Differential production of interleukin-6 and tumor necrosis factor-α in primary rat astrocyte cultures using two distinct methods of microglia elimination

Zoltan Barany1 | Istvan Toth1 | Gergely Jocsak1 | Laszlo V. Frenyo1 | Tibor Bartha1 | Agnes Sterczer2 | David S. Kiss1

Abstract
Hepatic encephalopathy (HE) is a neurocognitive disorder due to acute or chronic liver failure as well as portosystemic bypass. Both neuroinflammation and oxidative stress have been strongly implicated in the pathogenesis of HE. An increasing body of evidence suggests that in the development of these mechanisms astrocytes may be involved. With regard of HE, this aspect of astrocytes is, however, neglected. While several studies focus on astrocytes related to the above-mentioned pathological processes, the applied in vitro models are generated by diverse methods, which make the results difficult to compare with each other. In addition, these astrocyte cultures frequently contain microglia as well, complicating the interpretation of results. Therefore, we applied enriched primary rat astrocyte cultures generated by microglia elimination via shaking or the combination of cytosine β-d-arabinofuranoside hydrochloride (AraC) and l-leucine methyl ester (LME). We examine whether some HE-relevant pathological factors, such as ammonia, manganese (Mn), lipopolysaccharide (LPS), and H2O2 could elicit oxidative stress and cytokine production in astrocytes. Mn exposure increased the oxidative stress in astrocytes in a time-dependent manner; furthermore, we could detect tumor necrosis factor (TNF)-α production in both cultures at resting state. In addition, it was around 10-fold higher in the cultures after shaking. LPS increased TNF-α secretion in cultures after shaking, but not in cultures after AraC + LME treatment. In contrast, interleukin (IL)-6 was detectable only in the AraC + LME-treated cultures. In conclusion, we have demonstrated that HE-relevant factors evoke oxidative stress and cytokine production, albeit depending on the elimination method used.

KEYWORDS
hepatic encephalopathy, LPS, manganese, neuroinflammation, oxidative stress
1 | INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric disorder associated with loss of liver function representing an essential clinical feature of decompensated stage of liver cirrhosis.\(^\text{1-4}\) Liver cirrhosis is a common cause of death worldwide\(^\text{5}\) and it is suggested that the relatively high mortality rate in cirrhosis is associated with the presence of HE as well.\(^\text{3}\) In addition, not only cirrhosis, but also acute liver failure (ALF) is frequently followed by HE.\(^\text{5,6}\) In acute condition, cerebral edema can develop leading to death in many cases.\(^\text{7,8}\)

No single mechanism explains every aspect of HE; however, it has been suggested that ammonia is one of the major factors involved in it.\(^\text{4}\) Ammonia enters the portal blood circulation from the intestinal tract, but the liver fails to process it; thus blood ammonia and consequently its cerebral metabolic rate increases.\(^\text{9-11}\) An elevated systemic ammonia level in patients with ALF could be linked with cerebral edema, a higher grade of HE, and mortality.\(^\text{7,8,12}\) Similarly, in patients with liver cirrhosis, blood ammonia level correlates with the severity of HE.\(^\text{13,14}\) Although ammonia has a pivotal role in the development of both acute and chronic HE, some results contradict to this relationship. Indeed, physiological ammonia level could also be observed during ALF\(^\text{15}\) and there is no significant difference in arterial ammonia level between patients with higher grades in cirrhosis.\(^\text{15}\) Moreover, arterial ammonia is increased only in the more serious stage of HE.\(^\text{16}\) These data suggest that beyond ammonia also other factors could be committed in the pathophysiology of HE.

It is suggested that beside ammonia, manganese (Mn) also contributes to the pathogenesis of HE. Mn concentration is increased in different brain regions during cirrhosis and/or portacaval shunt\(^\text{17}\) and significantly higher Mn concentration could be measured both in the cortex and in the basal ganglia in HE.\(^\text{18}\) Thus, it is not surprising that in rats with minimal HE, reduction of Mn intake improves the cognitive and locomotor functions.\(^\text{19}\)

Besides the elevated ammonia and Mn, systemic inflammatory response syndrome and altered cytokine levels in the blood are associated with the severity of HE in cirrhosis;\(^\text{14,15}\) moreover, sepsis is one of the major factors leading to death in ALF.\(^\text{20}\) It is well established that inflammation is linked to reactive oxygen species (ROS) generation by cell signaling pathways.\(^\text{21}\) Oxidative stress is characterized by an imbalance between the production of free radicals and the antioxidant capacity.\(^\text{21,22}\) Increased ROS production is present in the brain in acute HE\(^\text{23}\) and it has been shown that ammonia and Mn enhance oxidative stress in astrocytes in acute\(^\text{24,25}\) and chronic\(^\text{26}\) cases. This oxidative stress is considered of high importance regarding the formation of astrocyte swelling, the main mechanism leading to brain edema during HE.\(^\text{27,28}\)

Besides, a growing body of evidence suggests that neuroinflammation is an important cerebral event during HE. Interleukin (IL)-1β mRNA expression is elevated in the frontal cortex of mice with ALF at coma stage; however, development of coma and brain edema delayed in the absence of IL-1 type 1 receptor.\(^\text{29}\) Hyperammonemic rats also showed activation of astrocytes and microglia as well as elevated IL-1β level in the cerebellum.\(^\text{30}\) Since then, it has been demonstrated that also other ILs (such as IL-6) are increased in the cerebral cortex of rats with portacaval shunts.\(^\text{31}\)

Microglia, as the resident brain macrophages, are key mediators of neuroinflammation, being responsible for the first-line immune response in the brain\(^\text{32}\) through their ability to phagocytosis, releasing a variety of pro- and anti-inflammatory cytokines, oxidants, and other mediators\(^\text{32-34}\) (reviewed by Brown and Vilalta\(^\text{35}\)).

