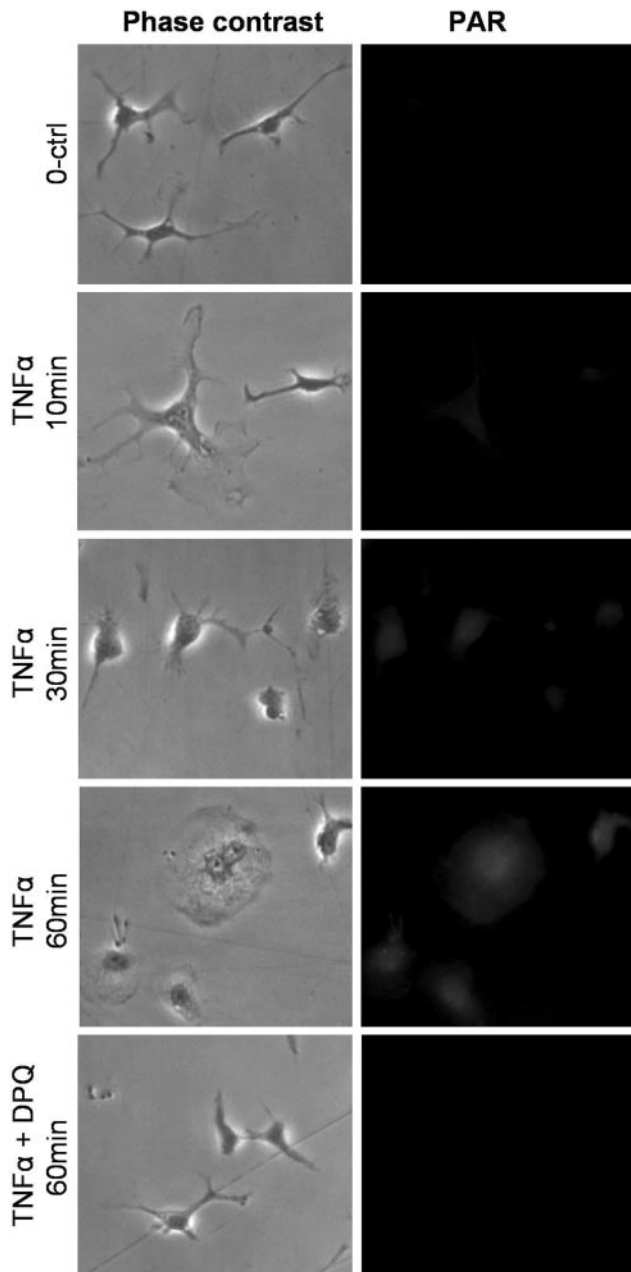


FIGURE 2. PAR activation in TNF- α -stimulated wt microglia. Representative phase contrast photomicrographs and poly(ADP-ribose) immunostaining show increasing PAR formation in wt microglia during 60-min incubation with TNF- α (15 ng/ml). Morphological changes are also apparent. TNF- α -induced PAR formation is reduced to basal levels by cotreatment with the PARP inhibitor, DPQ (25 μ M). Photographs are representative of two studies with similar results.

Because TNF- α stimulation failed to increase MMP-9 release from PARP-1^{-/-} microglial, we used the PARP inhibitor DPQ to determine whether PARP-1 enzymatic activity is essential for the MMP-9 response. As shown by Western blotting (Fig. 3*b*), DPQ prevented the TNF- α -induced release of MMP-9 from wt microglia. In agreement with this result, DPQ also reduced MMP-9 activity in the culture medium of TNF- α -treated wt microglia. This effect was evident in both zymography (Fig. 3, *c* and *d*) and biochemical assays (Fig. 3*e*). These approaches complement one another because zymography can distinguish between specific MMP family members, but may not be truly quantitative, whereas the biochemical assay provides reliable quantitation of the MMP ac-

