Article

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HMGB1 Triggers Astrocyte Inflammation, Leading to Neurotoxicity through the TLR2 and 4 Pathways

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Background: Astrocytes are the most abundant cell type in the central nervous system (CNS) and play a crucial role in neuroinflammatory responses to damage and disease in the CNS. High mobility group box 1 (HMGB1) is an immuno-adjuvant factor that acts as a ligand for toll-like receptor (TLR)2/4 and is the receptor for advanced glycation end products (RAGE). It is associated with chronic neurodegeneration and neuroinflammation. We conducted a study to investigate the impact of HMGB1 on astrocytic cytotoxicity and inflammatory evolution.

Method: We cultured rat astrocytes and treated them with lipopolysaccharide (LPS) (TLR4 ligand), zymosan (TLR2 ligand), and HMGB1 or left them untreated as a control. Then, we blocked the activity of TLR2 and TLR4 with monoclonal antibodies. Using Luminex, we conducted measurements of cytokines interleukin-6 (IL-6), interleukin-1β (IL-1β), and interleukin-10 (IL-10) at various time intervals. Also, we performed a Flow Cytometry analysis to check for any signs of apoptosis or necrosis in the collected cells. Finally, we tested the effect of astrocytes conditioned medium on neurons after TLRS agonists and antagonists treated them.

Results: Our findings showed that the rate of apoptosis was highest after stimulation by HMGB1 (38.8%). However, the antagonists of TLR4 (25.8%) and TLR2 (31.2%) slightly reversed the rate. HMGB1 activated astrocytes to express higher levels of IL-6 and IL-1β, like bacterial ligands. Conversely, the anti-inflammatory cytokine IL-10 was reduced after prolonged interaction with HMGB1. Our data also showed that TLRs antagonists reacted differently according to time intervals for blocking cytokine release after astrocytes were stimulated with HMGB1.

Conclusions: HMGB1 exhibits diversity in astrocyte activation. It can trigger TLR pathways, resulting in inflammation and subsequent neurotoxicity, or it can be involved in tissue regeneration at some stage.

Keywords: HMGB1; TLR; astrocytes; apoptosis; inflammation

Introduction

The body employs inflammation as a defense mechanism, aiming to protect itself, dispose of dead cells, and initiate healing [1,2]. Astrocytes play a crucial role in responding to central nervous system (CNS) damage and disease with neuroinflammation, making them the second most critical cell type in the central nervous system for normal neurological function [3]. Astrocytes play a diverse role in controlling CNS disorders. Once this diversity is well understood, they can participate in treating neurodegenerative diseases [4].

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PRRs) that play a vital role in initiating innate and adaptive immunity by recognizing invading microorganisms [2,5]. There are ten known types of TLRs in humans, while mice have nine types. Each class specializes in identifying specific pathogen's molecular structures that are conserved. For instance, TLR4 recognizes gram-negative lipopolysaccharide (LPS), while TLR2 identifies gram-positive peptidoglycan lipoproteins and zymosan [1].

Researchers found that while microglia in rodents express mRNA for all TLRs, astrocytes express TLR2, TLR3, and TLR4 more selectively [6]. Stimulating toll-like receptors with their corresponding microbial molecules activate the NF-κB pathways [7]. These pathways activate chemokines and cytokines, causing an immune response even without infection [8,9]. A molecule called high mobility group box 1 (HMGB1), a protein that binds to DNA produced by various cells in the central nervous system is one of the ligands [10]. When a cell is damaged, HMGB1 can be released into the space outside of the cell. If released by a dead or dying cell, HMGB1 can act as a cytokine that promotes inflammation. It binds to TLRs on cells and stimulates the NF-κB pathway.

It has been shown that binding of HMGB1 to its surface receptors TLR4 and advanced glycation end products (RAGE) leads to the release of inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [10]. Also, these inflammatory cytokines are considered the central cytokines

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of systematic inflammation that disrupt the blood-brain barrier (BBB), leading to neurological degeneration [11]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that reduces BBB impairment and protects its permeability [11,12].

