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Inhibition of Microglial Phagocytosis Is Sufficient To Prevent Inflammatory Neuronal Death

Jonas J. Neher,* Urte Neniskyte,* Jing-Wei Zhao,[†] Anna Bal-Price,[‡] Aviva M. Tolkovsky,*[†] and Guy C. Brown*

It is well-known that dead and dying neurons are quickly removed through phagocytosis by the brain's macrophages, the microglia. Therefore, neuronal loss during brain inflammation has always been assumed to be due to phagocytosis of neurons subsequent to their apoptotic or necrotic death. However, we report in this article that under inflammatory conditions in primary rat cultures of neurons and glia, phagocytosis actively induces neuronal death. Specifically, two inflammatory bacterial ligands, lipoteichoic acid or LPS (agonists of glial TLR2 and TLR4, respectively), stimulated microglial proliferation, phagocytic activity, and engulfment of ~30% of neurons within 3 d. Phagocytosis of neurons was dependent on the microglial release of soluble mediators (and peroxynitrite in particular), which induced neuronal exposure of the *eat-me* signal phosphatidylserine (PS). Surprisingly, however, *eat-me* signaling was reversible, so that blocking any step in a phagocytic pathway consisting of PS exposure, the PS-binding protein milk fat globule epidermal growth factor-8, and its microglial vitronectin receptor was sufficient to rescue up to 90% of neurons without reducing inflammation. Hence, our data indicate a novel form of inflammatory neurodegeneration, where inflammation can cause *eat-me* signal exposure by otherwise viable neurons, leading to their death through phagocytosis. Thus, blocking phagocytosis may prevent some forms of inflammatory neurodegeneration, and therefore might be beneficial during brain infection, trauma, ischemia, neurodegeneration, and aging. *The Journal of Immunology*, 2011, 186: 4973–4983.

Inflammation occurs in many neurologic diseases and is believed to exacerbate neuronal loss in conditions such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, meningitis, AIDS dementia, brain trauma, and stroke (1, 2). In particular, the production of reactive oxygen and nitrogen species (RONS) as part of the inflammatory response has been shown to contribute to neurodegeneration, both in acute and chronic neurodegenerative conditions (2–4).

Microglia are key regulators of brain inflammation, and their activation causes neuronal loss in models of neurodegenerative disease in vitro and in vivo (2, 5, 6). The number of dying neurons found in these disease models and in brains of patients with neurodegeneration is often low (see, for example, Refs. 5, 7), which is generally attributed to rapid phagocytic removal of neurons by microglia (8) after neurons are terminally committed to

die. In principle, however, phagocytosis could be the cause, rather than the consequence, of neuronal death.

Phagocytosis of neurons appears to be highly regulated through a sequence of events often starting with the neuronal release of soluble factors (such as ATP), which induce microglial chemotaxis. On reaching the target neuron, microglia recognize surface signals (so-called *eat-me* signals) such as externalized phosphatidylserine (PS), which induce attachment and/or cell uptake (for review, see Ref. 9). Phagocytic recognition of externalized PS can be enhanced by soluble adaptor proteins, including milk fat globule epidermal growth factor-8 ([MFG-E8] also known as lactadherin), which binds PS on target cells and the vitronectin receptor (the heterodimeric $\alpha_v\beta_3/5$ integrin) on phagocytes, thus bridging phagocyte and target cell and improving phagocytic recognition (10).

We have previously investigated inflammatory neurodegeneration induced by the bacterial cell wall components lipoteichoic acid (LTA) or LPS in primary mixed neuronal-glial cultures (5, 11). In these studies, we noticed that the neurons were disappearing without any increase in apoptotic or necrotic cells, and we attributed this to rapid phagocytosis of dead or dying cells (5). However, we now report that this inflammatory neuronal loss is executed by microglial phagocytosis of the neurons. We show that when phagocytosis itself is blocked, without interfering with inflammation, the neurons survive rather than die, as would have been the case had phagocytosis occurred subsequent to cell death. In particular, neuronal loss results from a sequence of events starting with microglial production of peroxynitrite. Peroxynitrite by itself is necessary and sufficient to cause reversible exposure of PS on neurons and their phagocytosis by microglia. Surprisingly, however, inhibition of phagocytosis prevents both neuronal loss and death (despite the ongoing inflammatory process). Thus, our data suggest that loss of neurons during inflammation can be executed by microglial phagocytosis, and accordingly that inhibition of this process can prevent neuronal death.

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Abbreviations used in this article: AD, Alzheimer's disease; cRAD, cyclo(Arg-Ala-Asp-D-Phe-Val); cRGD, cyclo(Arg-Gly-Asp-D-Phe-Val); FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride; LME, L-leucine-methyl ester; LTA, lipoteichoic acid; MFG-E8, milk fat globule epidermal growth factor-8; MnTBAP, manganese(III) tetrakis(4-benzoic acid) porphyrin chloride; NBD, nitro-benzoxadiazole; PS, phosphatidylserine; RONS, reactive oxygen and nitrogen species; SIN-1, 3-morpholininosydnonimine; SOD, superoxide dismutase; TMRM, tetramethylrhodamine-methyl ester; TW, transwell insert.

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Materials and Methods

Materials

Purified (endotoxin-free) LTA was from Invivogen, LPS (*Salmonella enterica* serotype typhimurium) and amyloid peptides (25–35) and (35–25) from Sigma, GM-CSF from R&D Systems, authentic peroxynitrite from Cayman, 3-morpholininosydnonimine (SIN-1) from Invitrogen, RGDS/RGES and cyclo(Arg-Gly-Asp-D-Phe-Val/Arg-Ala-Asp-D-Phe-Val) (cRGD/cRAD) from Bachem, C2A protein domain was a kind gift of Dr. Kevin Brindle, and 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) and manganese(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP, a superoxide dismutase [SOD] mimetic) were from Calbiochem. Goat anti-MFG-E8 blocking Ab and goat control IgG were from Santa Cruz Biotechnology, rabbit anti-cleaved caspase-3 from Cell Signaling Technology, and Alexa 488-labeled *Griffonia simplicifolia* isolectin-B4 from Invitrogen. Secondary Abs goat anti-rabbit-Cy3, donkey anti-goat-Cy3, and Fc-region-specific anti-goat F(ab')₂ fragment were purchased from Jackson Immunolaboratories. Annexin V-EGFP was from Cambridge Bioscience. Nitrobenzoxadiazole (NBD)-labeled PS (1-oleoyl-2-[6-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)hexanoyl]-sn-glycero-3-phospho-L-serine ammonium salt, 16:0–12:0) was obtained from Avanti Polar Lipids. Carboxylate-modified fluorescent microspheres were from Invitrogen. All other materials were purchased from Sigma.

Primary cell culture

All experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Primary neuronal-glial cultures from postnatal days 5–7 rat cerebella were prepared as described previously (5). Cells were plated at a density of 5×10^5 cells/24-well plate and stimulated after 7–9 d in vitro. At this point, the culture composition of these “mixed cultures” was $85 \pm 5\%$ neurons, $7 \pm 3\%$ astrocytes, and $5 \pm 3\%$ microglia (see Supplemental Table I and Ref. 11 for characterization of cell types in these cultures). When desired, microglia were selectively eliminated after 6 d in vitro by treatment with L-leucine-methyl-ester ([LME] 50 mM) for 4 h and medium was replaced with conditioned medium from sister cultures (these cultures will be referred to as “microglia depleted”). Independent glial and pure microglial cultures were prepared as described previously (11). For prestimulation of microglia, 5×10^5 cells were plated into six-well plates (Nunc), left to adhere for 24 h, and stimulated with LTA (50 μ g/ml). For transwell experiments, $2.5\text{--}3 \times 10^4$ of these LTA-stimulated microglia were then either plated onto transwell membrane inserts (pore size, 0.4 μ m; referred to as “transwell cultures”) or directly onto the LME-treated cultures below transwell inserts (TWs) for 6 h (these cultures are referred to as “reconstituted”).

Culture treatment

Purified LTA was used at 50 μ g/ml and LPS at 100 ng/ml for all studies. A β peptides were dissolved in DMSO to a concentration of 2.5 mM, aliquoted, and stored at -80°C . For culture treatment, A β stocks were first diluted 1:100 in double-distilled H₂O and then 1:100 in neuronal culture medium (final concentration, 250 nM). Authentic peroxynitrite and SIN-1 were used at the concentrations indicated in the text. For SIN-1 experiments, cultures were frequently shaken gently after addition of the reagent to avoid local hypoxia (because SIN-1 degradation consumes oxygen).

Reagents were added either together with LPS/LTA/SIN-1/peroxynitrite/A β_{25-35} (RGDS/RGES, cRGD/cRAD, C2A protein domain, IgG, or anti-MFG-E8 Abs) or 2 d after stimulation (SOD, FeTPPS, MnTBAP, L-NAME, catalase). MFG-E8 blocking Ab and goat control IgG buffers were exchanged for PBS by repeated washing using Amicon Ultra-4 centrifugal filter devices (30-kDa cutoff; Millipore) and blocked with a 5-fold molar excess of Fc-region-specific anti-goat F(ab')₂ fragment (0.5 h, 37°C) to prevent recognition of the Abs through their Fc domains by microglial Fc γ Rs. A β_{25-35} was first preincubated with polymyxin B (1000 U/ml, 0.5 h, 37°C) and then diluted into the culture medium (final concentration of polymyxin B, 10 U/ml), to test for LPS contamination. These conditions were found to block LPS (100 ng/ml)-mediated microglial activation and neuronal loss in mixed neuronal/glial cultures (data not shown).

Quantification of cell densities

Cell densities were assessed in live cultures at 3 or 7 d after stimulation. For live-cell counts, cultures were incubated with the nuclear stains Hoechst 33342 (5 μ g/ml) and propidium iodide (1 μ g/ml); Alexa 488-tagged isolectin-B4 (1 μ g/ml) was used to identify microglia. Healthy and apoptotic (chromatin-condensed) neurons were recognized by their distinct nuclear morphology, whereas propidium iodide-positive cells (indicating membrane permeabilization) were scored as necrotic. Cell densities were

assessed using a Zeiss Axiovert S100 microscope. Four microscopic fields per well (between 150 and 200 neurons per field in control wells) in two wells per condition were quantified for a single experiment. Experiments performed with cultures from at least three independent culture preparations were used for statistical analysis.

