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Neuroinflammation Mediated by Glia Maturation Factor Exacerbates Neuronal Injury in an *in vitro* Model of Traumatic Brain Injury

Mohammad Ejaz Ahmed,[1–3,*] Govindhasamy Pushpavathi Selvakumar,[1–3,*] Duraisamy Kempuraj,[1–3] Sudhanshu P. Raikwar,[1–3] Ramasamy Thangavel,[1–3] Kieran Bazley,[1,2] Kristopher Wu,[1,2] Osaid Khan,[1,2] Klaudia Kukulka,[1,2] Bret Bussinger,[1,2] Iuliia Dubova,[1–3] Smita Zaheer,[1,2] Raghav Govindarajan,[1,2] Shankar Iyer,[1–3] Casey Burton,[4] Donald James,[4] and Asgar Zaheer[1–3]

Abstract

Traumatic brain injury (TBI) is the primary cause of death and disability affecting over 10 million people in the industrialized world. TBI causes a wide spectrum of secondary molecular and cellular complications in the brain. However, the pathological events are still not yet fully understood. Previously, we have shown that the glia maturation factor (GMF) is a mediator of neuroinflammation in neurodegenerative diseases. To identify the potential molecular pathways accompanying TBI, we used an *in vitro* cell culture model of TBI. A standardized injury was induced by scalpel cut through a mixed primary cell culture of astrocytes, microglia and neurons obtained from both wild type (WT) and GMF-deficient (GMF-KO) mice. Cell culture medium and whole–cell lysates were collected at 24, 48, and 72 h after the scalpel cuts injury and probed for oxidative stress using immunofluorescence analysis. Results showed that oxidative stress markers such as glutathione and glutathione peroxidase were significantly reduced, while release of cytosolic enzyme lactate dehydrogenase along with nitric oxide and prostaglandin E2 were significantly increased in injured WT cells compared with injured GMF-KO cells. In addition, injured WT cells showed increased levels of oxidation product 4-hydroxynonenal and 8-oxo-2′-deoxyguanosine compared with injured GMF-KO cells. Further, we found that injured WT cells showed a significantly increased expression of glial fibrillary acidic protein, ionized calcium binding adaptor molecule 1, and phosphorylated ezrin/radixin/moesin proteins, and reduced microtubule associated protein expression compared with injured GMF-KO cells after injury. Collectively, our results demonstrate that GMF exacerbates the oxidative stress–mediated neuroinflammation that could be brought about by TBI-induced astroglial activation.

Keywords: ezrin/radixin/moesin proteins; glia maturation factor; neurodegeneration; neuroinflammation; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is a sudden damage to the brain caused by mechanical forces leading to death and disability among young and older people worldwide. Over 10 million hospitalizations annually are directly attributed to TBI and approximately 57 million people worldwide have experienced such a brain injury.[1] Brain damage following TBI results from direct impact (primary injury) and indirect impact (secondary injury). The secondary injury progressively initiates an acute inflammatory response, including breakdown of the blood–brain barrier, infiltration of peripheral blood into the brain, activation of resident inflammatory and immunocompetent cells, and eventually to the release of several kinds of cytokines and chemokines that subsequently leads to secondary damage of brain.[2-4]

The involvement of multiple cell types like glial cells, neuronal cells, inflammatory cells, and endothelial cells complicates the understanding of TBI. Numerous TBI models have been established over the last decades that have been invaluable in understanding the mechanism of TBI pathology. The *in vitro* injury model is very valuable to understand the changes in cellular functions after injury and characterizes important biomarkers that are relevant in evaluating the in vivo TBI model.[5] Different *in vitro* TBI models have been developed, such as static mechanical injury, dynamic mechanical injury, hydrodynamic model, and cell stretch model, to convincingly solve the many aspects of the post-traumatic events in

[1]Department of Neurology and [2]Center for Translational Neuroscience, School of Medicine, University of Missouri, Columbia, Missouri, USA.
[3]Harry S Truman Memorial Veterans Hospital, Columbia, Missouri, USA.
[4]Phelps Health, Rolla, Missouri, USA.
*These authors contributed equally to this manuscript.

cell culture system, including cellular ultra-structural changes, ionic alteration, and free radical production after injury to provide a platform for the repeatable evaluation and well-controlled environmentally isolated experiments.[6,7]

The ezrin/radixin/moesin (ERM) family of proteins is responsible for the remodeling of tissue and linking of membrane surface integral proteins with the cortical actin cytoskeleton, and participates in signal transduction pathways in the brain.[8] Any alteration in expression or functional changes in ERM eventually triggers changes to the actin cytoskeleton followed by disruption of cellular interactions, abnormal cell shape, and rupture of membranous structure, with massive trafficking inside the cell and apoptosis.[9] Expression of ezrin is shown to be up-regulated following brain trauma *in vivo.* It has been found that phosphorylation of ERM occurs promptly in response to astroglial activation following TBI.