Research has shown that HMGB1 plays a role in developing various neurological diseases involving inflammation, such as stroke, epilepsy, and traumatic brain injury [13–16]. This occurs through the activation of innate immune mechanisms, as HMGB1 is part of a group of molecules known as damage-associated molecular patterns (DAMPs) [13]. Study has shown that after a traumatic brain injury, damaged neuronal cells release HMGB1, causing microglial activation [14]. A recent study found that tau oligomers cause neuroinflammation in astrocytes via HMGB1, indicating a crucial role in tauopathies like Alzheimer's [15]. Research has demonstrated that HMGB1 contributes to the development of Huntington's disease, a genetic neurodegenerative disorder, by causing inflammation of neurons and cell death [16].

This study aimed to investigate the impact of HMGB1 on astrocyte function, including cytotoxicity and release of inflammatory mediators, and whether its TLR2/4 antagonists can reverse these effects.

Materials and Methods

Animals

The Wister rats (Vital River Laboratories is located in Beijing, China) were sacrificed following the Biomedical Ethics Research Committee guidelines at King Abdul-Aziz University (Reference No 335 l6) for primary animals with a minimized number of rats, and unnecessary duplication of yielded isolated cultured cells from the animal was avoided. Isoflurane (2 mg/kg) was used for anesthesia, followed by cervical dislocation for euthanasia, ensuring minimal distress and pain for the mouse.

Astrocytes Isolation, Culturing, and Treatments

Two male rat pups, both two weeks old, were used to establish astrocyte cultures obtained from the KFRC animal facility [17]. After dissection, the cortical tissue was placed on two separate Petri dishes with cold earls balanced salt solution (EBSS) (100 mL EBSS + 1 mL $MgSO_4$ + 0.3 g BSA + 0.25 glucose), (E6267, M2643, A9418, 50-99-7, respectively, Sigma Aldrich, Saint Louis, MO, USA). The tissue was then chopped into small pieces and transferred to a 30 mL sterile solution. Solution 1 (2.5 mg trypsin (T9201, Sigma Aldrich, Saint Louis, MO, USA) + 10 mL EBSS solution) was added and agitated thrice for 15 minutes in a 37-degree Celsius water bath. Solution 2 (1.6 mL of solution 1 + 8.4 mL EBSS solution) was added and left for 5 minutes before removing the supernatant. Solution 3 (100 µL DNase (D5025, Sigma Aldrich, Saint Louis, MO, USA) + 12 mg trypsin inhibitor (T9003, Sigma Aldrich, Saint Louis, MO, USA) + 100 µL $MgSO_4$ + 100 mL EBSS solution) was added and titrated before transferring the suspension to a fresh tube. Finally, solution 4 (0.4 g BSA + 80 µL $MgSO_4$) was added and centrifuged for 8 minutes.

The pellet was re-suspended in 20 mL culture medium (500 mL, dulbecco's modified eagle's medium (DMEM)/Glutamax I + 75 mL fetal calf serum + 5 mL Gentamycin + 5 mL fungizone), (31966-021, 10108-165, 15710-049, 15290-026, respectively, Invitrogen, Shanghai, China). It was then transferred to a large flask with a total volume of 50 mL culture media and incubated in a primary incubator. After two days, the media was changed, aspirated after ten days, and replaced with 50 mL of shaking media (500 mL DMEM/Glutamax I + 50 mL fetal calf serum + 5 mL Gentamycin + 5 mL fungizone) and placed the flask in a shaking incubator overnight. After removing the medium containing unwanted cells, they were washed with PBS and trypsinized, then re-suspended the cells in culture media. The mycoplasma test was performed and followed a manufactured sheet of a lookout one-step mycoplasma PCR detection kit (MP0050, Sigma Aldrich, Saint Louis, MO, USA) to ensure damaging mycoplasma contamination using a conventional polymerase chain reaction in cultured cells before proceeding to our experiments. Lastly, the cells were plated in a poly-D-lysine (P6407, Sigma Aldrich, Saint Louis, MO, USA) coated six-well plate and placed on a shaker for 24 hours at 37 degrees before treatment with TLR agonists with or without antagonists.