Phagocytosis assay

Pure microglia from glial cultures were plated at a density of 5×10^4 cells on poly-L-lysine-coated 24-well plates. Cells were left to adhere overnight and stimulated with 50 μ g/ml LTA, 100 ng/ml LPS, or 50 ng/ml GM-CSF for 24 h. Three microliters of a 1:10 dilution of 1 μ m carboxylate-modified microspheres was added and incubated for 2 h (37°C , 5% CO₂). The medium was removed, ice-cold culture medium was added to arrest bead uptake, and cells were detached by mechanical agitation. Cells were spun down at $150 \times g$ for 7 min at room temperature and resuspended in 50 μ l DMEM containing isolectin-B4 (1 μ g/ml), Hoechst 33342 (5 μ g/ml), and propidium iodide (1 μ g/ml). The number of beads per cell was counted for >60 cells from at least three independent experiments on a Zeiss Axiovert S100 microscope.

Measurement of PS exposure using NBD-PS

Neurons from transwell cocultures were incubated with 10 μ g/ml NBD-labeled PS (16:0–12:0) in HBSS for 0.5 h at 37°C . For some experiments, cytochalasin D (1 μ M, 0.5 h) was added to the cultures before washing. Cells were washed and incubated with ice-cold HBSS containing 25 mM sodium dithionite for 2 min to reductively destroy (“quench”) exoplasmic NBD-fluorophores (12). Cells were washed extensively and analyzed by confocal microscopy (Olympus Fluoview 300 microscope). Fluorescence intensity of neuronal plasma membranes of individual cells was determined for >450 cells/condition from more than three independent experiments using ImageJ software.

Immunostaining

Cells were grown on 13-mm glass coverslips coated with 0.0005% polylysine. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.3% Triton X-100, blocked with 10% normal serum of the secondary Ab host species, and incubated overnight at 4°C with the primary Ab. Secondary Ab was added in PBS containing 4% serum and 0.3% Triton-X 100. Nuclei were counterstained with Hoechst 33342. As reported for macrophages (13), MFG-E8 staining was detergent sensitive, so Triton X-100 was omitted throughout. Live cultures were washed with HBSS and incubated with HBSS containing the anti-MFG-E8 Ab (4 μ g/ml) and 5% donkey serum for 1 h at 37°C , 5% CO₂ before fixation. Coverslips were mounted in Vectashield mounting medium (Vector Laboratories) and analyzed by confocal microscopy (Olympus Fluoview 300 microscope).

Annexin V staining

To assess neuronal PS exposure after peroxynitrite treatment, we incubated cultures with Annexin V-EGFP (1:500), Hoechst 33342 (5 μ g/ml), and propidium iodide (1 μ g/ml), and washed with ice-cold HBSS. Images were taken on a Leica DMI6000 microscope and quantified as described earlier.

Assessment of inflammatory mediators

Levels of IL-1 β and TNF- α were assessed in culture media at 3 d after stimulation using rat Quantikine ELISA Kits (R&D Systems). NO levels were determined by measuring nitrite levels using the Griess reaction as described previously (5), and calibrated using nitrite standard solution in culture medium. Note that for experiments using the RONS scavengers FeTPPS and MnTBAP, nitrite levels could not be determined because of the color of the reagents.

Statistical analysis

All data shown are expressed as the mean \pm SEM for at least three independent experiments (*n*). Results were analyzed using SPSS software. Normality of data was verified (Shapiro–Wilk test) before further analysis. Means were compared by one-way ANOVA and post hoc Bonferroni test. The *p* values <0.05 were considered significant.

Results

Inflammatory activation enhances microglial phagocytic activity and causes loss of neurons by phagocytosis

To examine the fate of neurons under inflammatory conditions, we treated primary cerebellar cultures (consisting of $85 \pm 5\%$ neurons,

$7 \pm 3\%$ astrocytes, and $5 \pm 3\%$ microglia; referred to hereafter as “mixed cultures”) with LTA, a bacterial TLR2 agonist, as described previously (5). LTA stimulation caused microglial proliferation (Fig. 1A) and production of TNF- α , IL-1 β , and NO (Supplemental Table I). Concomitantly, there was a loss of $\sim 40 \pm 5\%$ of neurons over 3 d without visible neuronal death by apoptosis or necrosis (Fig. 1A). We had previously assumed that this neuronal loss was due to phagocytic removal of dead or dying neurons by microglia (5). To test this assumption, we first examined whether LTA-induced neuronal loss was dependent on the presence of microglial cells. To this end, microglia were selectively eliminated from mixed cultures by treatment with the lysosomotropic reagent LME (14) (referred to hereafter as “microglia-depleted” cultures) (Fig. 1A), without reducing astrocyte (Supplemental Fig. 1) or neuron numbers (Fig. 1A). Importantly, selective elimination of microglia completely prevented LTA-induced neuronal loss (Fig. 1A), thereby implicating microglia as the primary mediators of this process.

To examine the dynamics of microglial–neuronal interaction, time-lapse video analysis was performed. Video imaging of LTA-stimulated mixed cultures revealed that neurons were being phagocytosed by microglia (Fig. 1D), in accordance with the absence of recognizable dying cells in LTA-treated cultures (Fig. 1A). Surprisingly, at the time of engulfment, neurons appeared identical to healthy neurons (e.g., there was no nuclear condensation) as far as could be discerned by light microscopy (Fig. 1D, Supplemental Video 1).

To test whether inflammatory activation of microglia would also enhance their phagocytic activity, we presented pure microglia (from glial cultures) with carboxylate-modified fluorescent microbeads (mimicking the negative surface charge of a cell exposing PS). Inflammatory activation of microglia with LTA or the bacterial TLR4-ligand LPS (15) strongly enhanced phagocytic uptake of microbeads (Fig. 1B). Microglial phagocytosis and proliferation are known to be stimulated by GM-CSF (16), which, however, does not increase microglial production of proinflammatory mediators such as TNF- α or IL-1 β (17). Although GM-CSF increased phagocytosis

of microbeads, as well as microglial proliferation in mixed cultures, it did not lead to neuronal loss (Fig. 1B, 1C). This result suggests that the phagocytic removal of neurons observed in LTA-treated mixed cultures is not due to stimulation of microglial phagocytosis alone, but also requires inflammatory signaling.

Inflammatory neuronal loss requires direct microglial–neuronal contact and neuronal exposure of eat-me signals

To test whether direct microglial–neuronal interaction was required for the execution of neuronal death, we added pure microglia back to microglia-depleted cultures either directly or physically separated from neurons on transwell membrane inserts (Fig. 2A). In the reconstituted cultures with direct microglial–neuronal contact, LTA induced $\sim 50\%$ of neuronal loss, similar to the loss observed in unmanipulated mixed cultures (Fig. 1A versus 2A, LTA). However, when the microglia were kept physically separated by a TW (referred to hereafter as “transwell coculture”), there was neither neuronal loss nor an increase in dying neurons below the transwell at 3 or 7 d after stimulation (Fig. 2A and data not shown). Importantly, transwell coculture did not prevent microglial inflammatory activation as judged by their strong proliferative response to LTA treatment (Fig. 2B) and by the increased production of the proinflammatory mediators TNF- α , IL-1 β , and NO (Supplemental Table I). This result indicated that, in the absence of direct microglial–neuronal interaction, soluble inflammatory mediators were not sufficient to cause neuronal death.

We wanted to test whether neurons in inflammatory-activated transwell cocultures would be lost when direct neuronal–microglial interaction was re-established. To this end, transwell cocultures and control cultures without microglia were first stimulated for 2 d with LTA. TWs with microglia were then removed, and pure LTA-activated microglia added to the neurons and incubated for 6 h (scheme in Fig. 2C). Despite the initial physical separation, $22 \pm 5\%$ of neurons were lost in cultures that had first been incubated with LTA for 2 d in the presence of transwell microglia. In contrast, there was no neuronal loss in nonactivated transwell

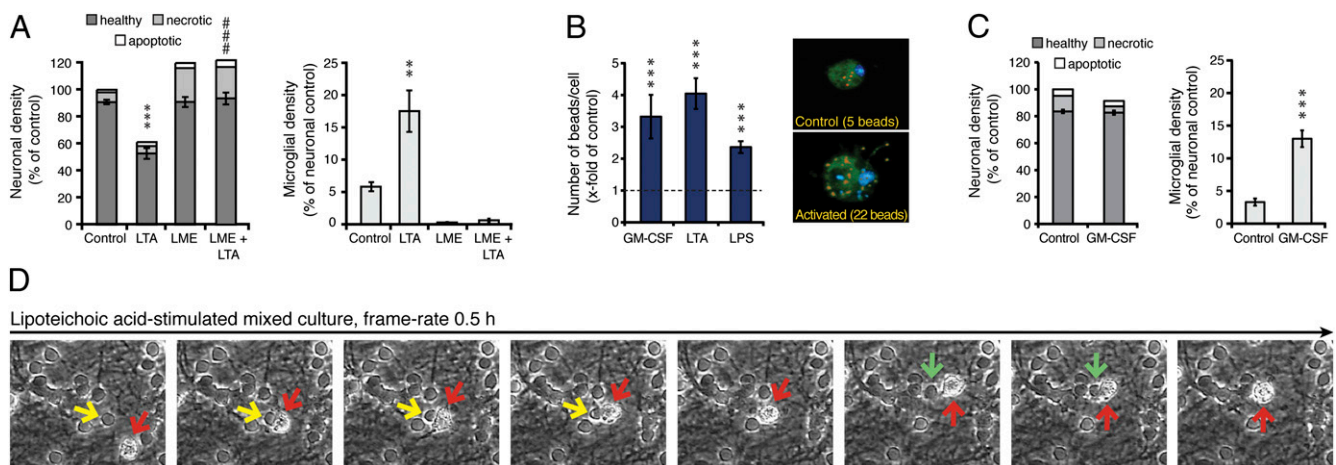


FIGURE 1. Inflammatory loss of neurons is mediated by microglial phagocytosis. *A*, LTA (50 μ g/ml) activation of mixed cultures for 3 d results in neuronal loss without death (*left panel*) and proliferation of microglia (*right panel*). Elimination of microglia with LME prevents neuronal loss (necrotic cells also include lysed microglia in LME-treated cultures). *B*, *Left panel*, Activation of microglia with LTA (50 μ g/ml), LPS (100 ng/ml), or GM-CSF (50 ng/ml) for 24 h strongly increases phagocytic uptake of carboxylate-modified microspheres (1 μ m), mimicking the negatively charged surface of PS-exposing cells. *Right panel*, Fluorescence image of microbead uptake (orange) by nonactivated and LTA-activated microglia stained with isolectin-B4 (green) and Hoechst 33342 (nuclei, blue). *C*, GM-CSF (50 ng/ml) stimulates microglial proliferation (*right panel*) and phagocytic activity (see *B*) but does not cause neuronal loss over 7 d (*left panel*). *D*, Single images from time-lapse video analysis of a mixed culture treated with LTA (frame rate, 0.5 h). Yellow and green arrows identify two neurons that are phagocytosed by a microglial cell (red arrows). Note the absence of neuronal morphological changes before engulfment. Data shown are means \pm SEM for three or more independent experiments; ** p < 0.01, *** p < 0.001 versus control, ### p < 0.001 versus LTA.

