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Critical Role of TLR4 Response in the Activation of Microglia Induced by Ethanol

Sara Fernandez-Lizarbe, Maria Pascual, and Consuelo Guerri

Microglial cells are the primary immune effector cells in the brain and play a pivotal role in the neuroinflammatory processes associated with a variety of neurological and pathological disorders. Alcohol consumption induces brain damage, although the neuropathological processes are poorly understood. We previously suggested that ethanol promotes inflammatory processes in the brain, up-regulating inflammatory mediators and signaling pathways associated with IL-1RI/TLR4 receptors. In the present study we investigate whether ethanol induces microglia activation by stimulating TLR4 response and whether this response causes neuronal death and contributes to ethanol-induced neuroinflammatory damage. We demonstrate that ethanol activates microglia and stimulates NF-κB, MAPKs, and MyD88-independent (IFN regulatory factor-3, IFN-β) pathways to trigger the production of inflammatory mediators, causing neuronal death. The inflammatory response induced by ethanol is completely abrogated in microglia of TLR4-deficient mice (TLR4−/−), thus supporting the role of these receptors in microglia activation and neuronal death. In accord with the in vitro findings, acute ethanol administration induces microglia activation (CD11b+ cells) in cerebral cortex of TLR4−/− mice, but not in TLR4−/− mice. Taken together, our results not only provide the first evidence of the critical role of the TLR4 response in the ethanol-induced microglia activation, but also new insight into the basic mechanisms participating in ethanol-induced neuroinflammatory damage. The Journal of Immunology, 2009, 183: 4733–4744.

Among these receptors, stimulation of TLR4 in microglial cells has been implicated with oligodendrocyte injury (10), neurodegeneration (11), and apoptosis of activated microglia (12, 13).

TLR responses involve the recruitment and activation of complex intracellular signaling cascades, which culminate with the induction of cytokines and other inflammatory mediators. Activation of TLRs leads to the recruitment of different Toll/IL-1R (TIR) domain-containing adaptor proteins (14, 15), including MyD88, MyD88 adaptor-like/TIR-associated proteins (MAL/TIRAP), TIR domain-containing adaptor-inducing IFN-β (TRIF), and Toll receptor-associated molecule (TRAM). These adaptors couple two distinct signaling pathways. One pathway, MyD88-dependent and MAL, activates the serine/threonine IL-1R-associated kinase (IRAK) and triggers a fast downstream stimulation of NF-κB and the induction of genes encoding inflammation-associated molecules and cytokines, as well as the activation of MAPK and AP-1 NF. The second, TRAM and TRIF, activates the IFN response transcription factor IFN regulatory factor-3 (IRF-3) and a number of IFN-β-dependent genes (16).

Recent evidence has suggested the role of TLR activation in brain injury and neurodegeneration (17). Alcohol is a neurotoxic compound and its abuse can cause brain damage (18, 19), and, in some cases, neurodegeneration (20). Although the neuropathological processes underlying these effects remain poorly understood, we have recently demonstrated that chronic ethanol abuse can increase cytokines and inflammatory mediators in the brain and cause cell death (21), suggesting neuroinflammatory damage (22). We further demonstrate that ethanol, at physiological relevant concentrations, is capable of activating TLR4 signaling not only in astrocytes (23), but also in RAW 264.7 cells (24), since blocking

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1 Abbreviations used in this paper: TRIF, Toll/IL-1R containing adaptor-inducing IFN-β; COX-2, cyclooxygenase-2; iNOS, inducible NO synthase; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; LTA, lipoteichoic acid; PMBS, polysaccharide B sulfate; TRAM, Toll receptor-associated molecule.

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TLR4 receptors abolishes the inflammatory signaling response, indicating that activation of TLR4 by ethanol could be an important mechanism of ethanol-induced neuroinflammation (22).

Therefore, considering the critical role of both microglia and TLR4 in the neuroinflammation and pathogenesis of many neurodegenerative disorders (11), and the interaction of ethanol with TLRs, this study was designed to assess whether ethanol induces microglia activation by stimulating TLR4 response and whether this response triggers neuronal death and contributes to ethanol-induced cell damage. Here we report that ethanol induces microglia activation and phagocytosis, and it triggers the production of inflammatory mediators and cytokines to contribute to the apoptotic observed in cortical neurons. Our results provide strong evidence of the role of TLR4 in ethanol-induced inflammatory response, since microglia of TLR4-deficient (TLR4$^{-/-}$) mice treated with ethanol induce neither inflammatory response nor neuronal death.

**Materials and Methods**

### Animals

Female Wistar rats (Harlan Ibérica) weighing 200–250 g, C57BL/6 wild-type mice (Harlan Ibérica), and the TLR4$^{-/-}$ knockout mice (C57BL/6 background, provided by Dr. S. Akira) were used. All animals were kept under controlled light/dark conditions (12/12 h), temperature (23°C), and humidity (60%). After mating, the dams were placed in separate cages during the gestation period. All animal experiments were conducted in accordance with the guidelines approved by the European Communities Council Directive (86/609/ECC) and by Spanish Royal Decree 1201/2005.