The astrocyte cultures were grown in a six-well culture plate until they reached semi-confluency. The cultured cells were then treated with different substances, including one µg/mL of LPS (TLR4 ligand) (L9641, Sigma Aldrich, Saint Louis, MO, USA), ten µg/mL of zymosan (TLR2 ligand) (14L17-MM, InvivoGen, Hong Kong, China), one µg/mL of HMGB1 (an endogenous TLR2/4 ligand) from (HM-120, HMGBiotech, Milano, Italy) or left untreated as a control. TLR2 and TLR4 antagonists were also used to block their activity. Astrocytes were preincubated with either 1 µg/mL of anti-TLR2-monoclonal antibody (10C05-MM, InvivoGen, Hong Kong, China) or 5 µg/mL of anti-TLR4-monoclonal antibody (ab22048, Abcam, Cambridge, UK) for at least 20 minutes before being stimulated by the TLRs agonists. After treatment, the cells were incubated at 37-degree Celsius and 5% CO_2 for 16 hours. At certain times (2 h, 6 h, and overnight), the supernatants were collected and frozen for further investigation, and subsequently, cells were harvested for flow cytometry.

Apoptosis/Necrosis Detection Assay

Annexin V detection assay uses a fluorescent together with Annexin V, which binds to the phosphatidylserine of apoptotic cells in a suitable binding buffer (Ca^{2+} dependent). The essay is accompanied by nuclei staining with propidium iodide (PI) to detect dead cells, making the membrane more permeable. The analysis is then performed by flow cytometry [18]. The cells isolated astrocyte cultures with or without treatments of TLR ligands were harvested

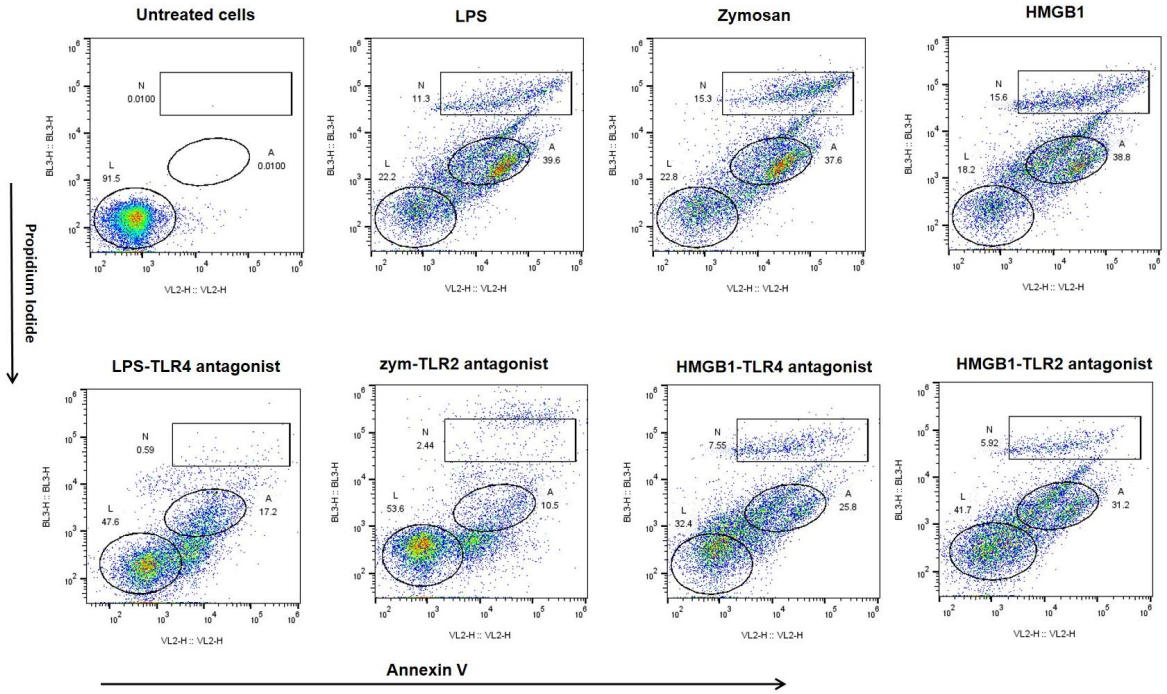

Fig. 1. Toll-like receptor (TLR) ligands induce astrocyte apoptosis and necrosis. Flow cytometry dot plot graphs representing astrocyte culture after staining with Annexin V/propidium iodide (PI) (apoptosis and necrosis markers) (left panel-healthy cells and suitable panel-unhealthy cells); the L is gated for live cells, A is gated for apoptotic cells, and N is gated for necrotic cells.

after 20 hours of incubation at 37 °C with 5% CO_2 with Trypsin. Then, cells were stained with an Annexin V/PI detection kit supplied by (V13241, Thermofisher, Shanghai, China) as described in the manual sheet. Stained cells were washed and analyzed using an Attune Flow Cytometer (A51848, Thermofisher, Shanghai, China). FlowJo software (Version v10.9, FlowJo™ Software and BD® Research, Ashland, OR, USA) was used to analyze the apoptosis and necrosis percentages in untreated and treated astrocyte cultures.