The role of central nervous system cells other than microglia was neglected related to neuroinflammatory processes, for a long time. As mentioned about the attributed cellular swelling, the astrocyte is the main affected cerebral cell type in patients with HE. Therefore, it is not surprising that a series of relatively novel studies have addressed the contribution of astrocytes to neuroinflammatory processes, highlighting their capability of producing different cytokines (IL-1β, IL-6, monocyte chemoattractive protein-1, TNF-α), even under physiological conditions\(^\text{36-39}\) as well as in the absence of oxygen\(^\text{40}\) at high glucose concentrations,\(^\text{41}\) after treatment with lipopolysaccharide (LPS),\(^\text{36,37,42,43}\) with cytokines,\(^\text{39,44}\) or with hydrogen peroxide, the latter of which is a relative stable type of ROS\(^\text{45}\) in different in vitro models.

Nevertheless, a considerable number of studies were published regarding the neuroinflammatory role of astrocytes (reviewed in Cekanaviciute and Buckwalter\(^\text{46}\)); the overwhelming majority of them was carried out on cultures not\(^\text{47}\) or inadequately purified from microglia and/or contaminated with other cellular elements\(^\text{40,48-50}\) or on a human astrocyte cell line originating from the astrocytoma.\(^\text{44}\) In some reports, culture composition was not even disclosed at all.\(^\text{51}\) In addition, there is evidence that not all astrocytes get stained positive for glial fibrillary acidic protein (GFAP), a commonly used specific marker for astrocytes.\(^\text{48,52}\) Cultures containing around 90% GFAP-positive cells produce detectable TNF-α\(^\text{40}\) and IL-1β\(^\text{52}\) even under physiological conditions, unlike cultures claimed to be fully purified from other cell types.\(^\text{53}\) Obviously, the pattern of cytokine production is different in astrocyte-enriched vs microglia-enriched cultures.\(^\text{54}\) The reason for this, on the one hand, is that the microglial cytokine production is measured instead of the astrocytes; on the other hand, is that microglial cytokines can induce the formation of reactive astrocytes type A1,\(^\text{55}\) a subpopulation capable of producing specific cytokines.\(^\text{56}\)

In conclusion, the evaluation of the results concerning the astrocytes’ specific role in neuroinflammation during HE is limited due to the potential influencing effects of other cell types; moreover the potential heterogeneity of the applied in vitro models does not allow comparison of the results.

The objectives of this study were to: (i) evaluate the composition of primary rat astrocyte cultures after two different methods of microglia elimination; (ii) to assess the ROS production in astrocytes after the treatment with some HE-relevant factors, such as ammonia, Mn, LPS, and H₂O₂; and (iii) finally to determine the TNF-α and IL-6 secretion in the astrocyte cultures in the two different models.
2 | METHODS

2.1 | Materials

Dulbecco’s modified Eagle’s medium (D5796), cytosine-β-d-arabinofuranoside (C1768), L-leucine methyl ester (LME) hydrochloride (L1002), Triton-X (T8787), anti-GFAP (ABIN115304), Mn (III) acetate dihydrate (215880), ammonium chloride (A9434), hydrogen peroxide (H1009), LPs from Escherichia coli O111:B4 (L4391), IL-6 (RAB 0311), TNF-α (RAB 0479), KCl (P-5405), KH₂PO₄ (P-5655), NaCl (S-5136) were purchased from Sigma. Lactate dehydrogenase (LDH) cytotoxicity detection kit (1164793001) was obtained from Roche. Ionized calcium-binding adapter molecule-1 (Iba-1, ab 178846) was purchased from Abcam. CM-H₂DCFDA (C6827) was purchased from Invitrogen.

2.2 | Preparation of astrocyte cultures

Mixed glial cell cultures were prepared from 2-day-old Sprague-Dawley rat pups as described previously. Briefly, brains were dissected, and meninges were removed, followed by mechanical dissociation and enzymatic digestion of the cells. Cells were grown in petri dishes, maintained in modified Eagle’s medium, which was changed in 1 and 2 days and then twice a week. After confluence was achieved at about 14 days, cells were transferred to 96-well plates. One day later, microglia were removed by two different methods applied independently from each other: either by incubating the cultures with β-d-arabinofuranoside hydrochloride (AraC) and LME (chemical elimination) or by shaking (mechanical elimination).

In case of the chemical elimination of microglia, cultures were treated with 10 μmol L⁻¹ AraC for 3 days, followed by 25 mmol L⁻¹ LME for 1 hour. AraC is an antimitotic agent that blocks the proliferation of microglia. LME is a lysomotropic agent internalized by microglia, wherein it causes disruption of lysosomes followed by apoptosis. After all treatments, the cultures were allowed to recover for 24 hours. In case of mechanical elimination, glial cultures were placed on an orbital shaker at 180 rpm for 30 minutes. After the supernatants were removed, cultures were further shaken at 240 rpm for 6 hours.

2.3 | Treatments

For assessing the cytotoxicity, ROS production, and IL-6 and TNF-α release, cultures were treated with H₂O₂ (5, 10, 50, 100, 150, 200, 300, and 400 μmol L⁻¹ and 1, 5, and 10 mmol L⁻¹ for 1, 6, and 18 hours), LPS (1, 10, 25, 50, 100, 1000, and 5000 ng for 1, 3 and 6 hours), Mn (5, 50, 100, and 500 μmol L⁻¹ for 1, 3, and 6 hours), and ammonia (10, 100, and 500 μmol L⁻¹ and 2, 5, and 20 mmol L⁻¹ for 1, 3, 6, 18, and 24 hours). For each condition, two to three identical experiments were performed, with three replicates in each experiment.