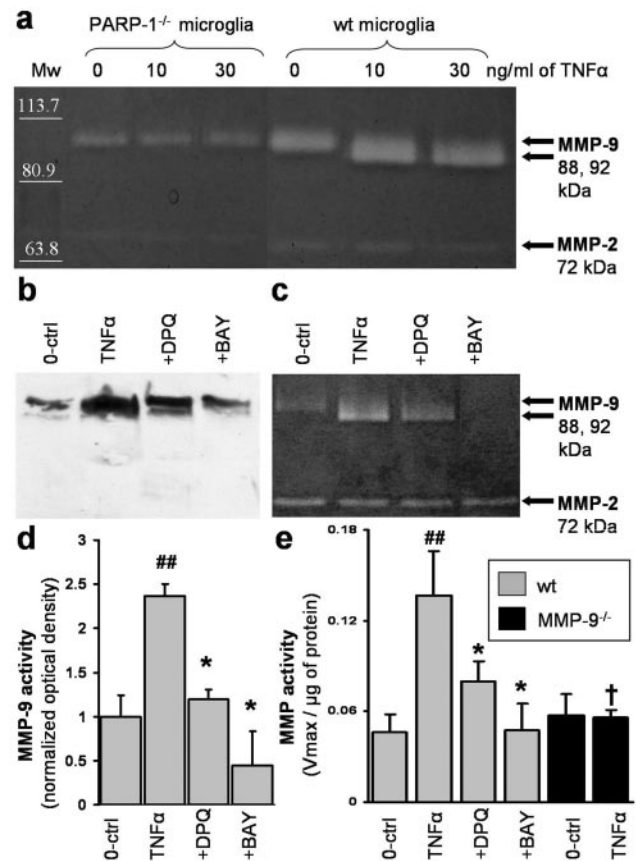


FIGURE 3. Microglial MMP activity in wt and PARP-1^{-/-} microglia. *a*, Zymograph showing MMP-2 and MMP-9 activity in medium from microglia incubated with TNF- α for 24 h at the designated concentrations. TNF- α induced cleavage of the inactive MMP-9 proform (92 kDa) to the active form (88 kDa) in wt microglia, but not in PARP-1^{-/-} microglia. MMP-2 release was greater in wt than in PARP-1^{-/-} cultures, but was unaffected in either cell type by TNF- α . *b*, Immunoblot showing increased MMP-9 protein levels in the medium of TNF- α -stimulated wt microglia. This increase was attenuated by the PARP inhibitor DPQ (25 μ M) and the NF- κ B inhibitor BAY 11-7082 (BAY 5 μ M). *c*, Zymography showing the inhibitory effects of the PARP-1 inhibitor (DPQ 25 μ M) and the NF- κ B inhibitor (BAY 5 μ M) on MMP-9 activity in medium from TNF- α -stimulated microglia. *d*, Quantitative assessment of MMP-9 zymography. *e*, Quantitative assessment of MMP activity as measured by colorimetric substrate assay. The lack of an increase in MMP-9^{-/-} cultures confirms the specificity of the assay conditions. Data are pooled from three independent experiments ($n = 3$). ##, $p < 0.001$ compared with control (0-ctrl). *, $p < 0.01$ compared with TNF- α . †, $p < 0.005$ MMP-9^{-/-} microglia compared with microglia from wt littermate controls.

tivity, but lacks absolute specificity for MMP-9. Together, these results indicate that the TNF- α -induced increase in extracellular MMP-9 activity is blocked by the PARP inhibitor DPQ. As a control, these studies were also performed using microglia from MMP-9^{-/-} mice to establish the specificity of the assay for MMP-9 activity. Microglia from MMP-9^{-/-} mice were prepared and treated in parallel with littermate, wt microglia. TNF- α induced no detectable increase in MMP activity in medium from the MMP-9^{-/-} cells, confirming that MMP-9 is the dominant species measured under these assay conditions (Fig. 3*e*).