### Culture of microglial cells

Primary rat and mouse cultures of cortical mixed glial cells were prepared as previously described (25). Cerebral cortices from 1- to 3-day-old neonatal rats or mice were dissected, carefully stripped of their meninges, and digested with 0.25% trypsin for 30 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium (DMEM/F-12; Invitrogen) with 14% FBS and 0.02% DNase I. The solution was pelleted, resuspended in culture medium, and brought to a single cell suspension by repeated pipetting following by passage through a 90-μm pore mesh. Cells were seeded at density of either 250,000 or 125,000 cells/ml for rat and mouse cultures, respectively. Cells were maintained at 37°C in humidified 5% CO2/95% air. Medium was replaced every 4–5 days, and cultures were used between 12 and 18 days in vitro.

Rat and mouse microglial cultures were prepared as previously described (26). Briefly, confluent mixed glial cultures were subjected to mild trypsinization (0.05%) in the presence of 0.25 mM EDTA and 0.5 mM Ca2+.

This results in the detachment of astroglial cells and leaves a population of firmly attached cells identified as >98–99% microglia, as determined by immunofluorescence using anti-CD11b, anti-GRF, and anti-mitogen-activated protein-2. Twenty-four hours after trypsinization, cells were rinsed and incubated in serum-free medium with N2 Supplement (Invitrogen) for 18 h, and then microglial cells were treated with either ethanol (10, 50, 100, or 200 mM; Merck Sharp and Dohme), LPS (50 ng/ml; Sigma-Aldrich), or LTA (1 μg/ml; Sigma-Aldrich), or LPS antagonist, polyoxyn B-sulfate (PMBS; 10 μg/ml; Sigma-Aldrich), was added to the cell medium 15 min before and during ethanol or LPS treatment, or to supernatants from TLR4$^{-/-}$ microglia treated with or without ethanol. PMBS is a cationic cyclic polypeptide that binds to the lipid A portion of the LPS in the cell membrane of Gram-negative bacteria, acting as a specific LPS antagonist.

### Morphological analyses

Rat microglia were stimulated with 50 mM ethanol for 7 or 24 h. Cells grown on 12-well culture plates were examined in a minimum of three wells for each experiment, and at least three experiments were conducted for each condition. Morphological differentiation was digitally recorded with a camera (model DFC-480; Leica Microsystems) mounted on a Leica microscope (model DM-6000B; Leica Microsystems).

### BrdU incorporation

Microglial cells, either treated with or without ethanol for 12, 24, and 48 h, were incubated with BrdU labeling reagent for 30 min at 37°C, following the manufacturer’s instructions (Roche Diagnostics). Briefly after postfixation with 70% ethanol, cells were incubated with 4 N HCl for 10 min at room temperature, and the staining with a monoclonal anti-BrdU Ab was coupled with FITC (Roche Diagnostics) for 45 min at 37°C. Finally, samples were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter).

### Phagocytosis

Rat microglial cells were cultured on glass coverslips and incubated with 2-μm-diameter Fluospheres at 0.01% solid mass (Molecular Probes) at 37°C for 30 min. After rinsing four times with PBS, cells were fixed with methanol for 5 min at −20°C and incubated for 1 h with FITC-labeled tomato lectin (1:500; Sigma-Aldrich). After a 5-min incubation with Hoechst 33342 (2.5 μg/ml in PBS; Molecular Probes), coverslips were mounted in fluorescent mounting medium (Difco). The results were digitally recorded with a camera (Ricoh XR-R 3000) mounted on a Zeiss microscope (Axioskop 2). To verify uptake by phagocytes, Z-stack images through the microglia cells were obtained by a Leica confocal microscope (model TCS-S2-POB) to corroborate that the fluorescein-labeled latex beads had been internalized and were not above the cell or adherent to the cell surface.

### Cytokine analysis

TNF-α and IL-1β levels were determined in cell culture media by ELISA kits from Diacletech Research (BioNova Científica) following the manufacturer’s protocols.

### Measurement of nitrite production

NO production was assessed by measuring the accumulation of nitrite in the culture medium by the Griess reaction using sodium nitrite as standard. Aliquots of culture medium were mixed with an equal volume of Griess reagent prepared by mixing equal volumes of 1% (w/v) sulfanilamide in 30% acetic acid and 0.5% (w/v) of N-1-naphthylethenediamine dihydrochloride in 60% acetic acid. Then, absorbance was measured at 540 nm in an automated microplate reader.