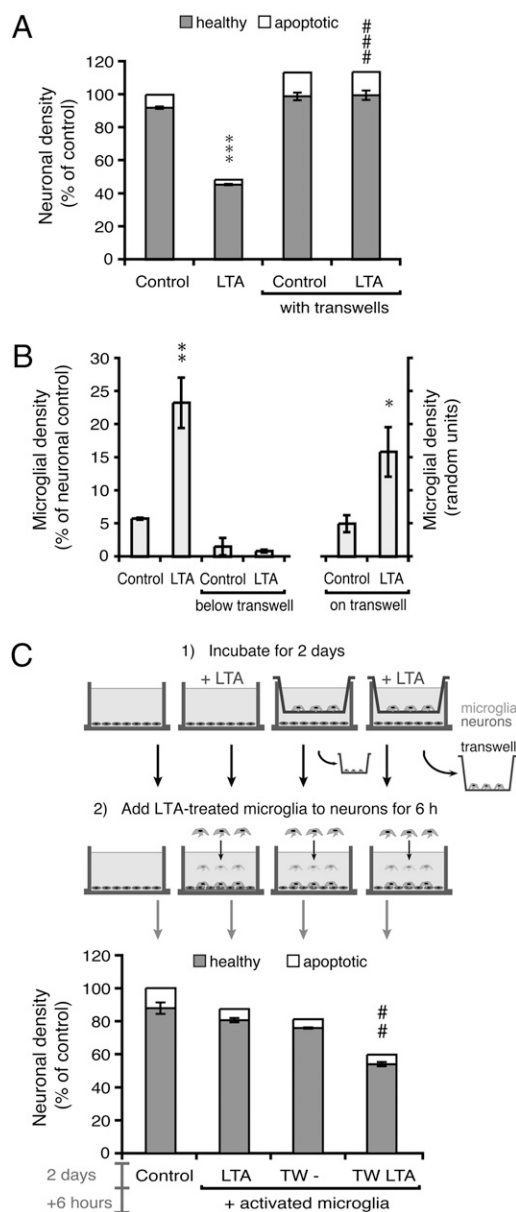


FIGURE 2. Neuronal loss requires release of soluble inflammatory mediators and direct neuronal-microglial contact. **A**, LTA-induced neuronal loss over 3 d is prevented by separation of neurons/astrocytes from microglia by TWs, without increasing the densities of dying cells (necrotic cells not shown, because LME treatment leads to microglial lysis and chromatin release; cf. Fig. 1). $***p < 0.001$ versus control, $###p < 0.001$ versus LTA. **B**, Microglial densities for experiment in **A**. $*p < 0.05$, $**p < 0.01$ versus respective controls. **C**, Separation of inflammatory soluble mediator release and direct microglial-neuronal contact using transwell cocultures (TW). *First column from left*, Microglia-depleted cultures were left untreated; *second column*, cultures were stimulated with LTA for 2 d, and LTA-activated microglia were then added for 6 h; *third column*, microglia-depleted cultures were exposed to nonactivated microglia on transwells for 2 d followed by addition of LTA-activated microglia for 6 h; *fourth column*, cultures were exposed to LTA-activated microglia on transwells for 2 d followed by LTA-activated microglia for 6 h. Delayed addition of LTA-activated microglia causes neuronal loss only if neurons were pre-exposed to soluble inflammatory mediators from LTA-activated transwell microglia. $###p < 0.01$ versus TW-. Data shown are means \pm SEM for three or more independent experiments.

cocultures (Fig. 2C), indicating that microglia were only capable of phagocytosing neurons that had been pre-exposed to soluble factors derived from LTA-activated transwell microglia leading

to the exposure of neuronal *eat-me* signals. Accordingly, pre-incubation of microglia-depleted cultures with LTA for 2 d (in the absence of transwell microglia) also failed to cause loss of neurons on subsequent addition of LTA-activated microglia (Fig. 2C). Hence LTA binding to neurons was not responsible for their removal, and LTA-activated astrocytes were not capable of inducing neurons to display *eat-me* signals.

Altogether, these results suggest that LTA-activated microglia contribute to the neuronal loss in two different ways: first, microglia release a soluble mediator(s), which leads to exposure of *eat-me* signals on neurons without committing them to death; and second, microglia recognize and phagocytose these neurons, only then inducing their death.

Inflammation-induced neuronal eat-me signaling is reversible

Because we had not observed any increase in neuronal apoptosis/necrosis in LTA-stimulated transwell cocultures, we asked whether *eat-me* signaling by neurons might be transient, thus allowing neurons to survive when separated from microglia. To test this possibility, we removed transwell microglia from cocultures 3 d after LTA stimulation, and the neurons were incubated for 1 d before the addition of exogenous, LTA-activated microglia (described in scheme in Fig. 3A). Under control conditions (presence of transwell microglia for 4 d), the addition of activated microglia to LTA-stimulated cultures caused the loss of $34 \pm 2\%$ of neurons (Fig. 3A). Strikingly, however, removal of transwell microglia for 1 d reduced neuronal loss to $14 \pm 3\%$ (Fig. 3A). This surprising finding implied that neuronal *eat-me* signaling was transient and that neurons were able to revert to a state that prevented their phagocytic removal, and therefore their death, when activated microglia were removed for only 1 d.

To assess directly whether a reversal of neuronal phagocytic signal exposure occurred after removal of transwell microglia, we analyzed one of the best characterized *eat-me* signals for phagocytic removal, the cell-surface exposure of PS. PS is located exclusively on the cytoplasmic leaflet of the plasma membrane in healthy cells (12), and PS exposure can be quantified by allowing cells to take up fluorescently labeled PS (NBD-PS) followed by selective quenching of exoplasmic (exposed) NBD-PS (12). Thus, PS exposure results in a decrease in fluorescence intensity after quenching. To first determine whether LTA-induced inflammation could mediate neuronal PS exposure, we added NBD-PS to neurons from transwell cocultures at 3 d after LTA stimulation. LTA-induced inflammation did not significantly change the total amount of NBD-PS integrated into the neuronal plasma membrane (before quenching, LTA: $87 \pm 13\%$ of control). However, LTA stimulation induced a $34 \pm 2\%$ decrease in intracellular (quenching-insensitive) NBD-PS fluorescence intensity (Fig. 3C), indicating that increased neuronal PS exposure occurs in the inflammatory environment.

To analyze whether this PS exposure was transient, we repeated the NBD-PS experiment, again using removal of the transwell microglia to allow neurons to recover for 1 d (cf. Fig. 3A). Whereas transwell cocultures that were incubated with LTA for 4 d showed a decrease in NBD-PS uptake of $32 \pm 4\%$ (equivalent to the value in cultures incubated with LTA for 3 d), removal of the transwell microglia for 1 d resulted in NBD-PS signal intensity being restored to $95 \pm 5\%$ of the value in untreated transwell cocultures (Fig. 3C). Thus, neuronal PS exposure was dependent on the continuous release of soluble mediators from activated microglia, and correspondingly, PS display was reversible when microglia were removed.

Altogether, these results show that soluble mediators released by inflammatory activated microglia are sufficient to cause neuronal