PARP-1 activation has previously been shown to facilitate DNA binding of NF- κ B to its gene promoter recognition sites in rat microglial cultures (6). Because MMP-9 expression is regulated by NF- κ B (42), we also examined whether MMP-9 secretion would

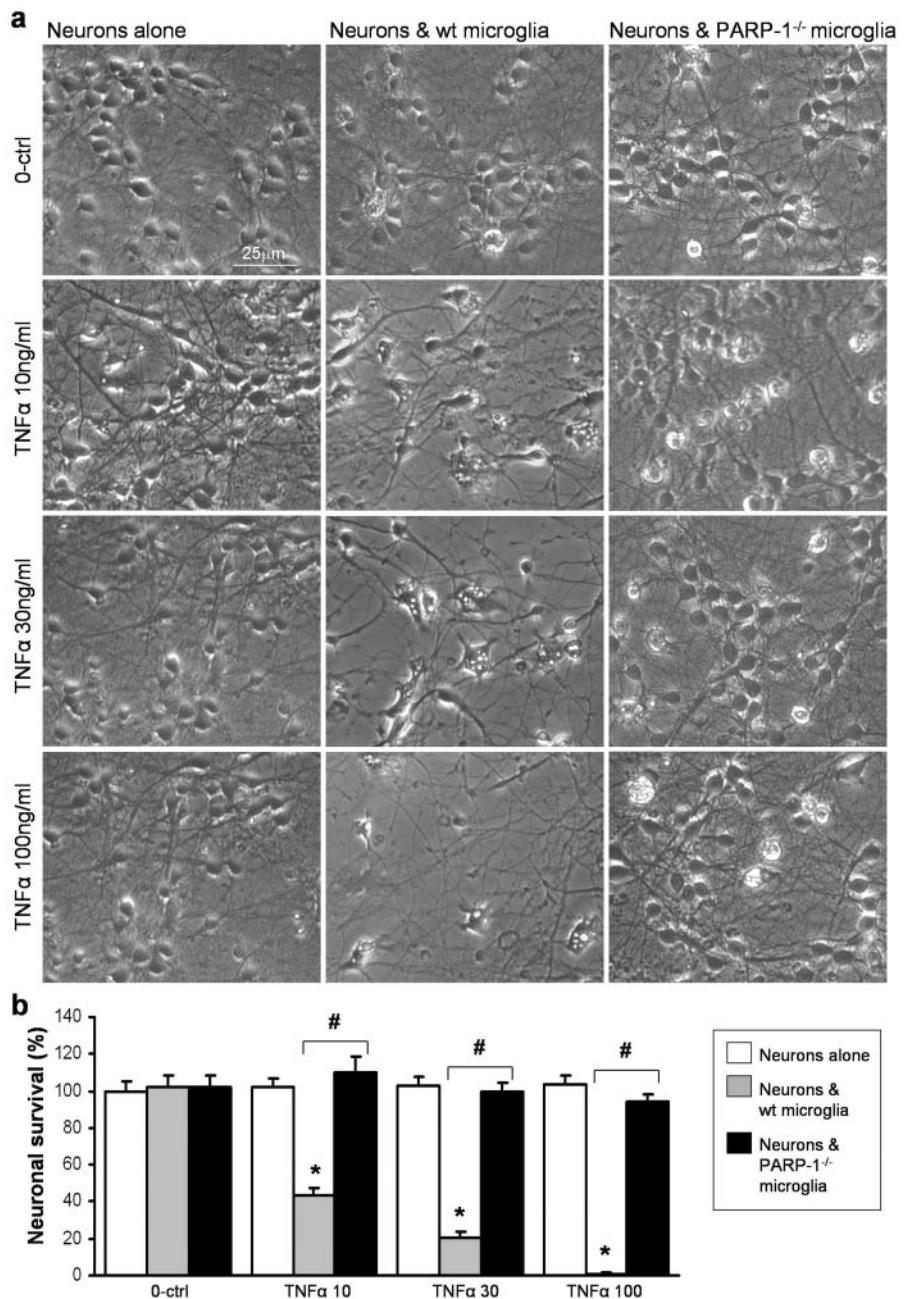


FIGURE 4. PARP-1 inhibition prevents microglial neurotoxicity. *a*, Representative microphotographs from wt neurons cultured alone, with wt microglia, or with PARP-1^{-/-} microglia exposed to 0–100 ng/ml TNF- α for ~40 h. TNF- α had no detectable effect in neuron viability in the absence of microglia or in neurons cultured with PARP-1^{-/-} microglia, but caused a dose-dependent neurotoxicity in the presence of wt microglia. *b*, Quantitative assessment of neuron survival under the same conditions as shown in *a*. Results are pooled from four independent experiments ($n = 4$), each with three to four culture wells per condition. *, $p < 0.001$ compared with neurons alone. #, $p < 0.001$ wt vs PARP-1^{-/-} microglia.

be down-regulated by BAY 11-7082, which inhibits I- κ B phosphorylation and thereby inhibits translocation of NF- κ B to the nucleus. BAY 11-7082 (BAY, 5 μ M) completely prevented the increase in MMP-9 secretion induced by TNF- α , but had no effect on MMP-2 (Fig. 3, *b–d*), consistent with the lack of an NF- κ B binding site in the MMP-2 promoter (42). As with the PARP inhibitor DPQ, the effects of BAY 11-7082 were quite similar in the zymograph and biochemical activity assays. Also like DPQ, BAY 11-7082 prevented TNF- α -induced microglial proliferation and morphological changes. Cells treated with TNF- α plus BAY 11-7082 showed a 1.14 ± 0.32 -fold increase in cell number over 24 h, compared with a 4.6 ± 0.27 -fold increase in cultures treated with TNF- α alone ($p < 0.01$).

Microglial neurotoxicity is mediated by MMP-9 and regulated by PARP-1

Microglia-neuron cocultures were prepared to determine whether PARP-1-dependent MMP-9 release from microglia can contribute

to neuron death. The presence of astrocytes in these cultures was minimized to avoid potential complicating effects of TNF- α on astrocytes, because TNF- α can induce astrocyte glutamate release and excitotoxic neuron death (43). Under basal conditions, there was no difference in the survival of neurons cultured with wt microglia, PARP-1^{-/-} microglia, or no microglia over the 48-h observation period. However, significant differences in microglial behavior and neurotoxicity were observed when the cultures were incubated with TNF- α . Cocultures containing wt microglia showed increasing neuron death with increasing TNF- α concentrations over the range of 10–100 μ g/ml, whereas neurons cocultured with PARP-1^{-/-} microglia showed no increase in neuron death (Fig. 4). TNF- α stimulation in neuron cultures devoid of microglia similarly had no effect on neuron viability (Fig. 4). Neuron death was also prevented in wt microglia-neuron cocultures in which DPQ was added with TNF- α (Fig. 5), although the interpretation of this result is complicated by the fact that neurons were also exposed to DPQ, which could affect neuron survival independent of its effects on microglia (44, 45).