### Neuronal culture and apoptotic quantification by flow cytometry

Primary cultures of rat or mouse cortical neurons were prepared as previously described (31), although some modifications were made for mice cortical neurons. Briefly, cortices from 17-day-old fetal wild-type mice were triturated and digested with 0.05% trypsin for 15 min at 37°C. Trypsinization was stopped with 14% FBS and 0.015% DNase I. Cells were harvested by centrifugation, resuspended in DMEM culture medium (Invitrogen), and passed through a 90-μm pore mesh. Cells were plated at a density of 1 × 105 cells/ml onto poly-n-lysine (12.5 μg/ml)-coated tissue culture wells. After 1 h of incubation in a humidified atmosphere of 5% CO2/95% air at 37°C, the cell medium was replaced by Neurobasal medium supplemented with B-27 (Invitrogen) and 1% glutamine.

At day 5 in vitro, the rat or mouse neuronal medium was removed and replaced by a conditioned medium from microglial cells treated with or without ethanol (50 mM) for 24 or 48 h. Neurons were incubated with microglia-conditioned medium for 1 and 2 days. Then, to analyze apoptosis and to differentiate between apoptosis and necrosis, cells were harvested and incubated with R-PE-conjugated annexin V (Molecular Probes) in combination with the cell-impermeant DNA fluorochrome 7-aminoactinomycin D (7-ADD; Molecular Probes) at room temperature for 15 min, following manufacturer’s protocol. Finally, samples were analyzed with a Cytomics FC500 flow cytometer (Beckman Coulter) and data (annexin V$^+$ and 7-ADD$^-$ cells) were illustrated by CXP software.
**Immunoprecipitation**

Microglial cells were treated with lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8), 4 mM sodium chloride, 40 mM sodium fluoride, and protease inhibitors) for 30 min on ice. Cellular extracts were pre-cleared with protein G-agarose for 1 h and then incubated overnight with anti-IRAK polyclonal Ab or with anti-STAT-1 polyclonal Ab (Santa Cruz Biotechnology) or preimmune serum. Then, protein G-agarose beads were added and samples were incubated for 1 h. Precipitated beads were solubilized by boiling in SDS buffer, and samples were analyzed by Western blot.

**Western blot analysis**

After ethanol, LPS, or LTA treatment, microglial cells were dissolved in lysis buffer for 30 min on ice. To analyze p65, subunit of NF-κB, the nuclear fraction was isolated as previously described (32). An equal amount of cell lystate of each sample was loaded onto SDS-PAGE and then blotted onto polyvinylidene difluoride membrane. Membranes were blocked with 5% dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), and then incubated overnight with the following Abs: anti-lamin A/C, anti-phospho (P)-IRAK1 (Ser172), and anti-P-IRF-3 (Ser396) from Cell Signaling Technology; and anti-IRAK1, anti-P-ERK-1/2, anti-ERK, anti-P-p38, anti-p38, anti-P-JNK, anti-JNK, anti-inducible NO synthase (iNOS), anti-cyclooxygenase-2 (COX-2), anti-p65, anti-P-serine, anti-IRF-1, anti-IRF-3, and anti-GAPDH from Santa Cruz Biotechnology. Membranes were then incubated with the appropriate secondary Ab, and were visualized with either ECL system (ECL Plus, Amersham Pharmacia Biotech) or alkaline phosphatase conjugate (Sigma-Aldrich). Band densities were quantified using the Alphalager 2200 software (Alpha Innotech).

**Native PAGE for IRF-3 detection**

To detect dimeric IRF-3, cells were lysed in whole microglia cell extract. After addition of native sample buffer (without SDS and 2-ME), cell extracts were run on a 10% separating gel and 4% stacking gel without SDS. Gels were pre-run for 20 min at 15 mA at 4°C. Electrophoretic run was conducted at 15 mA and 4°C using a Tris-glycine running buffer (27 mM Tris (pH 8)/186 mM glycine), resulting in a separation without SDS. Gels were pre-run for 20 min at 15 mÅ at 4°C. Afterward the tissue was rinsed in TBST and incubated with a biotinylated anti-IRF-3 antibody for 1 h. These sections were incubated with fluorescein-labeled latex beads for 30 min and incubated with streptavidin-peroxidase. Scale bar, 10 μm. B. Western blot analysis of phospho-p65 (P-p65) using a specific Ab for P-p65. The Western blot analysis of phospho-p65 stained with an antibody specific to P-p65. The results were analyzed using the ImageJ software with data normalized to the mRNA level of the cyclophilin A. Data analyses were done using the LightCycler 480 relative quantification software. Quantification was assessed by measuring the percentage of threshold area occupied by CD11b staining relative to the whole area of tissue in cerebral cortex sections of nontreated (control) and ethanol-treated animals. Results were expressed as fold increase over control values. Approximately 18–20 fields of the cerebral cortex from three to four coronal brain sections of three to four animals per group were analyzed.

**Results**

Ethanol changes the morphology of microglial cells

Microglial cells, the resident macrophage population of the brain, are activated in response to different stimuli, which lead to morphological and functional changes. Therefore, to investigate whether ethanol activates the morphology of microglia, cells were incubated with ethanol (50 mM) for either 7 or 24 h. Fig. 1A shows that ethanol treatment induced an activated state presenting a rod-like bipolar morphology with elongated cell bodies. Additionally, ethanol-treated cells also showed bushy morphology characterized by multiple processes radiating from the central cell body (arrows) and ameboid or macrophage-like morphology (arrowheads).