2.4 | Immunocytochemistry

Cell cultures were characterized by immunofluorescence labeling for GFAP and for Iba-1 to detecting astrocytes and microglia, respectively. Procedure was carried out as described elsewhere. Images were acquired using inverted fluorescence microscope (Zeiss Axio Vert.A1). Images were analyzed using Image J software (NIH Image).

2.5 | Analysis of cell viability

Two separate methods of cell viability were applied for examining the cytotoxicity of the test compounds, as we measured LDH activity and cellular neutral red uptake using commercial kits.

2.6 | Measurement of intracellular formation of ROS

Changes in ROS levels were determined by CM-H₂DCFDA as described previously. For this measurement, cells in 96-well plates were first incubated with 10 μmol L⁻¹ CM-H₂DCFDA in serum-free loading medium for 30 minutes at 37°C and then washed twice with PBS (0.1 mol L⁻¹, pH 7.4, 37°C), and afterward exposed to different concentrations of H₂O₂, ammonia, LPS, and Mn. Immediately after the end of incubation period, fluorescence was measured with excitation at 485 nm and emission at 538 nm.

2.7 | Enzyme-linked immunosorbent assays

IL-6 and TNF-α were measured from the culture medium. Cell culture supernatants were collected, and the cytokines released into the culture medium were analyzed using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions.

2.8 | Statistical analysis

Data were analyzed with analysis of variance (ANOVA) with Dunnett’s post hoc comparison. Statistical differences were detected when P < .05. All data are presented as mean ± SEM and all statistical analysis was performed using Prism software (version 5.0, GraphPad Software).

3 | RESULTS

3.1 | Characterization of the astrocyte cultures

In this study we verified by immunocytochemistry that both of our purification procedures resulted in highly purified astrocyte cultures
with an almost complete elimination of microglia. The purity of astrocyte cultures without microglia elimination was 90% (Figures 1 and 2), while the ratio of astrocytes after both chemical and mechanical eradication of residual microglia exceeded 98% confirmed by the astrocyte marker GFAP with a prominent decrease in the microglia marker Iba-1 (Figures 3 and 4).

3.2 | Cytotoxicity induced by the test compounds

Both the neutral red uptake and the LDH activity assays demonstrated that H2O2 exposure induces a noticeable decrease in cell viability. If measured by neutral red assay, the decrease reached a significance level in case of 100 µmol L⁻¹ and 1, 5, and 10 mmol L⁻¹ H2O2 after 18 hours of incubation and 10 µmol L⁻¹ H2O2 after 24 hours of incubation. Other test compounds did not affect cell viability as LDH release and neutral red uptake were not affected neither by LPS nor by Mn and ammonia.

If measured by LDH assay, the astrocytes’ viability was significantly reduced in cultures treated with H2O2 for 18 hours (100 µmol L⁻¹ and 1 mmol L⁻¹) and 24 hours (10 and 100 µmol L⁻¹, 1 mmol L⁻¹). Interestingly, some concentrations of H2O2, LPS, ammonia, and Mn caused a significant decrease of the LDH release.

3.3 | Intracellular ROS accumulation induced by ammonia, Mn, LPS, and H2O2

In mixed astrocyte-microglia cultures, oxidative stress was elevated after the treatment with 100 µmol L⁻¹ Mn for 3 and 6 hours and with 1000 ng LPS for 1 hour, moreover, all of the applied concentrations of H2O2 increased ROS production (Figure 5). In cultures, after chemical microglia elimination, the exposure of Mn, applied with a concentration of 20 µmol L⁻¹ for 3 and 6 hours and with 100 µmol L⁻¹ for 6 hours, significantly increased CM-H2DCFDA fluorescence. Furthermore, Mn at 20 µmol L⁻¹ induced significant elevation of oxidative stress in time-dependent manner. In ammonia- , LPS- , and H2O2-exposed astrocytes there was no significant increase in fluorescence intensity when compared to control; however, in some LPS- and ammonia-treated and all of the H2O2-treated groups, a slight, but not significant increasing in ROS production could be revealed (Figure 6).

The ROS-generating activity of Mn could be measured in cultures after mechanical microglia elimination as well. Both 20 and 100 µmol L⁻¹ Mn caused intracellular ROS accumulation after 3 and 6 hours. Interestingly, both LPS and H2O2 provoked increased ROS production, however, not in the higher concentrations (Figure 7).

3.4 | Cytokine production in primary astrocyte cultures after mechanical microglia elimination

We have found that nonstimulated resting astrocytes were able to produce TNF-α after 18 hours (Figure 8); conversely, the proinflammatory cytokine IL-6 was undetectable in the culture medium. Following both an 18- and a 24-hour exposure to LPS, the TNF-α protein expression was significantly elevated (Figure 8), whereas the level of IL-6 protein remained below the level of detection after the incubation of either of the test substances (data not shown).

3.5 | Cytokine production in primary astrocyte cultures after chemical microglia elimination

Astrocyte cultures purified by AraC and LME treatment produced the inflammatory cytokines TNF-α (Figure 9) and IL-6 (Figure 10) without addition of any substances (ammonia, Mn, LPS, and H2O2). In addition, TNF-α level was around 10-fold higher in the culture medium after chemical microglia elimination (Figure 9) than after shaking (Figure 8). Furthermore, it has been shown that neither of the treatments were able to induce increases in release of TNF-α and IL-6 from microglia-depleted cultures.