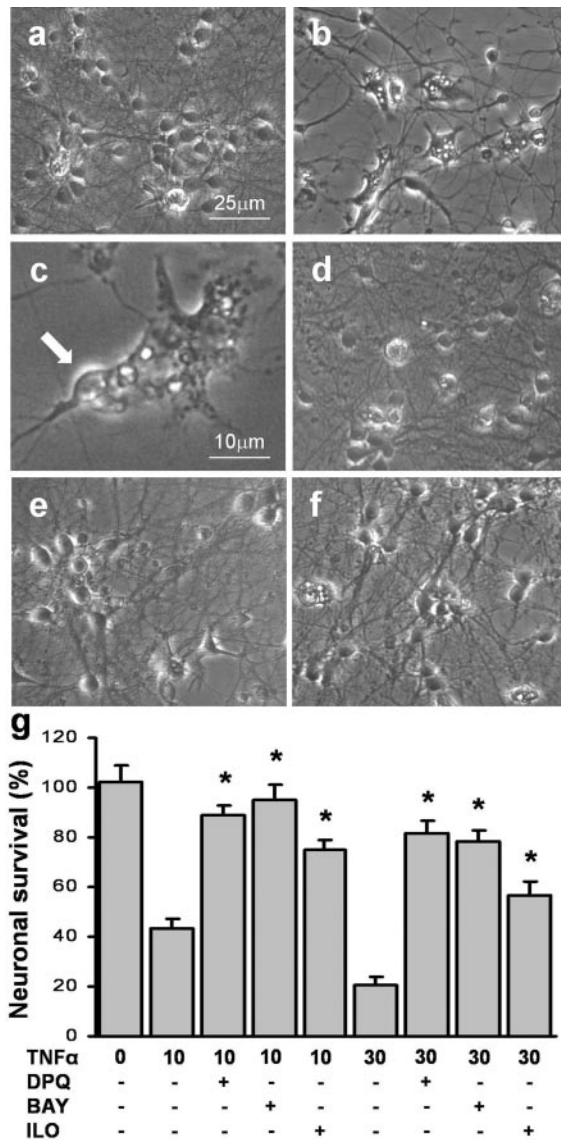


FIGURE 5. Inhibition of PARP activity, NF- κ B nuclear translocation, and MMP activity each block TNF- α -induced microglial neurotoxicity. *a-f*, Photomicrographs from cocultures of wt neurons and wt microglia. Cultures not stimulated with TNF- α show normal microglial and neuron morphology (*a*), whereas cultures stimulated with 30 ng/ml TNF- α (*b* and *c*) show activated microglia and degenerating neurons. In *c*, the arrow points to a neuron cell body undergoing phagocytosis by a microglial cell approaching from right upper corner. TNF- α -induced microglial neurotoxicity was significantly reduced in the presence of: *d*, the PARP inhibitor DPQ (25 μ M); *e*, the inhibitor of NF- κ B, BAY 11-7082 (BAY 5 μ M); and *f*, the MMP inhibitor ILO (10 μ g/ml). *g*, Quantitative assessment of neuron survival in TNF- α -treated neuron-microglia cocultures. Results are pooled from three independent experiments ($n = 3$), each with three to four wells per condition. *, $p < 0.001$ compared with TNF- α alone at the designated concentration.

Because TNF- α induced microglial activation and MMP-9 release, and because MMP-9 can cause neuron death (18, 20), we also used the microglia-neuron coculture system to determine whether MMP-9 is a significant neurotoxic factor released by wt microglia. Cocultures were prepared with neurons from wt mice and microglia from either MMP-9 $^{-/-}$ mice or littermate wt mice and subsequently incubated with TNF- α . As shown in Fig. 6, neuron death was substantially reduced in the cocultures prepared with MMP-9 $^{-/-}$ microglia. MMP-9 $^{-/-}$ microglia responded to TNF- α

with morphological changes and increased proliferation in a manner indistinguishable from wt microglial, supporting a direct effect of MMP-9 release on neuron viability. In addition, neuronal death was similarly reduced in wt microglia cocultures treated with the MMP inhibitor ILO (Fig. 5). Neuron death was also reduced by BAY 11-7082 (Fig. 5), further supporting a link between PARP-1 activation, NF- κ B nuclear translocation, and MMP-9 release.

Discussion

These results suggest that microglia become activated and release MMP-9 in response to TNF- α , and that these responses to TNF- α require PARP-1 enzymatic activity. Genetic and pharmacological inhibition of microglial PARP-1 prevented TNF- α -induced microglia activation, microglial release of MMP-9, and microglia-induced neuron death. The microglia-induced neuron death was also reduced by genetic and pharmacological inhibition of MMP-9. These findings identify MMP-9 as a mediator of microglia-mediated neurotoxicity and establish PARP-1 enzymatic function as an important factor regulating microglial MMP-9 release.