To assess whether the changes in morphology in the ethanol-treated microglia were associated with changes in cell proliferation, we incorporated BrdU into the microglial cells treated with or without 50 mM ethanol for 12, 24, and 48 h. The results revealed that, in the absence of serum, very low levels in the BrdU incorporation were detected in either cells treated with or without ethanol for 12, 24, and 48 h, and no differences were noted between control and ethanol-treated cells (data not shown). These results indicate that the changes in morphology were not associated with changes in cell proliferation.
indicating that ethanol stimulates the phagocytic machinery of microglia cells, which were capable of ingesting latex beads.

**Ethanol activates microglial cells to release inflammatory mediators, which induces neuronal cell death**

As one of the characteristic of activated microglia is to secrete proinflammatory mediators, we determined the production of TNF-α, IL-1β, and NO in the medium of microglial cells treated with ethanol, at different concentrations (10, 50, and 100 mM), for 7, 24, and 48 h. As a positive control, we used microglial cells stimulated with LPS (50 ng/ml) for 24 h. Fig. 2A shows that both LPS and ethanol, at either low or high concentrations, stimulated microglia to produce cytokines and NO. In general, ethanol stimulated the release of TNF-α, IL-1β, and NO in microglial cells from 7 h onward at all the concentrations used (10, 50, and 100 mM), although the maximum increase was observed at 24 h. At this time, the production of IL-1β was similar in cells treated with either ethanol or LPS. Nonetheless, the levels of TNF-α and NO were ~2-fold higher in LPS (TNF-α, 188 ± 10 pg/ml; NO, 7.72 ± 1.3 μM) than in the ethanol-treated microglia (TNF-α, 65–84 pg/ml; NO, 2.4–3.2 μM). The cytokine and NO levels were in the previously described range for LPS-activated microglia (33, 34).

We next addressed whether the production of cytokines and other inflammatory mediators secreted by activated microglia can induce neuronal damage. For these experiments cortical neurons (at 5 days in vitro) were cultured with microglia-conditioned medium obtained from those cells treated with 50 mM ethanol for either 24 or 48 h, in which maximal release of inflammatory mediators was observed (Fig. 2A). Neurons were cultured with the microglia-conditioned medium for 1 and 2 days and neuronal apoptosis was measured by flow cytometry. Furthermore, since the microglia-conditioned medium contained some alcohol, we used neurons incubated with a medium in which ethanol was added at the same concentrations found in the conditioned medium of ethanol-treated microglia as control neurons. The ethanol concentrations found were 4.8 ± 1.1 and 2.5 ± 0.7 mM for the supernatant of the microglia treated with ethanol for 1 or 2 days, respectively. The results in Fig. 2B show a significant apoptosis in the neurons cultured for 2 days with the supernatant of microglia treated with 50 mM ethanol for 24 or 48 h. However, higher levels of apoptosis were observed in neurons incubated with conditioned medium of 48-h ethanol-treated microglia than in those neurons exposed to the conditioned-medium of microglia treated for 24 h with ethanol. The results also revealed that the addition of 2.5 or 4.8 mM ethanol to the culture medium did not significantly affect the natural apoptosis observed in those neurons cultured without alcohol (data not shown). These results indicate that microglia produced inflammatory mediators in response to ethanol and that the mediators accumulated in the medium were capable of inducing neuronal death.

**Ethanol activates IRAK and MAPK pathways in microglia**

Our previous results demonstrate that ethanol activates macrophages by stimulating TLR4 response, initiating an inflammatory
processes (24). We therefore determined whether acute ethanol treatment could stimulate TLRs in microglial cells, by analyzing the activation of IRAK, ERK, p38, and JNK (35) in astroglia treated with 50 mM ethanol over different times. To assess IRAK phosphorylation, this kinase was immunoprecipitated and then detected with anti-P-IRAK1 (Ser376) by Western blot. Fig. 3A shows that the phosphorylation of IRAK occurred rapidly at 10 min, increased at 30 min, and then remained high up to 3 h. Similarly, phosphorylation of ERK, p38, and JNK also occurred as early as 10 min, although maximal activation was noted between 30 and 60 min and decreased thereafter (Fig. 3A). Furthermore, when cells were treated with different ethanol concentrations for 30 min, we observed a dose-dependent increase in the activation of ERK, JNK, and p38, although no significant differences in phosphorylation of these kinases were noted between 50 and 100 mM ethanol (Fig. 3B).