Glia maturation factor (GMF), an inflammatory protein containing 141 amino acids, was discovered, isolated, and cloned in our laboratory and is mainly expressed by astrocytes and some neuronal cells in the brain.[10-12] We have previously shown that there is an increase in the level of GMF in the neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and experimental autoimmune encephalomyelitis.[12–15] The potential role of GMF in TBI has not yet been explored. In the present study, we used the cell culture scratch injury model on mixed cultures containing primary neurons, astrocytes, and microglia from wild type (WT) and GMF-deficient (GMF-KO) mice and followed the expression of proteins in the cells and the extracellular cell culture media following 24, 48, and 72 h after injury. We propose that induction of scratch cellular injury in the cells isolated from the brains of pups from WT and GMF-KO mice would help elucidate the potential role of GMF in neuro-degeneration, neuroinflammation, and oxidative stress following the scratch injury *in vitro* cell culture model of TBI. In the present study, we hypothesize that knocking down of GMF should provide neuroprotection and reduction in neuroinflammation and oxidative stress by limiting astroglial activation following injury.

Methods

Mouse primary neural cell culture

Mouse primary neural cultures were prepared by using fetal brains obtained from wild type (WT; C57BL/6) mice and GMF-KO (C57BL/6) mice as described previously.[16,17] Brain tissues were harvested and the cells were isolated and used to grow *in vitro* mixed cell culture that contains astrocytes, microglia, and neurons. Briefly, brain tissues were obtained from E16 mouse brain. Cells were dissociated by the enzymatic digestion method using a 0.25% trypsin/ethylenediaminetetraacetic acid solution. These mixed neural cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco, Thermo Scientific),1% 1 penicillin/streptomycin (Gibco Thermo Scientific), 2 mM L-glutamine (Gibco Thermo Scientific), at 37°C in humidified 5% CO_2 and 95% air. Cultures (0.3×106 cells/well) were grown on the top of the poly-D-lysine–coated cover glass (Millipore, Billerica, MA) in six-well tissue culture plates for 2 weeks.[18] These cell cultures represent a mixed neural and glial population based upon morphological observation under a microscope. Cultures prepared according to this culture condition were found to contain 54% neurons and 46% glial cells at maturity as determined by immunofluorescence staining for glial fibrillary acidic protein (GFAP; astrocytes), ionized calcium binding adaptor molecule 1 (IBA1; microglia), and microtubule associated protein (MAP2; neurons). The mice used in this study were maintained and the experiments were performed in accordance with the National Institutes of Health (NIH) guidelines approved by the Animal Care and Use Committees at the University of Missouri (Columbia, MO).

In vitro *model of traumatic brain injury*

An *in vitro* scratch mixed cellular culture injury model was used in this study. Cellular Injury was induced using 22 size scalpel blade cuts (20-20 times) in differentiated mixed culture 10 times perpendicularly in either direction approximately 2 mm apart as described previously.[19] The scratch model produces an injury in the cells that is clearly separated from the surrounding uninjured cells in the cell culture. Injured cultures and uninjured control cultures were then incubated for 24, 48, and 72 h at 37°C, 5% CO_2 in humidified air. The medium was collected from both the WT and GMF-KO injured cultures at different time-points, centrifuged at 4°C for 10 min at $10,000 \times g$ to remove any cells and debris, and stored at -80°C until analyzed. The adherent cells were mechanically removed with a cell lifter and saved separately in low bind Eppendorf tubes after removal of any remaining liquid by centrifugation in 4°C for 10 min at 10,000 g.

Lactate dehydrogenase release assay

The amount of lactate dehydrogenase (LDH) released into the culture medium after the cellular injury was analyzed based on the conversion of a tetrazolium salt into red formazan product according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI; Cat No. 601170). Briefly, cells were subjected to scalpel injury and the effect of treatment on cells was examined by assaying LDH release at different time-points (i.e., 0, 24, 48, and 72 h following scratch injury). The absorbance that is proportional to the number of lysed cells was measured at 490 nm. The blank LDH levels were subtracted from the cellular injury LDH values, and results were expressed as pg/mL. The amount of LDH released by the cells in the presence of 1% Triton X100 was considered as maximal LDH release absorbance.[20] The results were expressed as % cytotoxicity.