TNF- α induces microglial PARP-1 activation

Immunostaining of microglia after TNF- α incubation showed a rapid, massive up-regulation of the PARP-1 enzymatic product PAR in the cell nuclei, which was blocked by coincubation with the PARP inhibitor DPQ. Additional evidence for TNF- α -induced activation of PARP, and activation of PARP-1 in particular, was provided by the abrogation of microglial morphology changes, proliferation, and MMP-9 release in PARP-1 $^{-/-}$ microglia. The possibility that developmental or genetic background differences other than PARP-1 gene disruption could account for these differences can be excluded by the observation that nearly identical results were obtained using the pharmacological PARP inhibitor, DPQ, in wt microglia. DPQ is a highly potent PARP inhibitor that has no detectable antioxidant properties (39, 46).

The mechanism by which TNF- α leads to PARP-1 activation is not established. PARP-1 is normally bound to nuclear DNA and becomes activated by DNA strand breaks or kinks (8). One possible mechanism for TNF- α -induced activation is cytokine-induced generation of reactive oxygen species in microglia, which in turn generate DNA strand breaks and PARP-1 activation (47). An alternative possibility is that PARP-1 is activated by mechanisms independent of DNA damage. PARP-1 activity has also been found to be influenced by Ca $^{2+}$, Mg $^{2+}$, phospholipase C, and polyamines (48, 49). Of note, there was no evidence of microglial death induced by TNF- α or LPS in these studies, with or without PARP inhibition. This is concordant with prior findings with rat microglia (6) and macrophages (13), but differs from results reported with the BV-2 microglial cell line (47). It is possible that this difference reflects the high basal proliferation rate of the microglial cell line and consequent susceptibility to apoptotic cell death.

PARP-1 interaction with NF- κ B promotes microglial activation and neurotoxicity

The ability of PARP-1 to enhance NF- κ B binding to DNA has been previously demonstrated (15). In microglia, this interaction has been linked to surface expression of CD11a, cytokine release, and inducible NO synthase-dependent NO release (5, 6). In the present study, a role for NF- κ B in TNF- α -induced microglial proliferation, morphology changes, MMP-9 release, and neurotoxicity is suggested by the inhibition of each of these events by BAY 11-7082, which inhibits activation and nuclear translocation of NF- κ B by blocking I κ B phosphorylation (50). More direct studies