**Ethanol treatment activates the NF-κB pathway and increases iNOS and COX-2 in microglial cells**

Activation of TLRs leads to a rapid downstream stimulation of NF-κB signaling (35), which triggers the transcriptional induction of some inflammatory mediators, including COX-2 and iNOS (36, 37). We therefore investigated the potential nuclear translocation of NF-κB following the stimulation of microglia with ethanol. For these experiments, microglial cells were treated with either ethanol (50 mM) or LPS (50 ng/ml) for 30 min, and the p65 subunit of NF-κB in the nuclear fraction was assessed. As shown in Fig. 4A, either ethanol or LPS was capable of activating NF-κB, as demonstrated by the increased levels of the NF-κB subunit, p65, in the nucleus. We then examined whether microglia expressed the inflammatory mediators iNOS and COX-2 in response to ethanol treatment. Fig. 4B shows that while the iNOS levels significantly increased at 1 and 3 h, and then decreased at 7 and 24 h of ethanol treatment, the COX-2 expression peaked 3 and 7 h of treatment, although these levels were still high after 24 h of the ethanol incubation. These results indicate that ethanol is capable of inducing a rapid response of NF-κB in microglia, triggering the expression of cytokines (e.g., TNF-α) and inflammatory mediators such as iNOS and COX-2.

To assess whether the ethanol-induced inflammatory signaling was due to the direct effects of ethanol and not to contaminating endotoxin, microglial cells were incubated with the LPS antagonist PMBS (10 μg/ml) 15 min before and during ethanol (50 mM) or LPS (50 ng/ml) treatment. The levels of ERK phosphorylation and COX-2 expression were evaluated. We found that while PMBS completely abolished the LPS-induced ERK phosphorylation and COX-2 expressions, treatment with PMBS did not significantly change the effects of ethanol on these proteins (Fig. 4C). These results confirm direct effects of ethanol on the activation of ERK and COX-2 expression, rather than to contaminating endotoxin.

**Ethanol induces the activation of both IRF-3 and the STAT-1/IRF-1 pathways in cortical microglial cells**

Recent evidence indicated that TLR4 and TLR3 responses trigger two distinct signaling pathways, MyD88-dependent and -independent (38). The second pathway utilized TRAM/TRIF adaptors and led to the activation of IRF-3 and to the induction of IFN-inducible genes (39, 40). Previous results in our laboratory demonstrated that ethanol promotes the activation of TLR4 signaling response in macrophages, since blocking these receptors abolishes the inflammatory response induced by ethanol (24). We therefore evaluated whether ethanol could stimulate a MyD88-independent pathway. Activation of IRF-3 by ethanol was assessed by detecting the formation of IRF-3 dimers, which results from the phosphorylation of IRF-3 at several residues in the C-terminal end (41). Fig. 5A shows the IRF-3 activation in microglial cells treated with 50 mM ethanol. Ethanol significantly increased the formation of IRF-3 dimers.
and the phosphorylation of IRF-3 after 3 h of ethanol treatment. Furthermore, because activation of IRF-3 induces the production of IFN-γ, which may in turn initiate STAT-1/IRF-1 signaling pathway in microglia (13), we assessed the effects of ethanol on STAT-1 phosphorylation (Fig. 5B) and IRF-1 expression (Fig. 5C). Ethanol stimulation increased both the STAT-1 phosphorylation and the IRF-1 expression at 1 and 3 h of ethanol treatment. These results suggest that ethanol is capable of activating a MyD88-independent pathway and the production of IFN-γ, which might initiate the STAT-1/IRF-1 pathway.

TLR4 response is critical for ethanol-induced microglia activation

Because our previous studies suggest that ethanol induces inflammatory signaling by activating TLR4 receptors (24, 42), we assessed the potential role of TLR4 response in the ethanol-induced microglia activation by using microglial cells of wild-type and TLR4−/− knockout mice. For these experiments, cells were stimulated with ethanol, and the production of cytokines and MAPK activation were assessed. Fig. 6A shows that microglia of wild-type mice produced TNF-α in response to ethanol (50 mM), as demonstrated by the significant increase noted in the mRNA levels of TNF-α after 3 h of ethanol treatment. Conversely, no changes in the TNF-α levels in response to ethanol were observed in TLR4−/− microglia. We then determined the activation of MAPKs induced by LPS or ethanol in microglial cells of wild-type and TLR4−/− mice. According to the results with rat microglia (Fig. 3), treatment with either LPS (50 ng/ml) or ethanol (10, 50, 100, and 200 mM) for 30 min induced the phosphorylation of ERK, JNK, and p38 (Fig. 6, B and C) in microglia of wild-type mice. However, activation of the above kinases does not occur in LPS- or ethanol-stimulated microglia of TLR4−/− mice. These results support the role of TLR4 response in the ethanol-induced activation of microglia cells.

To investigate whether TLR4-deficient cells could respond to other TLRs ligands, microglial cells were treated with LTA, a TLR2 agonist, and the activation of ERK, JNK, and p38 phosphorylation was measured upon 30-min and 2-h LTA treatment. Fig. 6D shows that LTA induced phosphorylation of ERK, JNK, and p38 upon 2 h of treatment in both TLR4 wild-type and TLR4-deficient microglia. These results reveal that TLR4-deficient cells are able to respond to TLR2 ligand.