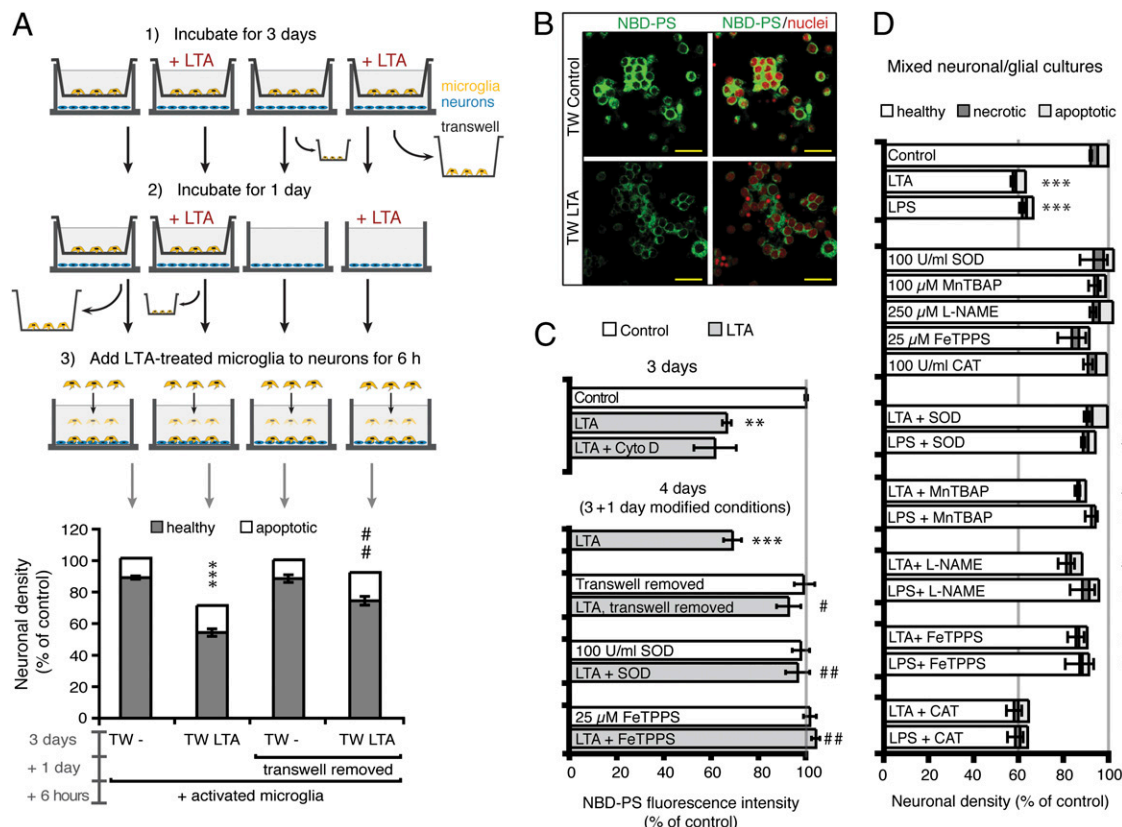


FIGURE 3. Inflammatory neuronal *eat-me* signaling is reversible and mediated by RONS. **A**, Removal of transwell microglia for 1 d after 3 d of LTA (50 μ g/ml) stimulation leads to decreased neuronal loss on addition of activated microglia (cf. Fig. 2), indicating reversibility of neuronal *eat-me* signaling. *** $p < 0.001$ versus TW-, ## $p < 0.01$ for TW LTA versus TW LTA – TW removed. **B**, Confocal micrographs of neurons below transwells (astrocytes lie in a layer underneath the focal plane) loaded with fluorescent PS (NBD-PS, green) at 3 d after LTA stimulation. Nuclei are counterstained with propidium iodide (red) after fixation. Note the reduced signal intensity of LTA-stimulated cultures (TW LTA) compared with control cultures (TW control) after quenching of exoplasmic NBD-PS, indicating increased PS exposure. Scale bars, 50 μ m. **C**, LTA stimulation of transwell cultures for 3 or 4 d increases PS exposure (quantified as a decrease in neuronal NBD-PS fluorescence after quenching of exposed NBD-PS). Blocking endocytosis with cytochalasin D (Cyto D) has no effect on NBD-PS levels. The LTA-induced PS exposure is reversed by removing the transwell microglia or by scavenging superoxide (using SOD) or peroxynitrite (using FeTPPS) for 1 d after 3 d of LTA stimulation. For all bars, the NBD-PS fluorescence of quenched neurons is expressed as percentage of NBD-PS signal for untreated transwell cultures. **D**, LTA- (50 μ g/ml) or LPS-induced (100 ng/ml) neuronal loss is prevented by delayed scavenging (added 2 d after LTA/LPS) of RONS using SOD, the SOD mimetic MnTBAP, or the peroxynitrite scavenger FeTPPS, or by inhibition of NO synthases using L-NAME, for 1 d. In contrast, catalase (CAT) has no effect. Neuronal density and death was quantified 3 d after LTA or LPS addition to mixed cultures. Microglial activation/proliferation was unaffected by all treatments (see Supplemental Table I). Data shown are means \pm SEM for three or more independent experiments. ** $p < 0.01$, *** $p < 0.001$ versus control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus LTA or LPS.

exposure of *eat-me* signals but are insufficient to directly induce neuronal apoptosis/necrosis. Only when microglia are allowed to directly interact with these neurons is neuronal death executed.

Peroxyntirite is necessary and sufficient for PS exposure and neuronal loss

The reversibility of PS exposure (within 1 d of microglial removal) was striking, indicating that a short-lived mediator(s) was responsible for neuronal exposure of this signal. RONS are inflammatory mediators with short half-lives and are consistently implicated in inflammatory neurodegeneration (2). Having found that LTA stimulates significant microglial superoxide (O_2^-) production (Supplemental Fig. 2) and NO release (Supplemental Table I) in mixed cultures, we investigated whether RONS contribute to PS exposure. Transwell cocultures were incubated with LTA for 3 d. Then either SOD (which degrades superoxide) or FeTPPS (a peroxynitrite decomposition catalyst) was added to the bottom of the well (without removing the transwell), and cultures were incubated for another day. Both treatments restored the intensity of the NBD-PS signal to control values ($95 \pm 5\%$ for SOD, $102 \pm 1\%$ for FeTPPS; Fig. 3C), similar to the reversal measured after re-

moval of transwell microglia ($95 \pm 5\%$). These results indicate that RONS released by activated microglia are essential for mediating neuronal PS display. Although it has previously been shown that RONS have direct neurotoxic effects (18), our results indicate that inflammatory activated glia release only sublethal levels of RONS, leading to reversible neuronal *eat-me* signal exposure and neuronal loss only on direct interaction with microglia.

We have previously shown that coapplication of the inducible NO synthase inhibitor 1400W, the SOD mimetic MnTBAP, or the peroxynitrite scavenger FeTPPS with LTA prevented neuronal loss (5). Because we have found subsequently that neuronal loss occurs predominantly between 2 and 3 d after inflammatory activation of mixed cultures (data not shown), and because it has become clear that RONS can mediate the inflammatory response (19), all inhibitors were applied here at 2 d after LTA treatment to first allow for inflammatory glial activation. Thus, if the protective effect of RONS scavengers were mediated through prevention or reversal of *eat-me* signal exposure, delayed addition should be sufficient for neuronal survival. Delayed addition of SOD and FeTPPS (which reversed PS exposure in the transwell system; see Fig. 3C), as well as MnTBAP (an SOD mimetic) and L-NAME (an

inhibitor of NO synthases), provided significant neuroprotection (Fig. 3D) without preventing microglial proliferation or the release of inflammatory cytokines (Supplemental Table I). In contrast, catalase (which degrades hydrogen peroxide) was ineffective (Fig. 3D). These results confirm that superoxide, NO, and their reaction product peroxynitrite are essential for neuronal loss in response to LTA, even on delayed addition when they do not interfere with the inflammatory response.

To examine whether we could generalize the conclusions drawn from LTA experiments, we analyzed the mechanism of neurodegeneration induced by LPS, a TLR4 ligand (15). In mixed cultures, treatment with LPS induced neuronal loss of a similar type and extent to LTA, as there was microglial proliferation (Supplemental Table I) and loss of $30 \pm 5\%$ of neurons within 3 d without any apparent increase in the number of apoptotic/necrotic cells (Fig. 3D). Time-lapse video analysis also confirmed that after LPS treatment, neurons were removed by microglial phagocytosis (data not shown). Furthermore, equivalent to LTA experiments, delayed addition of all RONS inhibitors interfering with NO, O_2^- , or peroxynitrite levels significantly protected against LPS-induced neuronal loss, whereas catalase was ineffective (Fig. 3D). Again, LPS-induced microglial proliferation and the release of inflammatory cytokines remained unaffected (Supplemental Table I).

These results indicate that the mechanism of neuronal loss might be similar for LTA and LPS, despite their acting through different receptors (i.e., TLR2 and TLR4, respectively). Furthermore, inflammatory neurodegeneration induced by LTA and LPS both require continuous production of O_2^- , NO, and their reaction product peroxynitrite (as shown by delayed addition of RONS inhibitors), indicating that only sublethal levels of RONS are

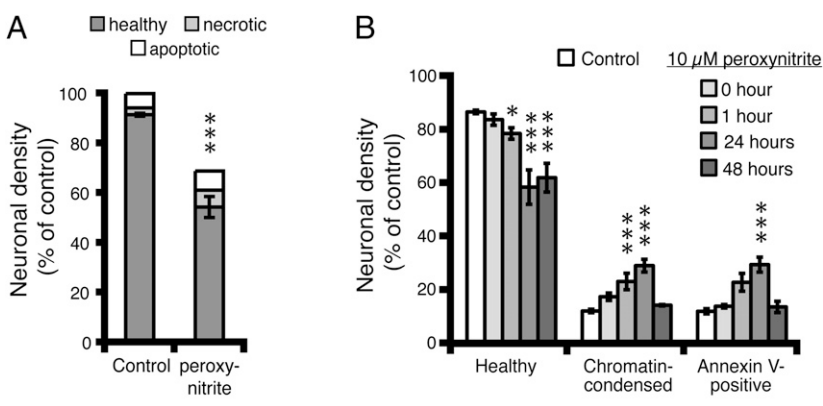
produced by activated microglia. To determine whether sublethal levels of peroxynitrite were sufficient to cause neuronal loss in the presence of microglia, we added low concentrations (10 μ M) of authentic peroxynitrite to mixed cultures. Similar to cultures treated with LPS or LTA (cf. Fig. 3D), peroxynitrite caused neuronal loss over 3 d without accumulation of dying cells (Fig. 4A). Interestingly, a significant increase from ~ 10 –25% in the numbers of chromatin-condensed and PS-exposing neurons (measured using Annexin V binding to PS) was found between 1 h and 1 d after treatment with peroxynitrite (Fig. 4B), and these cells had disappeared 2 d after peroxynitrite addition, concomitant with a $\sim 20\%$ decrease in the number of healthy neurons (Fig. 4B). Importantly, although the profile of PS exposure and chromatin condensation was similar in microglia-depleted cultures (Fig. 4D), the decrease in the number of healthy neurons was reversible, and these cells then remained alive for up to 7 d after stimulation (Fig. 4C). Thus, peroxynitrite was sufficient to induce reversible chromatin condensation and exposure of PS, as well as neuronal loss, but only in the presence of microglia, equivalent to the mechanism of neuronal loss during inflammation induced by LPS or LTA.

Inhibition of phagocytosis prevents LTA-, LPS-, and peroxynitrite-induced neuronal loss

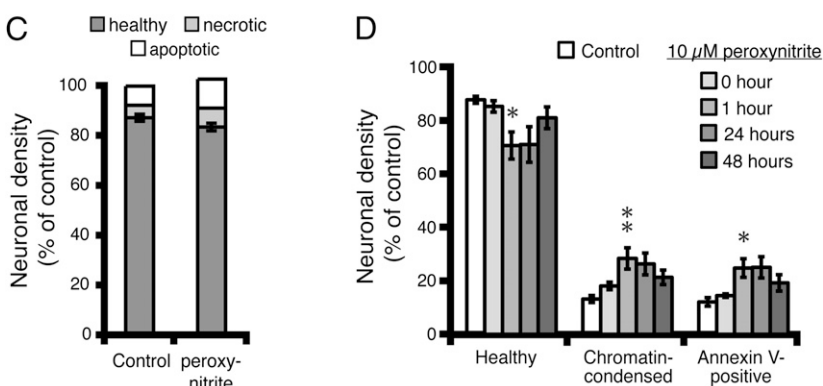
Because our results had indicated that neurons in inflammatory activated cultures were only committed to die if microglial-neuronal interaction was possible (Figs. 2C, 3A), we wanted to test whether blocking phagocytosis itself was sufficient to prevent neuronal death. To block microglial recognition of PS displaying neurons, we masked the exposed PS by adding the C2A domain of the protein synaptotagmin I (C2A) that binds PS tightly (20).