Luminex

Canine cytokine multiplex assay assessed IL-6, IL-1β, and IL-10 cytokines (CCYTOMAG-90K, Merk Millipore, Darmstadt, Germany). In addition, frozen supernatant samples (from astrocytes treated with TLR2/4 ligands and their antagonists) were evaluated for cytokines levels in duplicate at one time by using a single plate, and the procedure was done according to the assay protocols by the manufacturer. The Luminex 200 machine (40-012, Merk Millipore, Darmstadt, Germany) and Milliplex Analyst software (Version 5.1, Merk Millipore, Darmstadt, Germany) were used for data analysis at the Neuroscience Unit in the KAU.

Neurotoxicity

We tested the effect of astrocytes conditioned medium on neurons after TLRs agonists and antagonists treated them. Therefore, we used an *ex-vivo* rat optic nerves (RON) model as in previously depicted experiments [19]. 5 µL of supernatants from astrocytic cultured treated with TLR ligands/antagonists were added to RON and incubated for 60 minutes at 95% O_2, 5% CO_2, 37 °C. Then, RONs were washed, fixed with 4% paraformaldehyde (P6148, Sigma Aldrich, Saint Louis, MO, USA)/0.1 M PBS and frozen at –80 °C in tissue tech.

Statistical Analysis

Data were analyzed with GraphPad Prism 9 software (Version 9.5.1, Prism, Boston, MA, USA). The variances of untreated and treated astrocyte cultures with different TLR agonists and antagonists were compared using a one-way analysis of variance (ANOVA; Bonferroni test). Significant differences were defined as p-values 0.05.

Results

HMGB1-Induced Astrocyte Apoptosis and Necrosis

This research examined how TLR ligands impact astrocyte apoptosis and necrosis caused by Annexin V and propidium iodide, as shown in Fig. 1. Analysis through flow cytometry revealed that lipopolysaccharide (a TLR4 agonist) and zymosan (a TLR2 agonist) resulted in higher rates of apoptosis and necrosis (39.6% and 11.3%, and 37.6% and 15.3%, respectively) compared to untreated cells (0.01% and 0.01% respectively). Interestingly, HMGB1 stimulation resulted in the highest rate of apoptosis (38.8%) and necrosis (15.6.3%), which was diminished by TLR2 and TLR4 antagonists (Fig. 1, Table 1).

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Table 1. Live, apoptosis, and necrosis cells treated by TLR4/2 agonists and antagonists or left untreated.

	Live mean ± SD	Apoptosis mean ± SD	Necrosis mean ± SD	Un-treated vs treated (*p*-value)	Agonist vs antagonist (*p*-value)
Un-treaded	91.17 ± 0.49	0.02 ± 0.027	0.01 ± 0.01		
LPS	22.04 ± 0.36	38.9 ± 0.69	10.9 ± 0.4	****	
LPS-TLR4 antagonist	46.8 ± 1.16	17.3 ± 0.35	0.6 ± 0.13		^^^
Zymosan	22.2 ± 0.71	37.4 ± 0.37	14.9 ± 1.04	***	
Zymosan-TLR2 antagonist	54.2 ± 0.87	10.8 ± 0.34	2.8 ± 0.75		^^
HMGB1	19.8 ± 1.24	38.2 ± 0.77	15.4 ± 0.5	***	
HMGB1-TLR4 antagonist	32.16 ± 0.73	26.9 ± 1.16	7.8 ± 0.46		^
HMGB1-TLR2 antagonist	41.8 ± 0.74	31.8 ± 0.75	5.07 ± 0.9		^

Comparisons were made between untreated cells control versus treated cells with TLR4/2 agonists ***$p < 0.001$, ****$p < 0.0001$; and TLR ligands versus TLR4/2 antagonists ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$. Statistical significance was one-way analysis of variance (ANOVA) (n = 5). LPS, lipopolysaccharide; HMGB1, high mobility group box 1; SD, standard deviation.