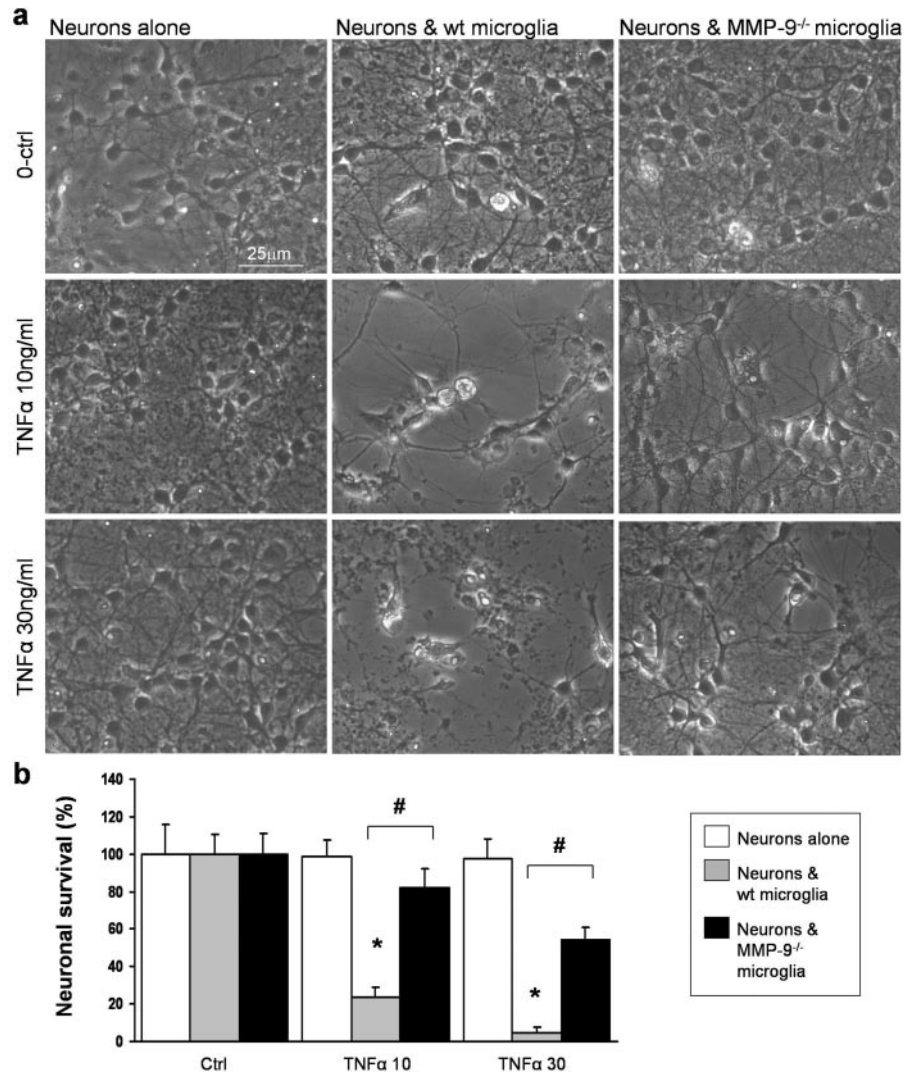


FIGURE 6. MMP-9 gene deletion reduces microglial neurotoxicity. *a*, Representative microphotographs from wt neurons cultured alone, with wt microglia, or with MMP-9^{-/-} microglia. In each case, the cultures were exposed to 0–30 ng/ml TNF- α for 40 h. TNF- α had no detectable effect on neuron viability in the absence of microglia, but caused a dose-dependent increase in neuron death in the presence of wt microglia. TNF- α -induced neuron death was substantially reduced in the cultures prepared with MMP-9^{-/-} microglia. *b*, Quantitative assessment of neuron survival under the same conditions as shown in *a*. Results are pooled from six independent experiments ($n = 6$), each with three to four culture wells per condition. *, $p < 0.001$ compared with neurons alone. #, $p < 0.005$ neurons in culture with MMP-9^{-/-} microglia vs neurons in culture with microglia from wt littermate controls.

of NF- κ B DNA binding are not feasible in primary mouse microglial cultures because of the limited number of cells that can be obtained from each experimental animal.

There is disagreement as to whether PARP-1 enzymatic activity is necessary for its function as a coactivator of NF- κ B. Pharmacological PARP inhibitors and reduced PARP-1 substrate (NAD⁺) have been shown to block the effect of PARP-1 on NF- κ B binding to DNA (6, 51). However, other studies have found no effect of PARP inhibitors on NF- κ B DNA binding, leading to the alternative proposal that PARP-1 acts by direct binding to NF- κ B, independent of catalytic activity (13, 14, 16). In the present studies, the effects of the NF- κ B inhibitor BAY 11-7082 were in every case mimicked by the PARP inhibitor DPQ, suggesting an essential role for PARP-1 enzymatic activity in its interaction with NF- κ B in the setting of TNF- α stimulation. It is possible that the differences reported in the literature may be attributable to the use of transformed cell lines in some studies, because PARP-1 expression and activity levels change significantly with cell differentiation (52).