Glutathione assay

Glutathione (GSH) levels were measured in the cell culture medium by using GSH assay kit (Cat No. 703002; Cayman chemical), according to the manufacturer's instruction. The level of GSH was calculated as μmol DTNB conjugate formed/mg protein using a molar extinction coefficient of $13.6\ 10^3\ M^{-1}\,cm^{-1}$ and the results were expressed picomoles of GSH per milligram of protein.

Glutathione peroxidase assay

The total activity of glutathione peroxidase (GPx) was measured in the supernatant of cell culture media by using the GPx assay kit (Cat No. 703102; Cayman chemical), as per manufacturer's instructions. GPx catalyzes the reduction of hydroperoxides by reduced glutathione and its activity is determined indirectly with a couple reaction with glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to $NADP^+$, followed as a rate of reduction in absorbance at 340 nm (A340) is proportional to the GPX activity. The total activity of GPx is defined as the amount of GPx required to oxidize one mmol of NADPH/min/mg of protein.

Nitric oxide assay

After mechanical scratch injury for 0 h, 24 h, 48 h, and 72 h, the accumulation of nitrite in the culture medium was measured as an indicator of nitric oxide (NO) production.[21] The aforementioned cell culture medium-supernatant media also were used to measure the levels of NO. The level of NO was determined colorimetrically using the Griess reagent in a NO assay kit (Cat No. 780001; Cayman

Chemical) and calculated as per the manufacturer's instructions. Fresh cell culture medium was used as a blank in every experiment and assayed at 540-550 nm. The total nitrite/nitrate concentrations were calculated as nitrite + nitrate (μM) = (NO) in medium and given as micromolar (μM) concentrations (means ± standard error of the mean. Nitrite/nitrate linear standard curves were determined by triple determinations ($n = 3$).

Prostaglandin E2 assay

The mixed cultures were subjected to mechanical injury by the scalpel CUTS. The media were removed from the cultures at 0, 24, 48, and 72 h after the injury was used to measure the level of prostaglandin E2 (PGE2) using enzyme-linked immunosorbent assay detection kit (Cat No. 514010; Cayman Chemical) that uses a PGE2-specific monoclonal antibody and calculated as per the manufacturer's instructions. Fresh cell culture medium was used as a blank in every experiment and assayed at 405-420 nm and the amount of PGE2 was expressed as picogram/ml of cell culture supernatant.

Immunofluorescence and confocal microscopy

The immunofluorescence staining and confocal microscopy were performed as we described before.[17,22-24] The mixed neuronal cells were seeded on Poly-D-Lysine pre-coated glass coverslips (Millipore, Billerica, MA). After the mechanical injury with scalpel, cells were fixed with 4% paraformaldehyde for 1 h and permeabilized by incubation in phosphate-buffered saline (PBS)/ 0.1% Triton X-100 for 15 min. Following three times wash in PBS, blocked with 5% normal goat serum for 30 min and finally incubated for 2 h at room temperature with primary antibody Iba1 (1:500 dilution; Cat No. ab5076, Abcam, Inc), GFAP (1:500 dilution; Cat No. ab7260, Abcam, Inc), MAP2 (1:500 dilution; Cat No. ab32454, Abcam, Inc.), ERM (1:250 dilution; Cat No. 3142, Cell Signaling Technology, Inc.), phosphorylated ezrin/radixin/moesin (pERM)1:250 dilution; Cat No. 3726, Cell Signaling Technology, Inc.), 4-hydroxynonenal (4-HNE) and 8-hydroxydeguanosine (8-OHdG; 1:200 dilution; Cat No. CAS 75899-68-2, Santa Cruz Biotech, Inc.).

Then, cells were incubated with appropriate secondary anti-rabbit and anti-mouse Alexa Fluor 488 (Cat No. A-11001, ThermoFisher Scientific) and Alexa Fluor 568 (Cat No. A-11011, ThermoFisher Scientific) for 1 h at room temperature. Finally, the cover-slips, VECTASHIELD with Antifade Mounting Medium with DAPI (Vector Laboratories), were mounted with Vinol (Sigma) onto microscope slides. Confocal imaging was performed on a Leica TCP SP8 laser scanning confocal microscope using a 63 × oil immersion objective (Molecular Cytology Core Facility, University of Missouri, Columbia, MO).