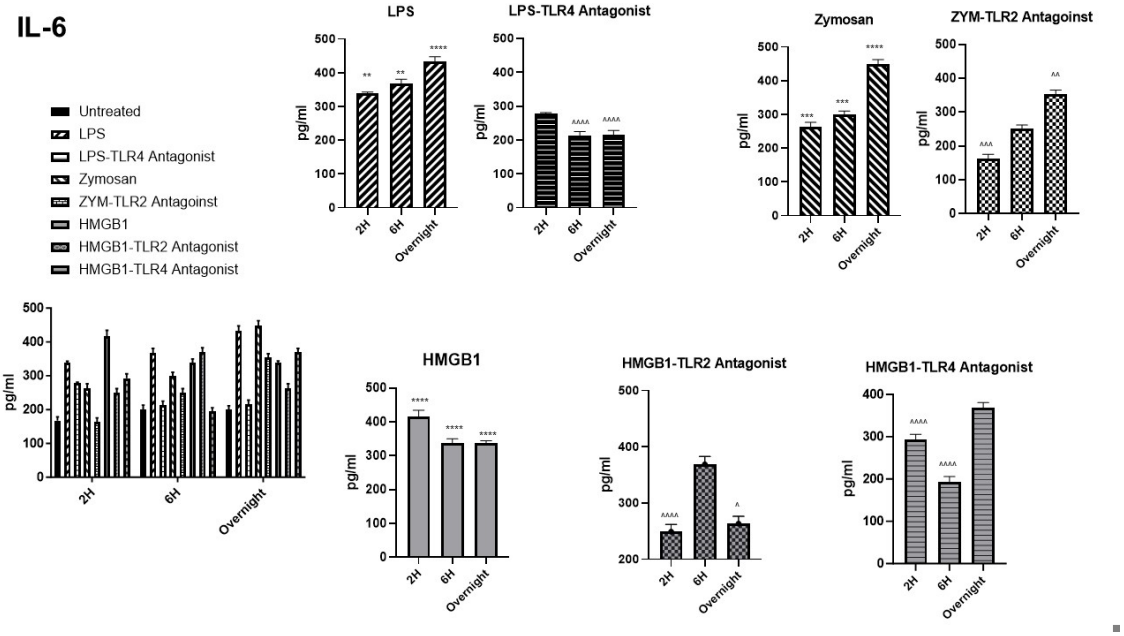

Fig. 2. HMGB1 induced interleukin-6 (IL-6) cytokine from astrocytes and blocked differently by TLR antagonists. Graphic representations of IL-6 levels of astrocytes in response to LPS, zymosan, and HMGB1 at different time intervals. Comparisons were made between untreated cells control versus stimulated cultures, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; and TLR ligands versus TLR4/2 antagonists ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$, ^^^^$p < 0.0001$. Values were illustrated as mean ± standard error of the mean (SEM). Statistical significance was one-way ANOVA (n = 5).

HMGB1 Induced IL-6 Cytokine from Astrocytes and Blocked Differently by TLR Antagonists

HMGB1, like the bacterial endotoxins, induced significantly higher amounts of IL-6 cytokine after two h, six h, and overnight stimulations (Fig. 2). The cytokine induction by TLRs-bacterial ligands was attenuated considerably with their blockers at corresponding periods. Interestingly, the TLR4 antagonist significantly blocked the IL-6 release at two h and six h of treatment of HMGB1, conversely to the effect of the TLR2 antagonist at six h.

HMGB1 Induced IL-1β Cytokine from Astrocytes but Unblocked by TLR Antagonists

LPS, zymosan, and HMGB1 significantly induced IL-1β cytokine at different time intervals (Fig. 3). While the TLR2/4 antagonists could block the induction of IL-1β with their bacterial ligands, they could not block IL-1β released by HMGB1.