4 | DISCUSSION

Studying astrocytes in culture is a powerful way to clarify their function; however, due to the presence of contaminating cells, such as microglia in astrocyte cultures, it is difficult to determine if a cellular effect is truly cell autonomous. To assess the separate astrogial function, there are various techniques used to eliminate microglia, including AraC treatment alone,63,64 LME treatment alone,59,65 combining AraC and LME,58 shaking alone,66 shaking combined with AraC treatment,67 or shaking combined with AraC and LME.68

In this study we established highly purified primary rat astrocyte cultures as well as mixed cultures. Moreover, microglia were...
eliminated by treatment with the combination of AraC and LME. Based on our preliminary results from immunocytochemical characterisation of both astrocytes and microglia, 10 µmol L⁻¹ AraC for 3 days and 25 mmol L⁻¹ LME for 1 hour were used, which resulted in 98% purity of the cultures. Besides, the other method of microglia elimination based on mechanical removing, which could be performed by several protocols.⁶⁷,⁶⁹ For the shaking, confluent glial cultures were placed on an orbital shaker at 180 rpm for 30 minutes and after supernatants were removed, cultures were shaking at 240 rpm for 6 hours. This method takes advantage of the much stronger attachment of astrocytes to the plastic of the culture flask relative to microglia.⁷⁰ According to our results, both the mechanical and the chemical purification procedure showed an equivalent efficiency in the removal of undesired cells.

As part of the protocol validation, LDH release and neutral red uptake assay were used to analyze the cell death attributed to the test compounds. These results were in accordance with each other: both methods revealed that H₂O₂, at certain concentrations, induced an LDH increase in the culture medium after 18 and 24 hours as well. Interestingly, based on LDH release, at the highest concentrations of

**FIGURE 2** Primary rat astrocyte culture without microglia elimination. Microglia show immunolabeling for Iba-1 (green); the cells were counterstained with DAPI (blue). Images were acquired using inverted fluorescence microscope (Zeiss Axio Vert.A1). Images were analyzed using Image J software (NIH Image, USA).

**FIGURE 3** Primary rat astrocyte culture after microglia elimination with the combination of 10 µmol L⁻¹ AraC for 3 days and 25 mmol L⁻¹ LME for 1 hour. Microglia show immunolabeling for Iba-1 (green); the cells were counterstained with DAPI (blue). Images were acquired using inverted fluorescence microscope (Zeiss Axio Vert.A1). Images were analyzed using Image J software (NIH Image).

**FIGURE 4** Primary rat astrocyte cultures after microglia elimination with shaking (orbital shaker at 180 rpm for 30 minutes; after the supernatants were removed, cultures were further shaken at 240 rpm for 6 hours). Microglia show immunolabeling for Iba-1 (green); the cells were counterstained with DAPI (blue). Images were acquired using inverted fluorescence microscope (Zeiss Axio Vert.A1). Images were analyzed using Image J software (NIH Image).

**FIGURE 5** Effect of ammonia, manganese, hydrogen peroxide, and lipopolysaccharide on intracellular ROS accumulation in mixed astrocyte-microglia cultures. Data show mean ± SEM of the experiments and analyzed by one-way ANOVA. Each experiment was replicated 4 times. The groups were comparable to their respective controls. *P < .05. **P < .01. ***P < .001.
The cytotoxicity remained under the control value. This result agrees with a previous finding where ROS inhibited LDH activity, probably due to the concentration-dependent effect of \( \text{H}_2\text{O}_2 \) diminishing LDH activity. In conclusion, although both methods rely on a suitable tool to assess the cytotoxicity, the application of LDH method should be considered carefully when testing \( \text{H}_2\text{O}_2 \).

A growing body of evidence points to the generation of oxidative stress in astrocyte cultures exposed to ammonia. In the present study, ammonia did not lead to ROS formation in astrocytes; nevertheless, we used higher concentration of ammonia for longer
sensitivity of astrocytes to the H\textsubscript{2}O\textsubscript{2} than in astrocytes. Furthermore, the data could not exclude oxidative stress can develop to a greater extent or more rapidly in microglia, than in astrocytes. Moreover, it has been described that graded concentrations and the redox state changes in a time-dependent manner after Mn treatment. However, it should be highlighted that other studies examined the ROS production on less purified cultures or the success of microglia elimination was not verified by immunostaining for microglia marker. One of our most important observations was that H\textsubscript{2}O\textsubscript{2} caused slight, not significant ROS production at either of the applied concentrations in astrocyte cultures after chemical microglia elimination, albeit we observed significant ROS generation in all of the examined concentrations of H\textsubscript{2}O\textsubscript{2} in mixed glial cultures. In other studies, H\textsubscript{2}O\textsubscript{2} treatment significantly increased the ROS level in astrocyte cultures; however, it has been described that graded concentrations of H\textsubscript{2}O\textsubscript{2} also provoked a dose-dependent rise of ROS production in astrocytes; in addition, the treatment conditions were similar to those we applied. The fact that H\textsubscript{2}O\textsubscript{2} did not elicit significant oxidative stress in AraC + LME-treated astrocyte cultures, but could provoke ROS production in astrocyte-microglia mixed cultures, suggests that oxidative stress can develop to a greater extent or more rapidly in microglia than in astrocytes. Furthermore, the data could not exclude the possibility that the presence of microglia might increase the sensitivity of astrocytes to the H\textsubscript{2}O\textsubscript{2}-evoked oxidative stress. Another study demonstrated that activated microglia cause the upregulation of antioxidative enzymes in astrocytes; thereby, a higher resistance to oxidative stress induced by H\textsubscript{2}O\textsubscript{2} was developed. Overall, our findings suggest that Mn causes oxidative stress in highly purified astrocyte cultures in a time-dependent manner.