FIGURE 4. Nontoxic levels of peroxynitrite cause reversible PS exposure and microglia-dependent neuronal loss. *A*, Low levels of peroxynitrite (10 μ M) cause loss of neurons over 3 d without accumulation of dying cells in mixed cultures. *B*, In mixed cultures, peroxynitrite increases neuronal PS exposure (Annexin V⁺ cells) and chromatin-condensation between 1 h and 1 d after treatment, returning to baseline at 2 d, concomitant with a decrease in healthy cells (0 h indicates staining immediately after addition of peroxynitrite). *C*, Peroxynitrite (10 μ M) does not cause significant neuronal death or loss in microglia-depleted cultures even 7 d after peroxynitrite addition (cf. *A*). *D*, In microglia-depleted cultures, peroxynitrite (10 μ M) induces reversible chromatin condensation and PS exposure, but no loss of neurons. Data shown are means \pm SEM for three or more independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

Mixed cultures



Microglia-depleted cultures



The addition of C2A to mixed cultures inhibited LTA-induced neuronal loss by ~50% (Fig. 5A) without affecting microglial proliferation (Supplemental Table I), indicating that PS exposure contributes to the phagocytosis of the neurons, possibly alongside other signals.

Phagocytic PS recognition can be mediated by so-called bridging proteins, including MFG-E8 (21). MFG-E8 is a secreted glycoprotein that binds exposed PS through its C1 and C2 domains, and the macrophage vitronectin receptor through an RGD motif, thus bridging the phagocyte and the target cell (10, 22). The addition of a function-blocking anti-MFG-E8 Ab (23) to LTA-stimulated mixed cultures significantly reduced neuronal loss by ~65% (Fig. 5A) without affecting microglial proliferation or the release of inflammatory mediators (Supplemental Table I), whereas the control IgG had no effect (Fig. 5A). Addition of the tetrapeptide RGDS (a broad-spectrum integrin receptor antagonist containing the RGD motif present in MFG-E8) significantly prevented neu-

ronal loss by ~80%, whereas the control peptide RGEs had no effect (Supplemental Fig. 3). Furthermore, cRGD, an integrin antagonist specific for the MFG-E8 receptor (24), also reduced neuronal loss over 3 d by ~80–90% (Fig. 5A) after inflammatory stimulation with either LTA or LPS. Importantly, the protective effect of cRGD was still significant at 7 d after LPS/LTA treatment (Fig. 5A), indicating that neurons did not undergo delayed cell death when their phagocytic removal was prevented. Furthermore, cRGD did not inhibit microglial proliferation in response to LTA or LPS, nor did it affect the release of inflammatory mediators (TNF- α , IL-1 β , or NO; Supplemental Table I). The control peptide cRAD, in contrast, did not prevent neuronal loss induced by either LTA or LPS (Fig. 5A).

To confirm the role of the PS–MFG-E8–vitronectin receptor system in neuronal removal by microglia, we also performed immunostaining for MFG-E8 in cultures treated with LPS \pm cRGD (Fig. 6A–O). As reported for macrophages (13), MFG-E8 staining

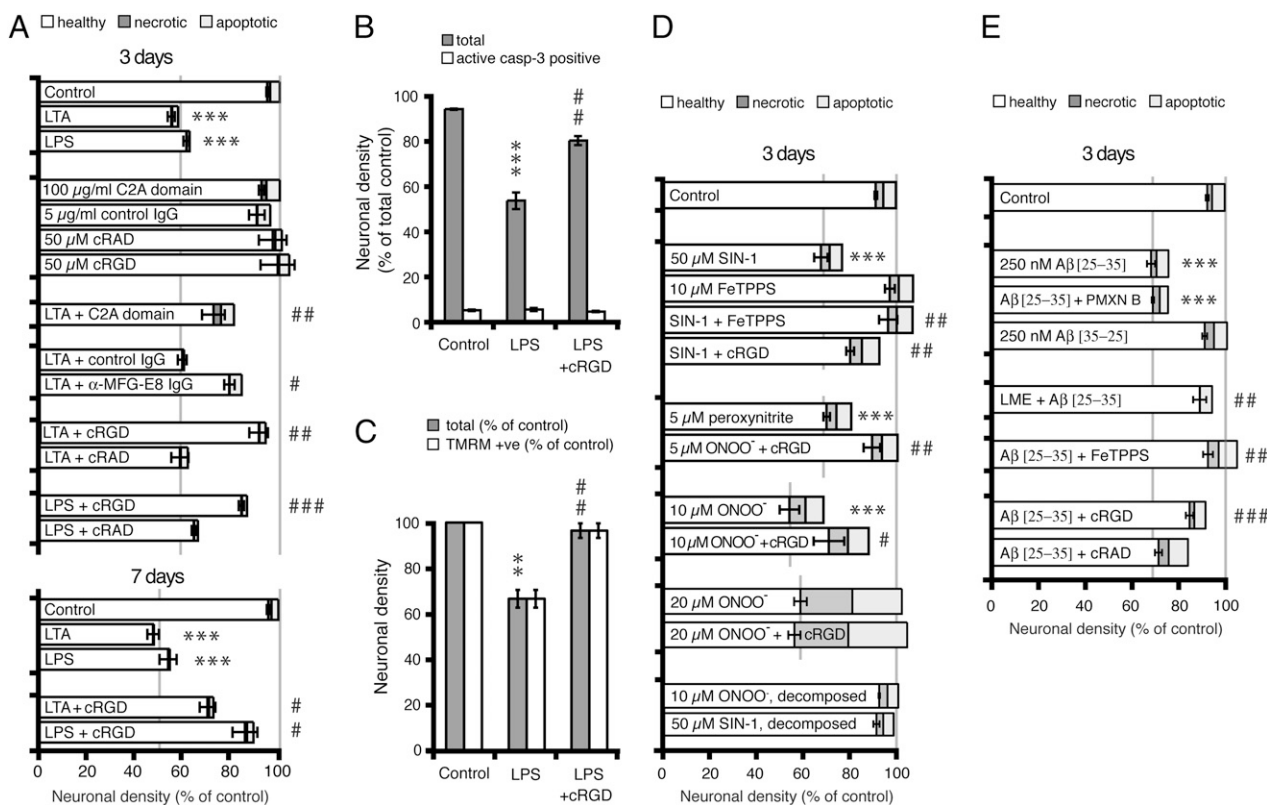


FIGURE 5. Inhibition of phagocytosis prevents LTA-, LPS-, peroxynitrite- and A β -induced neurodegeneration, leaving behind healthy neurons. **A**, Neuronal loss induced by LTA (50 μ g/ml) or LPS (100 ng/ml) is reduced by inhibiting the PS–MFG-E8–vitronectin receptor system. Significant neuroprotection is provided by: 1) preventing PS recognition through addition of C2A domain, 2) blocking MFG-E8 bridging of PS-exposing cells and the vitronectin receptor on microglia using a MFG-E8 blocking Ab, or 3) using the vitronectin receptor-specific blocking peptide cRGD to prevent MFG-E8 recognition. In contrast, a control IgG and cRAD have no effect. **B**, Protection provided by cRGD (50 μ M) against LPS (100 ng/ml)-induced neuronal loss does not lead to accumulation of caspase-3⁺ cells 3 d after stimulation. **C**, Loading with TMRM (3 nM) indicates that the plasmalemmal and the mitochondrial membrane potential are intact in neurons protected from LPS (100 ng/ml)-induced neuronal loss by cRGD (50 μ M). Dark bars represent the number of healthy neurons as a percentage of that in the untreated control culture; light bars represent the total number of neurons positive for TMRM as percentage of that in the untreated control culture. Thus, in each treatment, virtually all neurons present are TMRM⁺. **D**, Treatment of mixed cultures with nontoxic levels of peroxynitrite and SIN-1 leads to significant neuronal loss after 3 d. This does not occur after decomposition of either reagent in culture medium (“decomposed”). The SIN-1 effect is mediated by peroxynitrite as shown by prevention of neuronal loss with the peroxynitrite scavenger FeTPPS. The vitronectin receptor antagonist, cRGD, protects against neuronal loss induced by low peroxynitrite (5 μ M), and partially protects against intermediate peroxynitrite (10 μ M) and SIN-1 (50 μ M), but not high peroxynitrite (20 μ M), which induces significant cell death by necrosis and apoptosis. **E**, Treatment of mixed cultures with the β -amyloid peptide (25–35) (A β [25–35], 250 nM) leads to significant neuronal loss over 3 d, whereas the reverse peptide A β (35–25) has no effect on neuronal density. A β (25–35)-induced neuronal loss is not prevented by incubation with polymyxin B (PMXN B), excluding LPS contamination. In contrast, elimination of microglia using LME treatment, delayed addition of the peroxynitrite scavenger FeTPPS (2 d after stimulation), or cotreatment with the vitronectin receptor antagonist, cRGD, significantly prevents A β (25–35) induced neuronal loss. The control peptide cRAD, in contrast, has no effect. Gray lines indicate 100% and the approximate level of neuronal loss for ease of comparison. Data shown are means \pm SEM for three or more independent experiments. ** p < 0.01, *** p < 0.001 compared with control; # p < 0.05, ## p < 0.01 compared with LTA/LPS/peroxynitrite/SIN-1/A β (25–35).

was detergent sensitive and located on microglial vesicles. In addition, MFG-E8–immunoreactive neurons were occasionally found in untreated cultures but were virtually absent in LPS-treated cultures, consistent with their phagocytic removal by activated microglia. However, in cultures treated with LPS + cRGD, MFG-E8–opsonized (coated) neurons were present and were isolated from any microglial processes (Fig. 6*F*, 6*I*, 6*L*), indicating that cRGD prevented their recognition through the microglial vitronectin receptor.

In summary, these results indicate that during inflammation, microglial phagocytosis of neurons is mediated by the PS–MFG-E8–vitronectin receptor pathway. Furthermore, blocking any of these molecules is sufficient to prevent neuronal loss and death, leaving behind healthy neurons with the capacity for long-term viability.