HMGB1 Altered IL-10 Cytokine from Astrocytes at Different Time Intervals and Responded Differently to TLR Antagonists

An anti-inflammatory cytokine, IL-10, was significantly increased with LPS and zymosan simulations at two h, six h, and overnight (Fig. 4). Similarly, IL-10 was in-

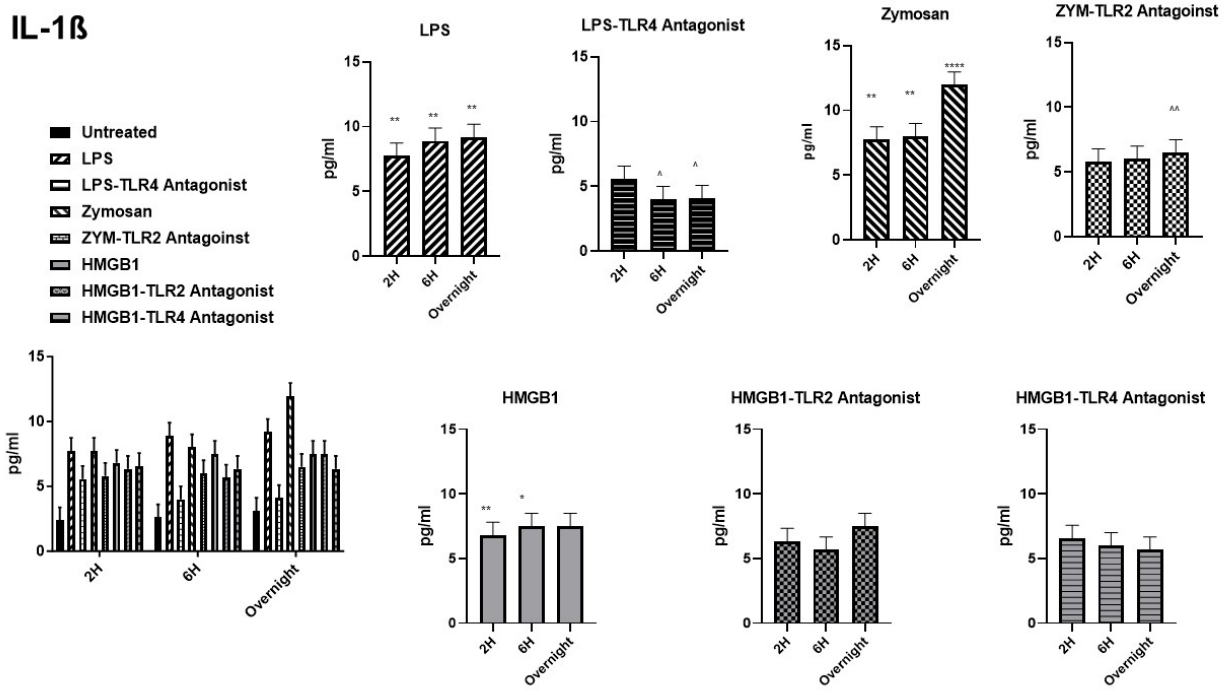

Fig. 3. HMGB1 induced interleukin-1β (IL-1β) cytokine from astrocytes but unblocked by TLR antagonists. Graphic representations of IL-1β levels of astrocytes in response to LPS, zymosan, and HMGB1 at different time intervals. Comparisons were made between untreated cells control versus stimulated cultures $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$; and TLR ligands versus TLR4/2 antagonists $\hat{}p < 0.05$, $\hat{}\hat{}p < 0.01$. Values were illustrated as mean ± SEM. Statistical significance was one-way ANOVA (n = 5).