It is well known that microglia play a key role in neuroinflammation by cytokine production. Several studies have described microglial release of TNF-\(\alpha\) and IL-6, and although astrocytes are also able to produce a variety of proinflammatory cytokines much less is known about the astrocytic IL secretion. Therefore, we measured the cytokine release into the medium in enriched astrocyte cultures generated by two different methods, as described earlier. Both cultures were observed to produce TNF-\(\alpha\) without adding any test substances. However, cultures after the chemical treatment produced around 10-fold more TNF-\(\alpha\) than those that underwent shaking. In addition, we could demonstrate that both applied concentrations of LPS at each incubation time enhanced the TNF-\(\alpha\) release from astrocyte cultures purified by shaking. In contrast, neither of the substances could intensify the TNF-\(\alpha\) secretion in AraC + LME-treated cultures. In line with these observations, adult astrocytes are able to produce TNF-\(\alpha\) that can be increased by both LPS and H\textsubscript{2}O\textsubscript{2}, albeit the cultures in this study contained 5% microglia. In another report, activated astrocytes also released TNF-\(\alpha\); however, the cellular composition of those cultures was not described. In accordance with this, TNF-\(\alpha\) could not be detected in supernatants of the LPS-induced human astrocyte cell line in human astrocyte culture with 99% purity and TNF-\(\alpha\) also could not be detected in microglia-free astrocyte cultures either under baseline conditions or after LPS treatment. Interestingly, TNF-\(\alpha\) release was below the detection limit under control conditions, but LPS increased the production of that in astrocyte cultures in which the percentage of astrocytes was not higher than 97%.

IL-6 was undetectable in the medium of astrocyte cultures after shaking, conversely basal IL-6 could be measured in cultures after AraC + LME treatment; however, it remained unchanged after activation of the cells with different test compounds. Astrocytes' IL-6 secretion has already been investigated by others as well; however, the results should be interpreted with caution due to the characteristics of the cultures. It has been shown that astrocytes could produce IL-6 after LPS treatment; however, it has been demonstrated on human astrocyte cell line originating from an astrocytoma. In another study, IL-6 release from activated astrocytes could be also observed traumatic and metabolic injury; however, the purity of the cultures was not described. In cultures containing more than 97% astrocytes, IL-6 could not be detected under baseline conditions; however, the secretion could be induced by LPS.

Overall, our data indicate that both methods of microglia elimination are appropriate for generating highly purified primary rat astrocyte cultures verified by immunocytochemistry. This study also demonstrated that enriched astrocyte cultures secrete both TNF-\(\alpha\) and IL-6, albeit the findings should be considered carefully when interpreting the neuroinflammatory role of astrocytes. It is assumable that cultures might contain more microglia or other cell types; moreover, the microglia elimination could directly influence the cytokines'
secrectome profile, which could lead to differences regarding TNF-α and IL-6 production. Furthermore, our observations support the evidence that Mn causes generation of ROS in astrocytes; in addition, at a concentration of 20 μmol L⁻¹, it causes oxidative stress in a time-dependent manner. We have also demonstrated that mixed glial cultures have higher sensitivity to the H₂O₂-evoked oxidative stress than cultures after chemical microglia elimination. In conclusion, examination of factors involved in the pathogenesis of HE showed that Mn causes oxidative stress; moreover, the TNF-α and IL-6 production of highly purified astrocyte cultures depends on the type of microglia elimination (mechanical vs chemical).

DISCLOSURE OF ETHICAL STATEMENTS
All animal experiments were conducted following the national and international guidelines and the relevant national laws on the protection of animals. The animals were treated according to the EC Council Directive of 24 November 1986 (86/696/EEC) and all procedures were reviewed and approved by the local ethical committee (Animal Welfare Board at University of Veterinary Medicine and regional animal welfare authority, ID: PEI/001/665-8/2015, Pest Megyei Kormányhivatal). No human participant was involved in this study.

ACKNOWLEDGEMENTS
This research was supported by OTKA K-115613 grant of the Hungarian Scientific Research Fund to Attila Zsarnovszky; 11475-4/2016/FEKUT, 11475-4/2016/FEKUT, 12190-4/2017/FEKUTSTRAT, 6199/2017/FEKUTSTART, EFOP-3.6.2-16-2017-00008 grants of the Hungarian Ministry of Human Resources.

The authors thank Zsuzsanna Szikora and Zsofia Osz for their excellent technical support, Jenő Reiczigel for his help in the statistical analysis, and Zsuzsanna Kornyey (Institute of Experimental Medicine) for her help in the generation of primary rat astrocyte cultures.

CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

ORCID
Zoltan Barany https://orcid.org/0000-0003-2823-5939

REFERENCES
1. Turco L, Garcia-Tsao G, Magnani I, Bianchini M, Costetti M, Caporali C, et al Cardiopulmonary hemodynamics and C-reactive protein as prognostic indicators in compensated and decompensated cirrhosis. J Hepatol. 2018;68(5):949–58.
2. Tapper EB, Parikh ND, Sengupta N, Mellinger J, Ratz D, Lok A-F, et al A risk score to predict the development of hepatic encephalopathy in a population-based cohort of patients with cirrhosis. Hepatology. 2018;68(4):1498–507.
3. D’Amico G, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. J Hepatol. 2006;44:217–31.
4. Tschochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014;383:1749–61.
5. Stravitz RT, Ellerbe C, Durkalski V, Schilsky M, Fontana RJ, Peterseim C, et al Bleeding complications in acute liver failure. Hepatology. 2018;67(5):1931–42.
6. Hahn M, Massen O, Nencii M, Pawlows J. Die Eck’sche Fistel zwischen der unteren Hohlvene und der Pfortader und ihre Folgen für den Organismus. Arch für Exp Pathol und Pharmakologie. 1893;32(4):161–210.
7. Bernal W, Hall C, Karvellas CJ, Auzinger G, Sizer E, Wendon J. Arterial ammonia and clinical risk factors for encephalopathy and intracranial hypertension in acute liver failure. Hepatology. 2007;46(6):1844–52.
8. Clemmesen JO, Larsen FS, Kondrup J, Hansen BA, Ott P. Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. Hepatology. 1999;29(3):648–53.
9. Weissborn K, Ahl B, Fischer-Wasels D, van den Hoff J, Hecker H, et al Correlations between magnetic resonance spectroscopy alterations and cerebral ammonia and glucose metabolism in cirrhotic patients with and without hepatic encephalopathy. Gut. 2007;56(12):1736–42.
10. Lockwood AH, Yap EWH, Wong WH. Cerebral ammonia metabolism in patients with severe liver disease and minimal hepatic encephalopathy. J Cereb Blood Flow Metab. 1991;11(2):337–41.
11. Keiding S, Sørensen M, Bender D, Munk OL, Ott P, Vilsstrup H. Brain metabolism of 13N-ammonia during acute hepatic encephalopathy in cirrhosis measured by positron emission tomography. Hepatology. 2006;43(1):42–50.
12. Bhata V, Singh R, Acharya SK. Predictive value of arterial ammonia for complications and outcome in acute liver failure. Gut. 2006;55(1):98–104.
13. Ong JP, Aggarwal A, Krieger D, Easley KA, Karafa MT, Van Lente F, et al Correlation between ammonia levels and the severity of hepatic encephalopathy. Am J Med. 2003;114(3):188–93.
14. Jain L, Sharma BC, Sharma P, Srivastava S, Agrawal A, Sarin SK. Serum endotoxin and inflammatory mediators in patients with cirrhosis and hepatic encephalopathy. Dig Liver Dis. 2012;44(12):1027–31.
15. Shawcross DL, Sharifi Y, Canavan JB, Yeoman AD, Abeles RD, Taylor NJ, et al Infection and systemic inflammation, not ammonia, are associated with Grade 3/4 hepatic encephalopathy, but not mortality in cirrhosis. J Hepatol. 2011;54(4):640–9.
16. Matkowskyj KA, Marrero JA, Carroll RE, Danilovich AV, Green RM, Benya RV. Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model. Am J Physiol Gastrointest Liver Physiol. 1999;277(2):G455–G462.
17. Rose C, Butterworth RF, Zayed J, Normandin L, Todd K, Michalak A, et al Manganese deposition in basal ganglia structures results from both portal-systemic shunting and liver dysfunction. Gastroenterology. 1999;117(3):640–4.
18. Li Y, Ji CX, Mei LH, Qiang JW, Ju S. Oral administration of trace element magnesium significantly improving the cognition and locomotion in hepatic encephalopathy rats. Sci Rep. 2017;7(1):1817.
19. Dolmans LM, Kamphuisen PR, van den Elst W, Zwaan J, Stroes ESG, Huisman M, et al Manganese-containing iron oxide as a therapeutic agent for ARDS. J Crit Care. 2012;27(4):559–65.
20. Rolando N, Wade J, Davalos M, Wendon J, Philpott-Howard J, Williams R. The systemic inflammatory response syndrome in acute liver failure. Gut. 2000;42(4):256–61.
21. Butterfield DA, Halliwell B. Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. Nat Rev Neurosci. 2012;13(1):24–33.
22. Sathyasaikumar KV, Swapna I, Reddy PVB, Murthy CRK, Dutta Gupta A, Senthilkumaran B, et al Fulminant hepatic failure in rats.
induces oxidative stress differentially in cerebral cortex, cerebellum and pons medulla. Neurochem Res. 2007;32(3):517–24.

24. Murthy CRK, Rama Rao KV, Bai G, Norenberg MD. Ammonia-induced production of free radicals in primary cultures of rat astrocytes. J Neurosci Res. 2001;66(2):282–8.

25. Jayakumar AR, Rao KVR, Kalaiselvi P, Norenberg MD. Combined effects of ammonia and manganese on astrocytes in culture. Neurochem Res. 2004;29(11):2051–6.

26. Görg B, Karababa A, Shafigullina A, Bidmon HJ, Häussinger D. Ammonia-induced senescence in cultured rat astrocytes and in human cerebral cortex in hepatic encephalopathy. Glia. 2015;63(1):37–50.

27. Jayakumar AR, Panickar KS, Murthy CRK, Norenberg MD. Oxidative stress and mitogen-activated protein kinase phosphorylation mediate ammonia-induced cell swelling and glutamate uptake inhibition in cultured astrocytes. J Neurosci. 2006;26(18):4774–84.

28. Rama Rao KV, Reddy PVB, Hazell AS, Norenberg MD. Manganese induces cell swelling in cultured astrocytes. Neurotoxicology. 2007;28(4):807–12.

29. Bémeur C, Qu H, Desjardins P, Butterworth RF. IL-1 or TNF receptor gene deletion delays onset of encephalopathy and attenuates brain edema in experimental acute liver failure. Neurochem Int. 2010;56(2):213–5.

30. Hernandez-Rabaza V, Cabrera-Pastor A, Taoro-Gonzalez L, Gonzalez-Usano A, Agusti A, Balzano T, et al. Neuroinflammation increases GABAergic tone and impairs cognitive and motor function in hyperammonemia by increasing GAT-3 membrane expression, Reversal by sulforaphane by promoting M2 polarization of microglia. J Neuroinflammation. 2016;13(1):83.

31. Cauli O, Rodrigo R, Piedrafita B, Boix J, Felipo V. Inflammation and inflammatory cytokine secretion by stimulated human astrocytes. Curr Alzheimer Res. 2017;14(7):731–41.

32. Masuch A, Shieh CH, van Rooijen N, van Calker D, Biber K. Nutritional and metabolic effects of ammonia on astrocytes. J Neurochem. 2012;120(6):1060–71.

33. Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. Brain Behav Immun. 2009;23(3):309–17.

34. Kirkley KS, Popichak KA, Afzali MF, Legare ME, Tjalkens RB. IPAF inflammasome is involved in interleukin-1β production from astrocytes, induced by palmitate: implications for Alzheimer’s Disease. Neurobiol Aging. 2014;35(2):309–21.