Furthermore, to evaluate the role of the TLR4-mediated MyD88-independent pathway in ethanol-induced stimulation of microglia, we analyzed the activation of IRF-3, the IFN-β mRNA levels, and...
microglia of TLR4−/− mice (Fig. 6, F and G). These results support the notion that ethanol-induced cytokine production, including IFN-β, is mediated by TLR4 activation via MyD88-dependent and -independent pathways.

A deficiency in the TLR4 function protects neurons from the effects of ethanol on microglia activation

We then assessed the relationship between the TLR4 function and the neurotoxicity associated with ethanol-induced microglia activation. To do this, we performed similar experiments as those described above, but in this case, the conditional medium from the wild-type or TLR4−/− microglia treated with 50 mM ethanol for 24 and 48 h were added to mice cortical neurons at 5 days in vitro. The murine neurons were cultured with the microglia-conditioned medium for 1 day and the neuronal apoptosis was measured by flow cytometry.

Fig. 7A shows a significant apoptosis in the neurons cultured for 1 day with the supernatant of wild-type microglia treated with 50 mM ethanol for 48 h. Conversely, we did not observe significant changes in neuronal survival when neurons were cultured with the supernatant of ethanol-treated TLR4−/− knockout cells. These findings support the notion that ethanol-induced neuronal death depends on the activated TLR4 pathway in microglial cells.

Finally, as microglia can be activated by endogenous TLR ligands (43, 44), we evaluated the possibility of ethanol being able to release endogenous TLR ligands from microglia, which could, on TLR4 wild-type microglia, act in an autocrine- or a paracrine-like manner, leading to the activation of the TLR4 pathway. To investigate this possibility, the supernatants from TLR4−/− microglia treated with or without 50 mM ethanol for 24 and 48 h were added to wild-type microglia plates in the presence or absence of PMBS, and P-ERK and iNOS levels were assessed after 30 min and 3 h of incubation, respectively. There were no significant differences in the levels of P-ERK and iNOS irrespective of the nature of the conditioned medium (control or ethanol-treated) or the presence or absence of PMBS (Fig. 7B). These results suggest that the culture supernatant of ethanol-treated TLR4−/− microglia does not contain endotoxin, endogenous TLR ligands, or other compounds that could activate wild-type microglia under our in vitro experimental conditions.

Role of TLR4 in ethanol-induced in vivo microglia activation

To provide more physiological relevance to our in vitro results on the role of TLR4 receptors in the ethanol-induced microglia activation, we performed in vivo experiments in which one i.p. injection of either saline or ethanol (4 g/kg) was administered to TLR4 wild-type and TLR4−/− knockout animals for 3 consecutive days. The activated microglia in coronal brain sections were analyzed for CD11b expression by immunohistochemistry. Representative micrographs from the cerebral cortex of wild-type and TLR4−/− knockout are shown in Fig. 8. Morphometric analysis revealed that ethanol administration induced greater CD11b immunoreactivity in the cerebral cortex of wild-type mice than in TLR4−/− knockout mice.

Discussion

Activation of microglia in infection, inflammation, or injury leads to the release of various toxic molecules, including cytokines and inflammatory mediators, contributing to the neuronal damage in various neurodegenerative disorders (4, 45). We have previously demonstrated that chronic ethanol treatment increases the signaling pathways associated with IL-1RI/TLR4 receptors in the brain and in astrocytes, and it triggers the production of cytokines and...
FIGURE 6. Effects of ethanol on TLR4 signaling in microglia of TLR4−/− and wild-type mice. A, Levels of TNF-α mRNA in microglial cells of wild-type and TLR4−/− mice treated with or without ethanol (50 mM) for 1, 3, and 7 h. B, Immunoblot analysis and (C) quantification of P-ERK, P-JNK, and P-p38 in microglial extracts of wild-type and TLR4−/− mice, treated either with ethanol (10, 50, 100, and 200 mM) or LPS (50 ng/ml)-treated cells of wild-type and TLR4−/− mice for 30 min. D, Immunoblot analysis of P-ERK, P-JNK and P-p38 from wild-type (WT) or TLR4−/− (KO) microglia treated with LTA (1 μg/ml) for 30 min and 2 h. A representative immunoblot from three different experiments is shown. E, To detect the formation of IRF-3 dimers, the microglia cells of TLR4−/− and wild-type mice, treated with or without ethanol (50 mM) for 1 and 3 h, were harvested and run on native gels as described in Materials and Methods. The monomeric and dimeric IRF-3 forms in the blots were detected by IRF-3 Ab, and then blots were reprobed with the P-IRF-3 (Ser396) Ab. F, The IFN-β mRNA levels of microglial cells of wild-type and TLR4−/− mice were treated with or without ethanol (50 mM) for 1, 3, and 7 h. G, Immunoblot analysis and quantification of IRF-1 in cell extracts of TLR4−/− and wild-type microglia treated with ethanol (50 mM) for different times (10 and 30 min and 1, 3, 7, and 24 h). Data represent means ± SEM; n = 7 independent experiments. *p < 0.05 and **p < 0.01 compared with the control value, according to a one-way ANOVA followed by Dunnett’s post hoc test. Blots were stripped, and the total quantities of ERK, JNK, p38, and GAPDH were also assessed. A representative immunoblot of each protein is shown.
inflammatory mediators and induces neural cell death (21). Although the molecular events from CNS injury leading to the activation of innate immunity are not well understood, we have proposed that ethanol activates IL-1RI/TLR4 receptors in glial cells and triggers neuroinflammation, which contributes to the pathogenesis of brain damage induced by ethanol consumption (22). However, whether microglia activation contributes to these events is presently unknown. We herein provide evidence that ethanol, at physiological relevant concentrations, induces morphological activation of microglia and is capable of activating TLR4 signaling to result in the secretion of cytokines and inflammatory mediators by microglia, which triggers neuronal apoptosis. We further show the lack of inflammatory response in ethanol-treated microglial cells of TLR4-/- mice, suggesting that TLR4 receptors play a key role in ethanol-induced microglia activation.