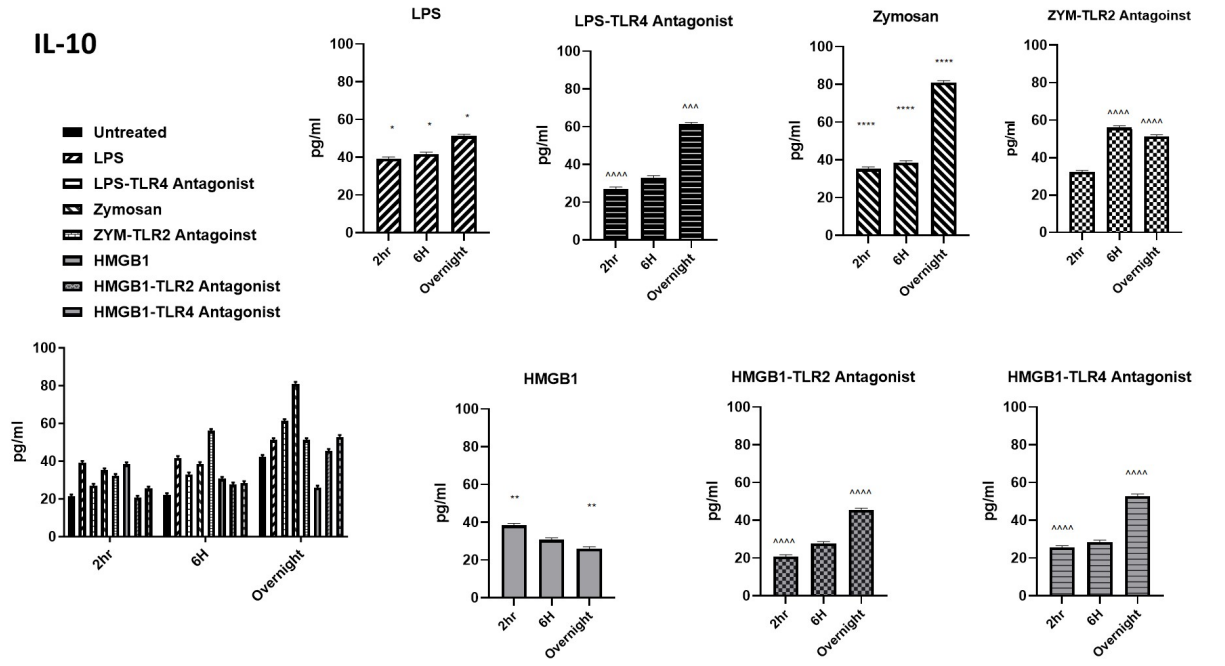

Fig. 4. HMGB1 altered interleukin-10 (IL-10) cytokine from astrocytes at different time intervals and responded differently to TLR antagonists. Graphic representations of IL-10 levels of astrocytes in response to LPS, zymosan, and HMGB1 at different time intervals. Comparisons were made between untreated cells control versus stimulated cultures. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$; and TLR ligands versus TLR4/2 antagonists, $\hat{}\hat{}\hat{}p < 0.001$, $\hat{}\hat{}\hat{}\hat{}p < 0.0001$. Values were illustrated as mean ± SEM. Statistical significance was one-way ANOVA (n = 5).

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creased dramatically with HMGB1 stimulation at two h but was significantly decreased overnight and remained unchanged at six h.

TLR4 antagonist reduced IL-10 levels against LPS induction at six h, although it augmented anti-inflammatory cytokine overnight. IL-10 levels were unchanged at two h, increased at six h, and decreased overnight with the TLR2 antagonist. For HMGB1, both TLR antagonists attenuated IL-10 release at two h, remained unchanged at six h, and augmented anti-inflammatory cytokine overnight.

Discussion

HMGB1, an immuno-adjuvant factor, acts as a ligand for TLR2/4 and RAGE1 [18]. A high level of HMGB1 has been linked to memory impairment, chronic neurodegeneration, and advanced neuroinflammation progression [13,20].

Evidence shows that upregulation of HMGB1 in spinal astrocytes activates astroglia and induces neuropathic pain that a TLR4 inhibitor can reverse [21]; HMGB1 enhances the production of sonic hedgehog via astrocytes from an animal model of multiple sclerosis [22], and HMGB1 promoted astrocytic neurotrophic response which is lost in amyotrophic lateral sclerosis model [23]. The studies mentioned above focus on models of neurodegenerative diseases that show the critical role of HMGB1 in astrocytes. However, this study aims to explore how HMGB1 affects the functions of astrocytes, such as neurotoxicity and the release of inflammatory mediators.

Our findings showed that astrocyte cultures treated with HMGB1 had a higher apoptosis rate than the control group. Additionally, TLR2 or TLR4 antagonists partially reversed the HMGB1-induced cell death. After a traumatic event, activating the TLR4 receptor by HMGB1 may trigger an innate immune response, but a different receptor, such as RAGE, might lead to cellular death [10,20]. A study supports the concept by showing that post-trauma, HMGB1, and RAGE are highly coexpressed in rats and humans [24]. In their model, the Xiao's team [22] showed that inhibiting RAGE was the only way to prevent the enhancement of astrocytic release of sonic hedgehog caused by HMGB1. That suggests that astrocytic death induced by HMGB1 could play a role in developing neurodegenerative diseases and the TLR signaling mechanism.