PARP-1 interaction with NF- κ B promotes microglial MMP-9 release and neurotoxicity

It has not previously been recognized that MMP-9 release can be a cause of microglia-induced neuron death. Results of the present studies show that TNF- α -stimulated microglia release MMP-9, that TNF- α -stimulated microglial cells kill neurons in coculture,

and that the neuron death can be substantially reduced by genetic or pharmacologic inhibition of microglial MMP-9. The MMP-9 inhibitor ILO also has effects on other MMP species and ectoenzymes (28–30). However, several lines of evidence suggest that MMP-9 is the primary cytotoxic factor. First, only MMP-2, MMP-3, and MMP-9 are known to be released by microglia, and of these, MMP-2 was shown in this study to be unaffected by TNF- α stimulation and MMP-3 may act primarily to activate MMP-9 (42). MMP-9 is the only MMP family member with a well-defined NF- κ B promoter element (53), although indirect or cell type-specific effects of NF- κ B on other MMP family members cannot be entirely excluded (54). More directly, studies with MMP-9^{-/-} microglia showed markedly reduced neuron death after TNF- α stimulation. Neuron death was also prevented by the manipulations that blocked microglial MMP-9 release from wt microglia, namely PARP-1 gene deletion and NF- κ B inhibition.

Of note, the zymography studies suggest that PARP-1^{-/-} microglia not only fail to up-regulate MMP-9 release in response to TNF- α , but also may fail to cleave basally released MMP-9 to its active form (Fig. 3*a*). This result may simply reflect the sensitivity limit of the zymography assay method, but failure to activate MMP-9 would suggest a concomitantly reduced release of molecules that cleave MMP-9, such as tissue-specific plasminogen activator (55, 56) or MMP-3 (19). Like MMP-9, both tissue-specific

plasminogen activator and MMP-3 may be regulated by transcription factors that interact with PARP-1 (13, 57, 58).

MMP-9 neurotoxicity in vivo

Increased levels of MMP-9 have been observed in blood from patients with Alzheimer's disease and stroke, and in blood, cerebral spinal fluid, and brain from patients with multiple sclerosis (59–62), but a contributory role for MMP-9 in these disorders has not been established. MMP-9 inhibition has been shown to reduce neuron damage after ischemia (63). This result was initially attributed to effects of MMP-9 on the blood-brain barrier (17), but more recent studies indicate that MMP-9 can also have direct neurotoxic effects. Jourquin et al. (18) demonstrated increased release and activity of MMP-9 after neurotoxic kainate stimulation in organotypic cultures and reduced neuron death by inhibition of MMP-9. Conversely, incubation with rMMP-9 induced neuron death in the organotypic cultures.

The in vitro systems used in this study facilitate the identification of factors that regulate microglial MMP release and demonstrate the potential for microglia-derived MMP-9 to contribute to brain injury. However, MMP-9 may also be released by other cell types, including neurons (42, 64, 65), and the relative importance of microglial MMP-9 release on brain injury in vivo may be strongly influenced by these other factors. For example, extracellular cleavage of MMP-9 to its active form is promoted by MMP-3 (19), and both MMP-9 and MMP-3 bind to tissue inhibitor of metalloproteinase-1 (TIMP-1) (66). Moreover, TIMP-3 can inhibit MMP-3 and other MMPs (67). Because MMPs and TIMPs are released at different time points and by several different cell types following brain injury (reviewed in Ref. 42), the net effect of microglial MMP-9 release may vary over time and in different brain regions.

In summary, the present studies suggest that: 1) TNF- α induces PARP-1 activation in microglia; 2) TNF- α -induced microglial activation and proliferation require activation of both PARP-1 and NF- κ B; 3) TNF- α -stimulated microglia release MMP-9; and 4) microglial release of MMP-9 can be directly neurotoxic. The studies also support prior reports that PARP-1 enzymatic activity is required for its function as a coactivator of NF- κ B. These findings may be germane to neurological disorders in which microglia and MMP-9 contribute to neural injury.

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