35. Zhang Y, et al. Evidence for miR-181 involvement in neuroinflammation during anoxia: secretome profile of cytokines and chemokines. PLoS One. 2014;9(4):e92325.

36. van Neerven S, Nemes A, Imholz P, Regen T, Denecke B, Andersson AK, Rönnbäck L, Hansson E. Lactate induces tumour necrosis factor-α and IL-6 in an in-vitro model of brain inflammation. J Neuroinflammation. 2017;14(1):99.

37. Cui YQ, Zheng Y, Tan GL, Zhang DM, Wang JY, Wang XM. 5R-5-hydroxytryptolide inhibits the inflammatory cascade reaction in astrocytes. Neural Regen Res. 2019;14(5):913–20.

38. Liang Y, et al. Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid. J Neuroimmunol. 2010;229(1–2):169–79.

39. Forshammar J, Block L, Lundborg C, Biber B, Hansson E. Naloxone and ouabain in ultralow concentrations restore Na+/K+-ATPase and cytoskeleton in lipopolysaccharide-treated astrocytes. J Biol Chem. 2011;286(36):31586–97.

40. Choi SS, Lee HJ, Lim I, Satoh JI, Kim SJ. Human astrocytes: secretome profiles of cytokines and chemokines. PLoS One. 2014;9(4):e92325.

41. Souza DG, Bellaver B, Souza DO, Quincozes-Santos A. Characterization of adult rat astrocyte cultures. PLoS One. 2018;13(8):e020822.

42. Horisuchi H, Parajuli B, Kawanokuchi J, Jin S, Mizuno T, Takeuchi H, et al. Oligomeric amyloid β facilitates microglial excitotoxicity by up-regulating tumor necrosis factor-α and downregulating excitatory amino acid transporter 2 in astrocytes. Clin Exp Neuroimmunol. 2015;6(2):183–90.

43. Wilms H, Sievers J, Rickert U, Rostami-Yazdi M, Mrowietz U, Lucius R. Dimethylfumarate increases microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 in a rat model of experimental autoimmune encephalomyelitis. Neurotherapeutics. 2016;13(4):685–701.

44. Liu L, Chan C. IPAF inflammasome is involved in interleukin-1β production from astrocytes, induced by palmitate: implications for Alzheimer’s Disease. Neurobiol Aging. 2014;35(2):309–21.

45. Forshammar J, Block L, Lundborg C, Biber B, Hansson E. Naloxone and ouabain in ultralow concentrations restore Na+/K+-ATPase and cytoskeleton in lipopolysaccharide-treated astrocytes. J Biol Chem. 2011;286(36):31586–97.

46. Chen Y, et al. Toll-Like Receptors 2, -3 and -4 Prime Microglia but not Astrocytes to induce Proinflammatory Gene Expression. Mol Med. 2015;21(1–12):472–82.

47. Kirkley KS, Popichak KA, Afzali MF, Legare ME, Tjalkens RB. Microglia amplify inflammatory activation of astrocytes in manganese neurotoxicity. J Neuroinflammation. 2017;14(1):99.

48. Xu J. New insights into GFAP negative astrocytes in Calbindin D28k immunoreactive astrocytes. Brain Sci. 2018;8(8):143.

49. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Oligomeric amyloid β facilitates microglial excitotoxicity by up-regulating tumor necrosis factor-α and downregulating excitatory amino acid transporter 2 in astrocytes. Clin Exp Neuroimmunol. 2015;6(2):183–90.

50. Wilms H, Sievers J, Rickert U, Rostami-Yazdi M, Mrowietz U, Lucius R. Dimethylfumarate increases microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 in a rat model of experimental autoimmune encephalomyelitis. Neurotherapeutics. 2016;13(4):685–701.

51. Wilms H, Sievers J, Rickert U, Rostami-Yazdi M, Mrowietz U, Lucius R. Dimethylfumarate increases microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 in a rat model of experimental autoimmune encephalomyelitis. Neurotherapeutics. 2016;13(4):685–701.

52. Xu J. New insights into GFAP negative astrocytes in Calbindin D28k immunoreactive astrocytes. Brain Sci. 2018;8(8):143.

53. Chistyakov DV, Azbukina NV, Astakhova AA, Gorainov SV, Chistyakov VV, Sergeeva MG. Sex-mediated differences in LPS-induced alterations of TNFα, IL-10 expression, and prostaglandin synthesis in primary astrocytes. Int J Mol Sci. 2018;19(9):2793.

54. Andersson AK, Rönnbäck L, Hansson E. Lactate induces tumour necrosis factor-α, interleukin-6 and interleukin-1β release in microglial- and astroglial-enriched primary cultures. J Neurochem. 2005;93(5):1327–33.

55. Pont-Lezica L, Colasse S, Bessis A. Depletion of microglia from primary cortical and pons medulla. Neurochem Res. 2007;32(3):517–24.

56. Pont-Lezica L, Colasse S, Bessis A. Depletion of microglia from primary cortical and pons medulla. Neurochem Res. 2007;32(3):517–24.

57. Gupta S, Knight AG, Gupta S, Keller JN, Bruce-Keller AJ. Saturated fatty acids activate inflammatory signaling in astrocytes. J Neurochem. 2012;120(6):1060–71.

58. Barany ET al. Modulation of interleukin-1β mediated inflammatory response of astrocytes, induces cell swelling and glutamate uptake inhibition in cultured astrocytes. J Neurosci. 2006;26(18):4774–84.