A further function of astrocytes, pro-inflammatory (IL-6 & IL-1β), and anti-inflammatory (IL-10) biomarkers were tested in the present study. At a short period (2 h) of incubation, the HMGB1 induces the highest levels of IL-6 from activated astrocytes, then reduced gradually over time. By contrast, IL-1β & IL-10 persisted after HMGB1 interacted for a longer time with astrocytes.

IL-6 and IL-1β are the critical mediators of inflammatory and immune responses induced in infection or tissue injury, and higher plasma levels of these cytokines have been reported in many neurological diseases [11,25]. In severe cases of epilepsy, the levels of HMGB1, TLR4, and IL-1β are higher than in mild cases [26]. Additionally, HMGB1 and IL-1β considered to be critical predictors of epilepsy prognosis [27]. Alzheimer's disease is associated with elevated levels of multiple inflammatory mediators, for instance, HMGB1, IL-1β, IL-6, TNF-α, and NF-κB [28]. Similarly, the higher expression of IL-1β and IL-6 due to stimulation of the HMGB1-TLR4-NF-κB pathway was approved in the MPTP-induced zebrafish Parkinson's disease model, and the associated with neuronal loss in the zebrafish brain, dyskinesia and dysangiogenesis [29]. IL-10 is an anti-inflammatory, which enhances the immunomodulation effect and has an essential role in the resolution phase [12]. IL-10 inhibits the proinflammatory cytokines, antigen presentation, and cell proliferation by reducing the monocyte and macrophage T-cell responses [12]. In the traumatic brain injury of animal experiments, the IL-10 expression in brain tissue was demonstrated to be raised immediately after injury, and it increased after 2–24 h [30]. Therefore, the role of inflammatory cytokines in the pathogenesis of neuroinflammatory disease has been gradually proven.

Many studies endorsed the concept that HMGB1 is not only a DAMP molecule produced after tissue damage as a danger signal but also a protein able to repair tissue [13,31]. It has been demonstrated that HMGB1 provokes a pro-inflammatory process to recruit stem cells in various diseases that promote new tissue regeneration [32,33]. This suggested that the HMGB1 might initially play a generative role in promoting astrocytes to neutralize the inflammatory activity until the macrophages and other leukocytes reach the neuronal system. On the other hand, our data indicated that if the HMGB1 persists for longer without clearance by the immune system, it might harm the astrocytes, leading to neurotoxicity [13].

Our data indicated that blocking HMGB1 action on astrocytes with TLR2/4 antagonists had different reactions depending on the time intervals, suggesting a potential for expanded therapeutic measures in future studies [34,35]. A recent study found that Quercetin can treat encephalitis in rats by blocking HMGB-1's interaction with TLR4, thus reducing neuronal cell apoptosis [36]. Additionally, researchers are exploring the potential of introducing the HMGB1-TLR pathway in anti-epileptic treatments [37].

New HMGB1 inhibitors to be utilized as therapeutic agents include small natural. Some examples of compounds that target HMGB1 include specific antibodies and synthetic molecules such as gabexate mesylate and glycyrrhizin [38].

Conclusions

In general, HMGB1 displays a range of effects on the activation of astrocytes. It can trigger pathways that cause inflammation and neurotoxicity or participate in tissue regeneration. Researchers must comprehend these cellular mechanisms to identify potential therapeutic targets for neurodegenerative ailments.

Availability of Data and Materials

Data are contained within the article.

Author Contributions

Both authors listed above qualify for authorship based on making substantial contributions to the following: (i) designed the experiment: EA & AA, (ii) data analysis and interpretation: EA & AA, and (iii) wrote and revised the manuscript: EA & AA, and both authors agreed on the manuscript final submission. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The study was designed with correspondence to the codes of the guidelines for Ethical Conduct in the Care and Use of Animals; experimental conduct and handling were authorized via the Animal Ethics division within the Biomedical Ethics Research Committee guidelines at King Abdul-Aziz University (Reference No 335 l6). The experiment was executed in consensus with the guidelines for dealing with experimental animals that are followed in KFMRC, KAU, Jeddah, Saudi Arabia, by the Canadian Council for Animal Safety and Health Care.

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Conflict of Interest

The authors declare no conflict of interest.

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