Experimental evidence demonstrated that phagocytic, cytotoxic, and proinflammatory functions are induced in microglia in a number of infectious and neurodegenerative disorders (46, 47). Under resting conditions microglia display a ramified morphology characterized by lack of endocytic and phagocytic activity. Once activated, microglia are committed to a particular behavioral phenotype, characterized by the swelling of the cell body, thickening of the proximal processes, and a reduction in distal ramification (7, 48). Activated microglia express a range of genes related to inflammation, such as inflammatory cytokines (e.g., TNF-α, IL-1β), proinflammatory enzymes, and free radicals. Here we demonstrate that ethanol, as other insults such as infection or brain injury, triggers the activation of microglia as demonstrated by (1) the change in its morphology, from ramified to an ameboid or activated macrophage-like shape, (2) the robust phagocytic response, and (3) the production of cytokines (TNF-α, IL-1β) and other inflammatory mediators such as NO. Elevated levels of TNF-α have been associated with demyelination (49) and neurodegeneration (50). Indeed, several studies using microglial cell culture and animal models have demonstrated that excessive quantities of inflammatory mediators produced by activated microglia can be deleterious to neurons (51, 52). According to these results, we demonstrate that conditioned medium of ethanol-treated microglia induces apoptosis in cultured neurons. Our results further support the role of TLR4 in the neurotoxicity induced by activated microglia since a deficiency in the TLR4 function protects neurons in culture from the damage induced by microglia treated with ethanol. Although

FIGURE 7. A deficiency in TLR4 function protects neurons from the effects of ethanol on microglial activation. A, Apoptosis analysis of murine cortical neurons assessed by flow cytometry. Neurons were incubated for 1 day with wild-type (WT) or TLR4 knockout (KO) microglia-conditioned medium obtained from ethanol-treated microglial cells (50 mM) for 24 and 48 h. Data represent means ± SEM; n = 7 independent experiments. *p < 0.05 compared with the control value, according to a one-way ANOVA followed by Dunnett’s post hoc test. B, Immunoblot analysis and quantification of P-ERK and iNOS in wild-type microglia incubated in presence or absence of PMBS (10 μM) for 30 min or 3 h with the supernatants of TLR4-/- microglia either untreated or treated with 50 mM ethanol for 24 or 48 h. CM, conditioned medium. Data represent means ± SEM of three independent experiments. Values of p were not significant compared with the control value, according to a one-way ANOVA followed by a Newman-Keuls post hoc test. Blots were stripped, and the total quantities of ERK and GAPDH were also assessed. A representative immunoblot of each protein is shown.
caution should be taken in extrapolating in vitro findings to in vivo situations, the present results suggest that the activation of TLR4 in microglia by acute ethanol intake could induce an inflammatory environment in the brain that may result in neuronal damage. According to this hypothesis, we have shown that acute ethanol treatment can cause microglial activation in the cerebral cortex of wild-type mice, but not in TLR4−/− mice. Moreover, other reports have demonstrated that both an acute dose and 10 daily doses of ethanol in experimental animals induce long-lasting increases of the proinflammatory cytokines and microglial activation in the brain (53).

Interestingly, a recent study also demonstrated an increased expression of the proinflammatory cytokine MCP-1 (or CCL2) and microglia activation in various regions of postmortem alcoholic brains (54). These results in humans highlight the present findings on the basic mechanisms by which ethanol intake can induce inflammatory mediators and neuroinflammation.