Statistical analysis

All the results obtained from the experiments were analyzed by GraphPad InStat 3 software. Results were given as mean ± standard error of the mean. Results were analyzed using a one-way analysis of variance followed by *post hoc* test Tukey-Kramer *post hoc* multiple comparison analysis to determine statistically significant differences. A $p < 0.05$ was considered statistically significant in all the experiments in this study.

Results

Absence of GMF decreases GFAP and Iba1 expression in mixed neural cells following in vitro *TBI injury*

Neuroinflammation is a major pathological process in the progression of secondary response after trauma. The inflammatory response is designated by the term sterile inflammation or inflammation in the absence of a pathogenic stimulus. To determine the glia activation after scratch injury,[25] a scalpel cut 20 times in two directions was used to injure the confluent monolayer of mixed primary neuronal cells. After the injury, plates were incubated for different time courses (0 h, 24 h, 48 h, and 72 h) and the cells were fixed and stained by immunofluorescence staining using specific antibodies for GFAP and Iba1. GFAP and Iba1, respective markers for reactive astrocytes and microglial, shows time course expression by double immunofluorescence staining of GFAP (green) and Iba1 (red; Fig. 1A, 1B). The intensity of staining gradually increased over the observed time course of 72 h in WT mice following injury. However, the absence of GMF in GMF-KO cells showed decreased GFAP and Iba1 expression over the observed time course of 72 h following TBI. Figure 1C and 1D shows the quantitative analysis of GFAP and Iba1 expression in WT and GMF-KO mice cells, respectively.

Absence of GMF preserves structural morphology of neurons through axonal regeneration following injury

Cytoskeleton-related proteins such as microtubule associated protein (MAP2) are part of the family of proteins responsible for stabilizing and remodeling neuronal shape by promoting microtubule synthesis and cross-linking with other components of the cytoskeleton. The mixed neuronal cultures following scalpel cut injury were incubated from 0 h to 72 h and stained with neuron-specific marker MAP2. Immunofluorescence staining for MAP2 of WT mice clearly showed neurite length per cell gradually becoming shorter over the observed time course of 72 h (Fig. 2A). However, neuronal cultures from GMF-KO mice showed a gradual increase in neurite length over the time course from 0 h to 72 h compared with WT mice (Fig. 2B).

Absence of GMF downregulates actin related proteins following scratch injury

Actin is the most abundant protein in the neuronal cells cell and plays an important role in maintaining cellular structure ''proliferation, phagocytosis and cell signaling.''[26] The ezrin, radixin, and moesin (ERM) family proteins bind with actin filament when phosphorylated and known to be upregulated following TBI.[19] To investigate whether the ERM proteins promote cellular re-modification after injury, we performed double immunofluorescence staining with antibodies against ERM, pERM, and GFAP. We found increased the expression of ERM along with its co-localization with GFAP over the time course of 0 to 72 h in the cells from WT mice following cell injury when compared with GMF-KO cells following scratch injury (Fig. 3A, 3B). Immunofluorescence staining of the phosphorylated form of ERM in cells from WT and GMF-KO mice demonstrate that in WT mice cells, astrocyte expresses more pERM over the time course 0 to 72 h (Fig. 4A). Further, we observed that pERM expression gradually decreased over the time course from 0, 24, 48, and 72 h following injury in GMF-KO cells when compared with WT cells (Fig. 4B).

Absence of GMF down-attenuates oxidative damage following cell culture scratch injury

Oxidative stress plays an important role in the progression of disease following TBI. Thus, we next examined the role of GMF in oxidative damage in the in vitro TBI cell culture model. Immunofluorescence staining of well-characterized markers of oxidative