Finally, we tested whether loss of neurons induced by RONS could also be prevented by inhibition of this pathway using cRGD. Mixed neuronal cultures were treated with peroxynitrite (5 and 10 μ M) or SIN-1, which spontaneously decomposes in aqueous solution to yield NO and O_2^- and their reaction product ONOO $^-$ (25). Addition of either peroxynitrite or SIN-1 to mixed cultures caused significant neuronal loss of 30–40% over 3 d without any increase in neuronal apoptosis/necrosis (Fig. 5*D*). Neuronal loss in response to SIN-1 was confirmed to be due to peroxynitrite production by coapplication of the peroxynitrite decomposition catalyst FeTPPS, which completely protected neurons for 3 d (Fig. 5*D*) and was also dependent on the presence of microglia (data not shown). In accordance with the results on PS exposure and neuronal loss induced by peroxynitrite (cf. Fig. 4), cRGD provided significant neuroprotection for 3 d after stimulation with 5–10 μ M peroxynitrite or SIN-1 (50 μ M). Importantly, neither peroxynitrite nor SIN-1 caused the release of the inflammatory mediators TNF- α or NO (data not shown). Hence the neuronal loss in response to peroxynitrite/SIN-1 stimulation was not due to secondary in-

flammation resulting from neuronal injury. Rather, peroxynitrite was directly causing neuronal loss.

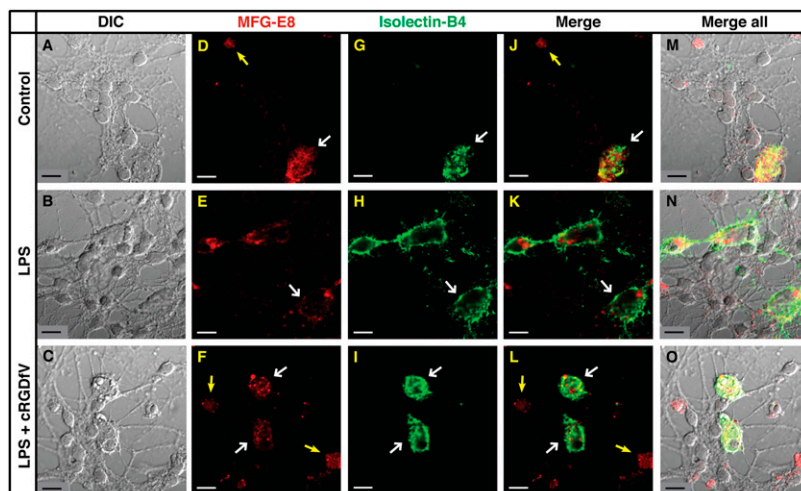
To exclude the possibility that cRGD had direct neuroprotective effects, we induced neuronal necrosis and apoptosis by levels of peroxynitrite (20 μ M; Fig. 5*D*) or *N*-methyl-D-aspartate (300 μ M; Supplemental Fig. 4) that were directly toxic to neurons. cRGD failed to rescue neurons after treatment with toxic concentrations of peroxynitrite or NMDA, indicating that it did not directly protect neurons.

These results indicate that sublethal concentrations of peroxynitrite are both necessary and sufficient to induce reversible neuronal *eat-me* signaling, leading to neuronal death through phagocytosis by microglia.

Neurons spared from phagocytic removal are viable

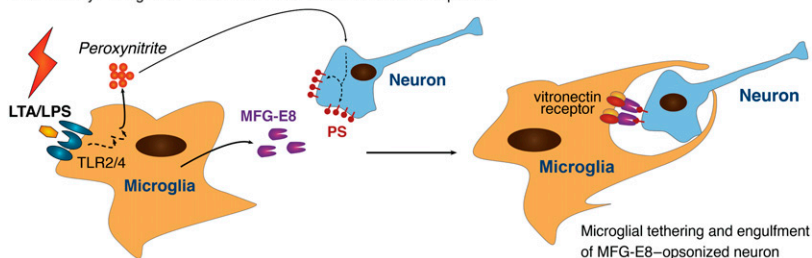
Neurons saved from phagocytic removal were not necrotic or chromatin condensed (Fig. 5*A*) and appeared morphologically identical to untreated neurons (data not shown, but see Fig. 1*D*). However, to ascertain the viability of the saved neurons, we immunostained mixed cultures stimulated with LPS \pm cRGD for cleaved (active) caspase-3. At 3 d, the density of neurons positive for cleaved caspase-3 was $\leq 5\%$ in all conditions (Fig. 5*B*), indicating that the protected neurons were not undergoing apoptosis. To further confirm that the rescued neurons were healthy, cultures treated for 3 d with LPS \pm cRGD were loaded with tetramethylrhodamine-methyl ester ([TMRM] 3 nM). At this concentration, dye uptake is dependent on both the plasmalemmal and mitochondrial membrane potential (26), two essential indicators of neuronal health. Excitotoxic glutamate caused depolarization of neurons measured by TMRM uptake (data not shown). In contrast, all the neurons saved from LPS-induced loss by inhibition of phagocytosis had normal TMRM loading (Fig. 5*C*), and therefore normal plasma membrane and mitochondrial membrane potentials, confirming that these neurons were viable.

FIGURE 6. MFG-E8 is located on microglia and neurons, and acts as a bridging molecule for phagocytic recognition. *A–O*, Differential interference contrast (DIC, *A–C*) and confocal fluorescent images showing live staining of mixed cultures for MFG-E8 (red) and microglia (isolectin-B4, green) 3 d after treatment. As reported for macrophages, MFG-E8 appears to be localized in vesicles on the microglial surface both in control and LPS (100 ng/ml)-activated cultures (white arrows). MFG-E8 opsonized cells (yellow arrows) are only rarely found in control cultures (*D*) and are absent in LPS-treated cultures (*E*), consistent with their phagocytic removal. In contrast, cells positive for MFG-E8 can be observed in cultures treated with LPS in combination with vitronectin receptor antagonist cRGD (cRGDfV, 50 μ M) (*F*), indicating inhibition of phagocytosis of MFG-E8–opsonized neurons. Scale bars, 10 μ m. The scheme shows the proposed mechanism of primary phagocytosis in inflammatory neurodegeneration.



Schematic mechanism of primary phagocytosis in inflammatory neurodegeneration

Inflammatory microglial activation and induction of neuronal PS-exposure



Inhibition of phagocytosis prevents neuronal loss induced by β -amyloid peptide (25–35)

AD is a neurodegenerative condition, characterized by extracellular plaques of aggregated β -amyloid protein. These plaques are associated with activated glia, and β -amyloid has been shown to induce microglial activation through TLR2 and TLR4 followed by the release of inflammatory mediators (including RONS) (27). A small peptide corresponding to amino acids 25–35 of β -amyloid ($A\beta_{25-35}$) is sufficient to cause ROS production and neuronal loss in mixed neuronal/microglial cultures equivalent to effects seen with LPS (28, 29). In accordance with these studies, we found that at low concentrations (250 nM), $A\beta_{25-35}$ caused loss of ~20% of neurons over 3 d. Importantly, this loss occurred in the absence of an increase in dying cells (Fig. 5E), analogous to the effects seen with LTA and LPS (Figs. 3, 5). To ensure that this loss was not mediated by LPS contamination of the peptide, $A\beta_{25-35}$ was preincubated with polymyxin B (which binds and inactivates LPS) (30). Polymyxin B did not prevent neuronal loss in response to $A\beta_{25-35}$, demonstrating that this loss was not mediated by LPS. Furthermore, the reverse peptide $A\beta_{35-25}$ had no effect on neuronal density, thus confirming an amyloid peptide-specific effect. Importantly, the $A\beta_{25-35}$ -induced loss was blocked by elimination of microglia (using LME treatment), demonstrating that microglia were crucial for neuronal loss and that $A\beta_{25-35}$ was not neurotoxic in their absence. Furthermore, delayed treatment (2 d after stimulation with $A\beta_{25-35}$) of mixed cultures with the peroxynitrite scavenger FeTPPS (25 μ M) significantly reduced neuronal loss (Fig. 5E). Most importantly, cotreatment of $A\beta_{25-35}$ stimulated mixed neuronal-glia cultures with the vitronectin receptor antagonist, cRGD (50 μ M), significantly inhibited neuronal loss (Fig. 5E). In contrast, the control peptide cRAD had no effect. These results demonstrate that equivalent to the bacterial TLR agonists LPS and LTA (cf. Figs. 3D and 5A), neuronal loss induced by the AD-related ligand $A\beta_{25-35}$ was microglia dependent, mediated by peroxynitrite, and executed by microglial phagocytosis of neurons.

Altogether, our data indicate that during inflammation, microglia execute neuronal death through phagocytosis of neurons that are otherwise viable. This may present a completely novel mechanism of inflammatory neuronal death in mammals because it has generally been assumed that neurons are removed by phagocytosis only after they are terminally committed to die. In contrast, we show in this article that blocking phagocytosis (without interfering with inflammation or neuronal cell death mechanisms) in itself is sufficient to rescue neurons during inflammation.

Discussion

Microglia are the professional phagocytes of the CNS and are vital for tissue homeostasis and injury responses. This includes the rapid phagocytic removal of dead or dying cells, which prevents the release of proinflammatory intracellular components and contributes to the resolution of inflammation (31). However, it is also becoming clear that during neuroinflammation, microglia can exacerbate neuronal loss (4, 32). The data presented in this article provide evidence that microglia can execute neuronal death during inflammation by a potentially novel mechanism of primary phagocytosis, as opposed to phagocytosis secondary to neuronal apoptosis or necrosis. Our results indicate that this process involves: 1) inflammatory activation of microglia and their phagocytic activity, 2) peroxynitrite-induced, reversible neuronal PS exposure, 3) opsonization of PS-exposing cells by the bridging molecule MFG-E8, and 4) recognition of MFG-E8 through the microglial vitronectin receptor (scheme in Fig. 6). Importantly, blocking any of these steps can prevent neuronal death.