The findings presented herein further demonstrate that ethanol-induced microglia activation occurs via TLRs receptors, archetypal pattern recognition receptors that mediate the general response of the innate immune system (38). The activation of these receptors by microbes and endogenous “danger signals” triggers, via a MyD88-dependent pathway, the stimulation of NF-κB and MAPKs pathways, which mediates many cellular functions, including activation of various transcription factors and production of proinflammatory and anti-inflammatory cytokines (16, 35, 53). Here we demonstrate that ethanol, at moderate acute concentrations such as 50 mM, is capable of inducing a rapid activation of ERK, JNK, and p-38, and also the translocation of NF-κB to the nucleus, as demonstrated by the nuclear expression of NF-κB subunit p65. Furthermore, our results strongly support the notion that TLR4 receptors are crucial in ethanol-induced inflammatory events in microglia since ethanol induces neither MAPK phosphorylation nor the production of TNF-α in microglial cells of TLR4-deficient mice. Several studies have demonstrated that endogenous molecules, such as injured and dying cells, can activate TLR4 via MyD88-dependent pathways during brain damage (43, 44). Under our in vitro conditions, however, this possibility did not seem to occur, because the supernatant of the ethanol-treated TLR4−/− microglia did not induce the TLR4 signaling in wild-type microglia.

Activation of the TLR4 by LPS can also trigger a second MyD88-independent pathway that uses TRAM-TRIF adaptor proteins. The stimulation of this pathway leads to the activation of IRF-3 (55, 56) and the production of IFN-β expression, which, in turn, could initiate STAT-1 signaling (13, 16, 57). Interestingly, we found that this pathway was also stimulated by ethanol, as demonstrated by the activation of IRF-3 and the IFN-β production. Ethanol also increases the levels of IRF-1 after 3–24 h of ethanol treatment, suggesting that the activation of this IFN regulatory factor is mediated by an IFN-3 or MyD88-dependent pathway that controls the expression of a large panel of genes (46), including the production of NO in microglia (58). Therefore, we propose that ethanol causes an inflammatory process in microglial cells by activating TLR4, MyD88-dependent, and MyD88-independent pathways to trigger the production of cytokines and other inflammatory mediators that induce the expression of IRF-1 and consequently the production of cytotoxic NO. Notably, the activation of this pathway has also been implicated in the pathogenesis of alcoholic liver disease (59, 60).

The modulatory effects of ethanol on the immune system are complex and depend on the dose, duration of ethanol treatment (acute vs chronic), and type of the cell and pathogen. In general, a suppressive effect of the immune response is observed in macrophages and monocytes during acute ethanol exposure or in ethanol-exposed monocytes stimulated with LPS. In these treatments, ethanol down-regulates the TLR4 response to LPS by limiting MAPK and NF-κB activation (61, 62), altered cytokine production, and decreased bacteria phagocytosis by alveolar macrophages (63, 64). In contrast, monocytes or macrophages exposed chronically to ethanol up-regulate cytokine production in response to LPS (65, 66). Although the differential effects of ethanol on the immune system are not well understood, it has been suggested that alcohol could modulate both the early and late steps of the TLR4-mediated activation (67). We proposed that ethanol, by interacting with the membrane microdomain lipid rafts, can activate or inhibit the TLR4 response (22). In accord with this proposal, we have demonstrated that either ethanol (at low/moderate concentrations) or LPS is capable of inducing translocation and clustering of TLR4 and signaling molecules (IRAK, MyD88, ERK) into isolated lipid rafts (24, 42). Furthermore, cell treatment with either high concentrations of ethanol or lipid raft disrupting agents (streptolysin O or saponin) inhibits ethanol-induced activation of the TLR4 signaling pathway (24). The effects of ethanol on TLR4 appear to be specific, because other short-chain n-alcohols do not induce TLR4 recruitment, although they slightly activate MAPKs signaling (23), and because other TLRs seems not to appear to be activated by ethanol (our unpublished results). We demonstrate, however, that ethanol activates TLR4/IL-1RI receptors in astrocytes (42). Nevertheless, ethanol-induced activation of IL-1RI seems not to be relevant in microglial cells, since ethanol does not induce inflammatory signaling in microglia of TLR4−/− mice.
Concluding the mechanisms by which ethanol induced activation of the two pathways associated with TLR4, recent studies suggest that TLR4 first induces MyD88-dependent signaling at the plasma membrane and is then endocytosed and activates TRAM-TRIF signaling from early endosomes (68). Therefore, it is possible that ethanol, by interacting with membrane lipids and/or phospholipids, makes the recruitment of the adaptor proteins MyD88 and TIRAP at the plasma membrane possible (69), and initiates TLR4 signaling via MyD88-dependent pathway as well as endocytosis. Then, ethanol could activate the TRAM-dependent signaling in endosomes.

In conclusion, the results presented herein provide the first evidence of the role of TLR4 in ethanol-induced microglia activation and response, and they suggest that the activation of microglial TLR4 signaling could trigger the release of inflammatory mediators, which could, in turn, induce white matter abnormalities and neuronal dysfunctions. These findings provide insight into the basic mechanisms participating in ethanol-induced neuroinflammation and brain damage (22, 54).

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Disclosures
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References