59. Barany ET al. Modulation of interleukin-1β mediated inflammatory response of astrocytes, induces cell swelling and glutamate uptake inhibition in cultured astrocytes. J Neurosci. 2006;26(18):4774–84.
microglia inflammatory profile improving antiagita activity. Brain Behav Immun. 2020;18(8):170–85.
62. Hamdi Y, Masmoudi-Kouki O, Kaddour H, Belhadj F, Gandofo P, Vaudry D, et al. Protective effect of the octadecaneuropeptide on hydrogen peroxide-induced oxidative stress and cell death in cultured rat astrocytes. J Neurochem. 2011;118(3):416–28.
63. Solenov E, Watanabe H, Manley GT, Verkman AS. Sevenfold-reduced osmotic water permeability in primary astrocyte cultures from AQP-4-deficient mice, measured by a fluorescence quenching method. Am J Physiol Cell Physiol. 2004;286(2):C426–C432.
64. Barbierato M, Facci L, Argentini C, Marinelli C, Skaper S, Giusti P. Astrocyte-microglia cooperation in the expression of a pro-inflammatory phenotype. CNS Neurol Disord Drug Targets. 2013;12(5):608–18.
65. Prajeeth CK, Kronisch J, Khorooshi R, Knier B, Toft-Hansen H, Gudi V, et al. Effectors of Th1 and Th17 cells act on astrocytes and augment their neuroinflammatory properties. J Neuroinflammation. 2017;14(1):204.
66. Takata-Tsui F, Choulanmountri N, Do LD, Philippot C, Novion Ducassou J, Couté Y, et al. Microglia modulate glitotransmission through the regulation of VAMP2 proteins in astrocytes. Glia. 2021;69(1):61–72.
67. Schilde G, Bohrer C, Beck K, Schachtrup C. Isolation and culture of mouse cortical astrocytes. J Vis Exp. 2013;71:50079.
68. Lau LT, Yu ACH. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. J Neurotrauma. 2001;18(3):351–9.
69. Milatovic D, Yin Z, Gupta RC, Sidoryk M, Albrecht J, Aschner JL, et al. Manganese induces oxidative impairment in cultured rat astrocytes. Toxicol Sci. 2007;98(1):198–205.
70. Chen CJ, Liao SL. Oxidative stress involves in astrocytic alterations induced by manganese. Exp Neurol. 2002;175(1):216–25.
71. Bi J, Jiang B, Liu JH, Lei C, Zhang XL, An LJ. Protective effects of catalpol against H2O2-induced oxidative stress in astrocytes primary cultures. Neurosci Lett. 2008;442(3):224–7.
72. Hamdi Y, Masmoudi-Kouki O, Kaddour H, Belhadj F, Gandolfo P, Vaudry D, et al. Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. Neurochem Res. 2008;33(1):8–15.
73. Görg B, Karababa A, Häussinger D. Hepatic encephalopathy and oxidative and inflammatory stress and increase the resistance of astrocytes to oxidative stress in vitro. Glia. 2008;56(10):1114–26.
74. Wang MJ, Huang HY, Chen WF, Chang HF, Kuo JS. Glycogen synthase kinase-3β inactivation inhibits tumor necrosis factor-α production in BV2 microglial cells. Brain Res. 2012;1435:15–23.
75. Wang B, Chen T, Wang J, Jia Y, Ren H, Wu F, et al. Methamphetamine modulates the production of interleukin-6 and tumor necrosis factor-alpha via the CAMP/PKA/CREB signaling pathway in lipopolysaccharide-activated microglia. Int Immunopharmacol. 2018;56:168–78.
76. Bi J, Jiang B, Liu JH, Lei C, Zhang XL, An LJ. Protective effects of catalpol against H2O2-induced oxidative stress in astrocytes primary cultures. Toxicol Sci. 2007;98(1):198–205.
77. Milatovic D, Yin Z, Gupta RC, Sidoryk M, Albrecht J, Aschner JL, et al. Manganese induces oxidative impairment in cultured rat astrocytes. Toxicol Sci. 2007;98(1):198–205.
78. Chen CJ, Liao SL. Oxidative stress involves in astrocytic alterations induced by manganese. Exp Neurol. 2002;175(1):216–25.
79. Bi J, Jiang B, Liu JH, Lei C, Zhang XL, An LJ. Protective effects of catalpol against H2O2-induced oxidative stress in astrocytes primary cultures. Neurosci Lett. 2008;442(3):224–7.
80. de Almeida LMV, Piñeiro CC, Leite MC, Broglese G, Leal RB, et al. Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. Neurochem Res. 2008;33(1):8–15.
81. Görg B, Karababa A, Häussinger D. Hepatic encephalopathy and oxidative and inflammatory stress and increase the resistance of astrocytes to oxidative stress in vitro. Glia. 2008;56(10):1114–26.
82. Song JH, Marszalec W, Kai L, Yeh JZ, Narahashi T. Antidepressants inhibit proton currents and tumor necrosis factor-α production in BV2 microglial cells. Brain Res. 2012;1435:15–23.
83. Lang MJ, Huang HY, Chen WF, Chang HF, Kuo JS. Glycogen synthase kinase-3β inactivation inhibits tumor necrosis factor-α production in microglia by modulating nuclear factor κB and MLK3/JNK signaling cascades. J Neuroinflammation. 2010;7:99.
84. Couto M, Coelho-Santos V, Santos L, Fontes-Ribeiro C, Silva AP, Gomes CMF. The interplay between glioblastoma and microglia cells leads to endothelial cell monolayer dysfunction via the interleukin-6-induced JAK2/STAT3 pathway. J Cell Physiol. 2019;234(11):19750–60.
85. Welser-Alves JV, Milner R. Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; Regulation by cytokines, lipopolysaccharide, and vitronecin. Neurochem Int. 2013;63(1):47–53.
86. Lu X, Ma L, Ruan L, Kong Y, Mou H, Zhang Z, et al. Resveratrol differentially modulates inflammatory responses of microglia and astrocytes. J Neuroinflammation. 2010;7(1):46.