In this study, we have focused on two bacterial components, LTA and LPS, which we and others (15, 33) have previously shown to induce an inflammatory response in glia (without any direct effects on neurons) through TLR2 and TLR4, respectively, and this, in turn, leads to secondary neuronal injury. LPS has been commonly used to model inflammation, as well as neurodegenerative disease, and can cause pathophysiological changes similar to those in systemic and central bacterial infections, as well as Parkinson's disease and age-related neurodegeneration (6, 34–36). In addition to LTA and LPS, activation of microglial TLR2 and TLR4 by β -amyloid (27) or other endogenous agonists released from stressed or dying neurons (such as heat shock protein 60 and high mobility group box 1 protein) (37–41) can result in inflammatory neurodegeneration. Many of these studies have also shown that neuronal loss is dependent on RONS (see, for example, Refs. 38, 39, 42), and oxidative stress has been implicated as a common final mediator of inflammatory neurodegeneration in many other models of neurodegenerative disease (for review, see Ref. 2). In accordance with these observations, we have shown in this article that RONS, and peroxynitrite in particular, are not only necessary, but also sufficient for mediating neuronal loss (Figs. 4, 5). However, in contrast with the general assumption that RONS cause direct neurotoxicity followed by phagocytic removal of dead or dying neurons, we show that the levels of RONS produced by activated glia are insufficient to cause neuronal death (Figs. 2, 3). Instead, sublethal doses of peroxynitrite, when added directly to cultures or when produced by microglia in response to TLR2/4 activation by LTA or LPS, led to reversible neuronal *eat-me* signal exposure, followed by the execution of neuronal death through microglial phagocytosis (Fig. 3). However, because of the reversibility of neuronal *eat-me* signaling, neurons were able to revert to a healthy state, and display long-term viability, when phagocytosis was blocked (Fig. 5). Interestingly, we have also found that low concentrations of a different oxidant, hydrogen peroxide (25 μ M), can induce reversible PS exposure without neuronal death (Supplemental Fig. 5), indicating that this mechanism is not limited to particular RONS. We do not currently know the mechanism of PS display. However, although PS exposure occurs during apoptosis, it can also be caused by calcium increase, ATP depletion, or oxidative stress (43), via activation of the scramblase or inactivation of the aminophospholipid translocase (the enzymes mediating PS externalization and internalization, respectively). Importantly, such PS exposure can be reversible and dissociated from cell death (44).

Although we have found in this study that low levels of peroxynitrite (5 and 10 μ M) caused neuronal death only indirectly through microglial phagocytosis, greater levels of peroxynitrite (20 μ M) caused apoptosis and necrosis, and this cell death was not prevented by addition of cRGD (Fig. 5D). Therefore, our data suggest that although RONS cause neuronal death by necrosis at high levels and by apoptosis at medium levels (as described in, for example, Ref. 18), at sublethal levels, RONS can cause neuronal death through phagocytosis. This type of scheme is supported by the findings that low levels of caspase activation in neurons can be reversible (45), and that phagocytosis actively contributes to developmental programmed cell death in *Caenorhabditis elegans* when caspase-3 activation is weak (46, 47). Furthermore, knock-out of the putative PS receptor in mice prevents developmental neuronal loss leading to pathologically enlarged brains (48), and microglia are known to mediate the loss of neurons in the developing mouse cerebellum and hippocampus (49, 50). These findings are suggestive of microglia or microglial phagocytosis contributing to neuronal death in mammals, and using immortalized cell lines, a recent study has reported the phagocytosis of

“normal” neurons by LPS- or amyloid-activated microglia (51). However, whether the inhibition of phagocytosis can prevent neuronal death has so far not been tested. Our data now show that inhibition of phagocytosis can prevent neuronal death under inflammatory conditions, and therefore that phagocytosis is the primary cause of death, rather than a secondary response to neuronal death caused by some other means.

As mentioned earlier, TLR2/4 can also be activated by endogenous ligands released from dying cells, and we cannot completely exclude the possibility that a small proportion of inflammatory-stressed neurons die and further activate microglia. However, inhibition of phagocytosis rescued the majority of neurons (Fig. 5), indicating that if secondary microglial activation contributed to neurodegeneration, this would also be through phagocytosis. Furthermore, we have shown that sublethal doses of peroxynitrite (either authentic or produced from SIN-1 decomposition; Figs. 4, 5) caused neuronal loss, which could be prevented by blocking phagocytosis. Importantly, this neuronal loss occurred in the absence of measurable inflammation, indicating that no secondary microglial activation caused by release of neuronal signals had taken place, and that peroxynitrite by itself was sufficient to induce neuronal loss.

Our results indicate a crucial role of the PS–MFG-E8–vitronectin receptor pathway in mediating neuronal loss through phagocytosis (Figs. 5, 6). MFG-E8 is one of a number of bridging molecules expressed by microglia (52, 53) that may enhance phagocytosis of neurons (21). In this study, we found that blocking the activity of MFG-E8 (using an MFG-E8 Ab) or preventing its interaction with neurons (using the C2A domain to block PS recognition) or microglia (using the RGD peptides to block the vitronectin receptor) significantly reduced inflammatory neuronal loss (Fig. 5). Therefore, although we cannot completely exclude the contribution of other signaling pathways, the PS–MFG-E8–vitronectin receptor system seems to have a nonredundant function in phagocytosis-mediated neuronal loss during LPS-, LTA-, and peroxynitrite-stimulated inflammation. Furthermore, we show in this article that the amyloid peptide, A β _{25–35}, also caused microglia-dependent neuronal loss, which could be prevented by blocking the microglial vitronectin receptor (Fig. 5E). This result indicates that the mechanism of primary phagocytosis described in this article is not limited to exogenous bacterial ligands, but may also apply to sterile inflammation induced by endogenous ligands as may occur during AD and other neurodegenerative conditions.

Our finding that inhibitors of phagocytosis prevented both neuronal loss and death indicates that the neurons must have been alive before engulfment. There was no evidence of apoptosis or necrosis (chromatin fragmentation, activated caspase-3, plasma membrane permeabilization) or dysfunction (loss of plasma membrane or mitochondrial membrane potential) in the neuronal population protected by inhibition of phagocytosis, suggesting that the spared neurons were normal. Other cellular changes that are sometimes associated with apoptosis (PS exposure, chromatin condensation) were induced in neurons by activated microglia but were fully reversible, indicating that the neurons were not committed to death.

The phagocytic removal of dying cells is generally believed to be a beneficial process, and failure to clear apoptotic cells and/or cellular debris can in itself lead to neuroinflammation and neurodegeneration (54). Furthermore, the phagocytic removal of apoptotic leukocytes by microglia has been shown to contribute to the resolution of autoimmune processes (55). It is therefore conceivable that inhibition of phagocytosis (as a therapeutic measure) would block these beneficial processes. However, different phagocytes use distinct phagocytic recognition systems depending on

tissue, inflammatory state, and which *eat-me* signals are present on target cells (56, 57). Thus, it is possible that specifically blocking the PS–MFG-E8–vitronectin receptor pathway may prevent inflammatory neuronal loss without preventing the beneficial roles of phagocytosis. However, this would require further investigation.

Analogous to macrophages, a classification of microglia based on distinct expression profiles, morphologies, and effector functions has been suggested (for reviews, see Refs. 58–60). The extreme points of this classification spectrum are the so-called classically activated M1 macrophage (induced by LPS and IFN- γ), and the alternatively activated M2 macrophage (induced by IL-4) or the deactivated M2 macrophage (induced by TGF- β or IL-10). Stimulation of microglia with LPS, LTA, or A β _{25–35} would, in all likelihood, result in their differentiation toward an M1 phenotype, and this is supported by their production of proinflammatory cytokines and RONS. However, the phagocytosis of PS-exposing cells is generally believed to be an anti-inflammatory event, which leads to the resolution of inflammation (56, 57), and LPS-stimulated microglia may release anti-inflammatory IL-10 after a delay of 48–72 h (51). Thus, the phenotype of our microglia is unlikely to be static but may be evolving from a mildly activated M1 to a deactivated M2. It will be of interest to test whether a switch to a microglial M2 phenotype occurs and whether this may be related to the phagocytic removal of viable neurons or its termination.

Inflammation, microglial activation through TLRs, and RONS-mediated neuronal injury have been implicated in many neurodegenerative conditions, including CNS infections, stroke, AD, and Parkinson's disease (for review, see Refs. 2, 4, 32, 61). Thus, our finding that neuronal death can be executed by microglial phagocytosis during inflammation may be relevant to a variety of brain pathologies.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Lobsiger, C. S., and D. W. Cleveland. 2007. Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. *Nat. Neurosci.* 10: 1355–1360.
2. Block, M. L., L. Zecca, and J. S. Hong. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat. Rev. Neurosci.* 8: 57–69.
3. Brown, G. C., and J. J. Neher. 2010. Inflammatory neurodegeneration and mechanisms of microglial killing of neurons. *Mol. Neurobiol.* 41: 242–247.
4. Glass, C. K., K. Saijo, B. Winner, M. C. Marchetto, and F. H. Gage. 2010. Mechanisms underlying inflammation in neurodegeneration. *Cell* 140: 918–934.
5. Kinsner, A., V. Pilotto, S. Deininger, G. C. Brown, S. Coecke, T. Hartung, and A. Bal-Price. 2005. Inflammatory neurodegeneration induced by lipoteichoic acid from *Staphylococcus aureus* is mediated by glia activation, nitrosative and oxidative stress, and caspase activation. *J. Neurochem.* 95: 1132–1143.
6. Choi, H. B., J. K. Ryu, S. U. Kim, and J. G. McLarnon. 2007. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. *J. Neurosci.* 27: 4957–4968.
7. Banati, R. B., S. E. Daniel, and S. B. Blunt. 1998. Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. *Mov. Disord.* 13: 221–227.
8. Nimmerjahn, A., F. Kirchhoff, and F. Helmchen. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308: 1314–1318.
9. Zitvogel, L., O. Kepp, and G. Kroemer. 2010. Decoding cell death signals in inflammation and immunity. *Cell* 140: 798–804.
10. Akakura, S., S. Singh, M. Spataro, R. Akakura, J. I. Kim, M. L. Albert, and R. B. Birge. 2004. The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin

- and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. *Exp. Cell Res.* 292: 403–416.
11. Bal-Price, A., and G. C. Brown. 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J. Neurosci.* 21: 6480–6491.
 12. Borisenko, G. G., T. Matsura, S. X. Liu, V. A. Tyurin, J. Jianfei, F. B. Serinkan, and V. E. Kagan. 2003. Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells—existence of a threshold. *Arch. Biochem. Biophys.* 413: 41–52.
 13. Oshima, K., N. Aoki, T. Kato, K. Kitajima, and T. Matsuda. 2002. Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles. *Eur. J. Biochem.* 269: 1209–1218.
 14. Giulian, D., K. Vaca, and M. Corpuz. 1993. Brain glia release factors with opposing actions upon neuronal survival. *J. Neurosci.* 13: 29–37.
 15. Lehnardt, S., L. Massillon, P. Follett, F. E. Jensen, R. Ratan, P. A. Rosenberg, J. J. Volpe, and T. Vartanian. 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc. Natl. Acad. Sci. USA* 100: 8514–8519.
 16. Giulian, D., and J. E. Ingeman. 1988. Colony-stimulating factors as promoters of amoeboid microglia. *J. Neurosci.* 8: 4707–4717.
 17. Suzumura, A., M. Sawada, and T. Marunouchi. 1996. Selective induction of interleukin-6 in mouse microglia by granulocyte-macrophage colony-stimulating factor. *Brain Res.* 713: 192–198.
 18. Lipton, S. A., Y. B. Choi, Z. H. Pan, S. Z. Lei, H. S. Chen, N. J. Sucher, J. Loscalzo, D. J. Singel, and J. S. Stamler. 1993. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364: 626–632.
 19. Block, M. L., and J. S. Hong. 2005. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog. Neurobiol.* 76: 77–98.
 20. Jung, H. I., M. I. Kettunen, B. Davletov, and K. M. Brindle. 2004. Detection of apoptosis using the C2A domain of synaptotagmin I. *Bioconjug. Chem.* 15: 983–987.
 21. Fuller, A. D., and L. J. Van Eldik. 2008. MFG-E8 regulates microglial phagocytosis of apoptotic neurons. *J. Neuroimmune Pharmacol.* 3: 246–256.
 22. Hanayama, R., M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu, and S. Nagata. 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature* 417: 182–187.
 23. Miksa, M., D. Amin, R. Wu, T. S. Ravikumar, and P. Wang. 2007. Fractalkine-induced MFG-E8 leads to enhanced apoptotic cell clearance by macrophages. *Mol. Med.* 13: 553–560.
 24. Haubner, R., R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, and H. Kessler. 1996. Structural and functional aspects of RGD-containing cyclic pentapeptides as highly potent and selective integrin α V β 3 antagonists. *J. Am. Chem. Soc.* 118: 7461–7472.
 25. Schrammel, A., S. Pfeiffer, K. Schmidt, D. Koesling, and B. Mayer. 1998. Activation of soluble guanylyl cyclase by the nitrovasodilator 3-morpholinodimethylamine involves formation of S-nitrosoglutathione. *Mol. Pharmacol.* 54: 207–212.
 26. Nicholls, D. G. 2006. Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. *J. Biol. Chem.* 281: 14864–14874.
 27. Reed-Geaghan, E. G., J. C. Savage, A. G. Hise, and G. E. Landreth. 2009. CD14 and toll-like receptors 2 and 4 are required for fibrillar A β -stimulated microglial activation. *J. Neurosci.* 29: 11982–11992.
 28. Combs, C. K., D. E. Johnson, S. B. Cannady, T. M. Lehman, and G. E. Landreth. 1999. Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. *J. Neurosci.* 19: 928–939.
 29. Combs, C. K., J. C. Karlo, S. C. Kao, and G. E. Landreth. 2001. beta-Amyloid stimulation of microglia and monocytes results in TNF α -dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J. Neurosci.* 21: 1179–1188.
 30. Muta, T., and K. Takeshige. 2001. Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 reconstitution of TLR2- and TLR4-activation by distinguishable ligands in LPS preparations. *Eur. J. Biochem.* 268: 4580–4589.
 31. Poon, I. K., M. D. Hulett, and C. R. Parish. 2010. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ.* 17: 381–397.
 32. Lucin, K. M., and T. Wyss-Coray. 2009. Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron* 64: 110–122.
 33. Kinsner, A., M. Boveri, L. Hareng, G. C. Brown, S. Coecke, T. Hartung, and A. Bal-Price. 2006. Highly purified lipoteichoic acid induced pro-inflammatory signalling in primary culture of rat microglia through Toll-like receptor 2: selective potentiation of nitric oxide production by muramyl dipeptide. *J. Neurochem.* 99: 596–607.
 34. Gao, H. M., J. Jiang, B. Wilson, W. Zhang, J. S. Hong, and B. Liu. 2002. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J. Neurochem.* 81: 1285–1297.
 35. Gavilán, M. P., E. Revilla, C. Pintado, A. Castaño, M. L. Vizuete, I. Moreno-González, D. Baglietto-Vargas, R. Sánchez-Varo, J. Vitorica, A. Gutiérrez, and D. Ruano. 2007. Molecular and cellular characterization of the age-related neuroinflammatory processes occurring in normal rat hippocampus: potential relation with the loss of somatostatin GABAergic neurons. *J. Neurochem.* 103: 984–996.
 36. Qin, L., X. Wu, M. L. Block, Y. Liu, G. R. Breese, J. S. Hong, D. J. Knapp, and F. T. Crews. 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55: 453–462.
 37. Park, J. S., D. Svetkauskaite, Q. He, J. Y. Kim, D. Strassheim, A. Ishizaka, and E. Abraham. 2004. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J. Biol. Chem.* 279: 7370–7377.
 38. Caso, J. R., J. M. Pradillo, O. Hurtado, P. Lorenzo, M. A. Moro, and I. Lizasoain. 2007. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation* 115: 1599–1608.
 39. Lehnardt, S., E. Schott, T. Trimbuch, D. Laubisch, C. Krueger, G. Wulczyn, R. Nitsch, and J. R. Weber. 2008. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. *J. Neurosci.* 28: 2320–2331.
 40. Babcock, A. A., M. Wrenfeldt, T. Holm, H. H. Nielsen, L. Dissing-Olesen, H. Toft-Hansen, J. M. Millward, R. Landmann, S. Rivest, B. Finsen, and T. Owens. 2006. Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation. *J. Neurosci.* 26: 12826–12837.
 41. Lehnardt, S., S. Lehmann, D. Kaul, K. Tschimmel, O. Hoffmann, S. Cho, C. Krueger, R. Nitsch, A. Meisel, and J. R. Weber. 2007. Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. *J. Neuroimmunol.* 190: 28–33.
 42. Walter, S., M. Letiembre, Y. Liu, H. Heine, B. Penke, W. Hao, B. Bode, N. Manietta, J. Walter, W. Schulz-Schuffer, and K. Fassbender. 2007. Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell. Physiol. Biochem.* 20: 947–956.
 43. Fabisiak, J. P., V. A. Tyurin, Y. Y. Tyurina, A. Sedlov, J. S. Lazo, and V. E. Kagan. 2000. Nitric oxide dissociates lipid oxidation from apoptosis and phosphatidylserine externalization during oxidative stress. *Biochemistry* 39: 127–138.
 44. Balasubramanian, K., B. Mirnikjoo, and A. J. Schroit. 2007. Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. *J. Biol. Chem.* 282: 18357–18364.
 45. Li, Z., J. Jo, J. M. Jia, S. C. Lo, D. J. Whitcomb, S. Jiao, K. Cho, and M. Sheng. 2010. Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization. *Cell* 141: 859–871.
 46. Reddien, P. W., S. Cameron, and H. R. Horvitz. 2001. Phagocytosis promotes programmed cell death in *C. elegans*. *Nature* 412: 198–202.
 47. Hoepfner, D. J., M. O. Hengartner, and R. Schnabel. 2001. Engulfment genes cooperate with ced-3 to promote cell death in *Caenorhabditis elegans*. *Nature* 412: 202–206.
 48. Li, M. O., M. R. Sarkisian, W. Z. Mehal, P. Rakic, and R. A. Flavell. 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science* 302: 1560–1563.
 49. Marin-Teva, J. L., I. Dusart, C. Colin, A. Gervais, N. van Rooijen, and M. Mallat. 2004. Microglia promote the death of developing Purkinje cells. *Neuron* 41: 535–547.
 50. Wakselmann, S., C. Béchade, A. Roumier, D. Bernard, A. Triller, and A. Bessis. 2008. Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAPI2 immunoreceptor. *J. Neurosci.* 28: 8138–8143.
 51. McArthur, S., E. Cristante, M. Paterno, H. Christian, F. Roncaroli, G. E. Gillies, and E. Solito. 2010. Annexin A1: a central player in the anti-inflammatory and neuroprotective role of microglia. *J. Immunol.* 185: 6317–6328.
 52. Milner, R., and I. L. Campbell. 2003. The extracellular matrix and cytokines regulate microglial integrin expression and activation. *J. Immunol.* 170: 3850–3858.
 53. Re, F., S. L. Belyanskaya, R. J. Riese, B. Cipriani, F. R. Fischer, F. Granucci, P. Ricciardi-Castagnoli, C. Brosnan, L. J. Stern, J. L. Strominger, and L. Santambrogio. 2002. Granulocyte-macrophage colony-stimulating factor induces an expression program in neonatal microglia that primes them for antigen presentation. *J. Immunol.* 169: 2264–2273.
 54. Napoli, I., and H. Neumann. 2009. Microglial clearance function in health and disease. *Neuroscience* 158: 1030–1038.
 55. Chan, A., R. Seguin, T. Magnus, C. Papadimitriou, K. V. Toyka, J. P. Antel, and R. Gold. 2003. Phagocytosis of apoptotic inflammatory cells by microglia and its therapeutic implications: termination of CNS autoimmune inflammation and modulation by interferon-beta. *Glia* 43: 231–242.
 56. Wu, Y., N. Tibrewal, and R. B. Birge. 2006. Phosphatidylserine recognition by phagocytes: a view to a kill. *Trends Cell Biol.* 16: 189–197.
 57. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2: 965–975.
 58. Ransohoff, R. M., and A. E. Cardona. 2010. The myeloid cells of the central nervous system parenchyma. *Nature* 468: 253–262.
 59. Martinez, F. O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu. Rev. Immunol.* 27: 451–483.
 60. Ransohoff, R. M., and V. H. Perry. 2009. Microglial physiology: unique stimuli, specialized responses. *Annu. Rev. Immunol.* 27: 119–145.
 61. Lehnardt, S. 2010. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia* 58: 253–263.