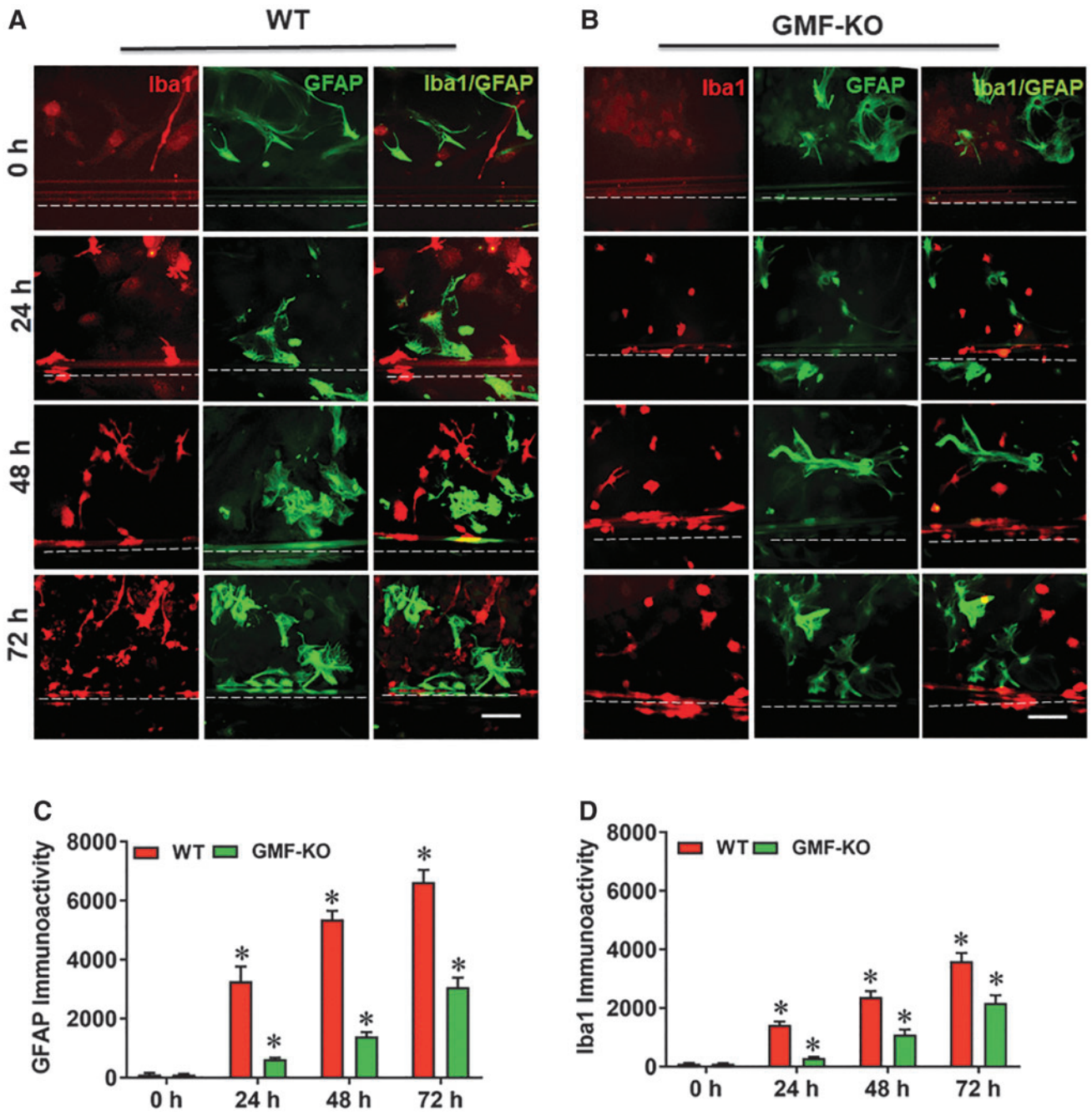

FIG. 1. The absence of glia maturation factor (GMF) attenuates *in vitro* injury-induced reactive gliosis. Representative immunofluorescence staining of glial fibrillary acidic protein (GFAP; green) and ionized calcium binding adaptor molecule 1 (IBA1; red) in mixed primary neurons and glial cells shows over the different time course effects (0, 24, 48 and 72 h) following post scratch induced injury. GMF-deficient (GMF-KO) mixed primary neurons and glial cells staining showed a gradually huge reduction in expression and co-localization of GFAP and compared with wild type primary mixed neuronal cells showed in **(A, B).** The immunoactivity associated with GFAP and Iba1 were quantified and shown as percentage changes versus 0 h control group **(C, D).** Scale bar = 50-μm and dash line represent the injury site. Data are presented as mean ± standard error of the mean ($n = 3$). *$p < 0.05$ versus uninjured control. Color image is available online.

damage 4-HNE and 8-OHdG, which are indicative of lipid peroxidation and DNA damage respectively. Our results showed a robust increase in the intensity of these oxidative stress markers in WT cell culture media. However, GMF-KO cell culture media showed gradual reduction in intensity of these oxidative stress markers over the time course 0 to 72 h following injury (Fig. 5A, 5B). The results highlight an important role for GMF in oxidative stress–mediated neuronal damage evident from the oxidative products released in the culture media following cell injury.

Absence of GMF reduces oxidative stress mediated neuronal injury following cell culture scratch injury

To determine whether the absence of GMF reduces oxidative stress–mediated neuronal injury, we used primary neuronal cells

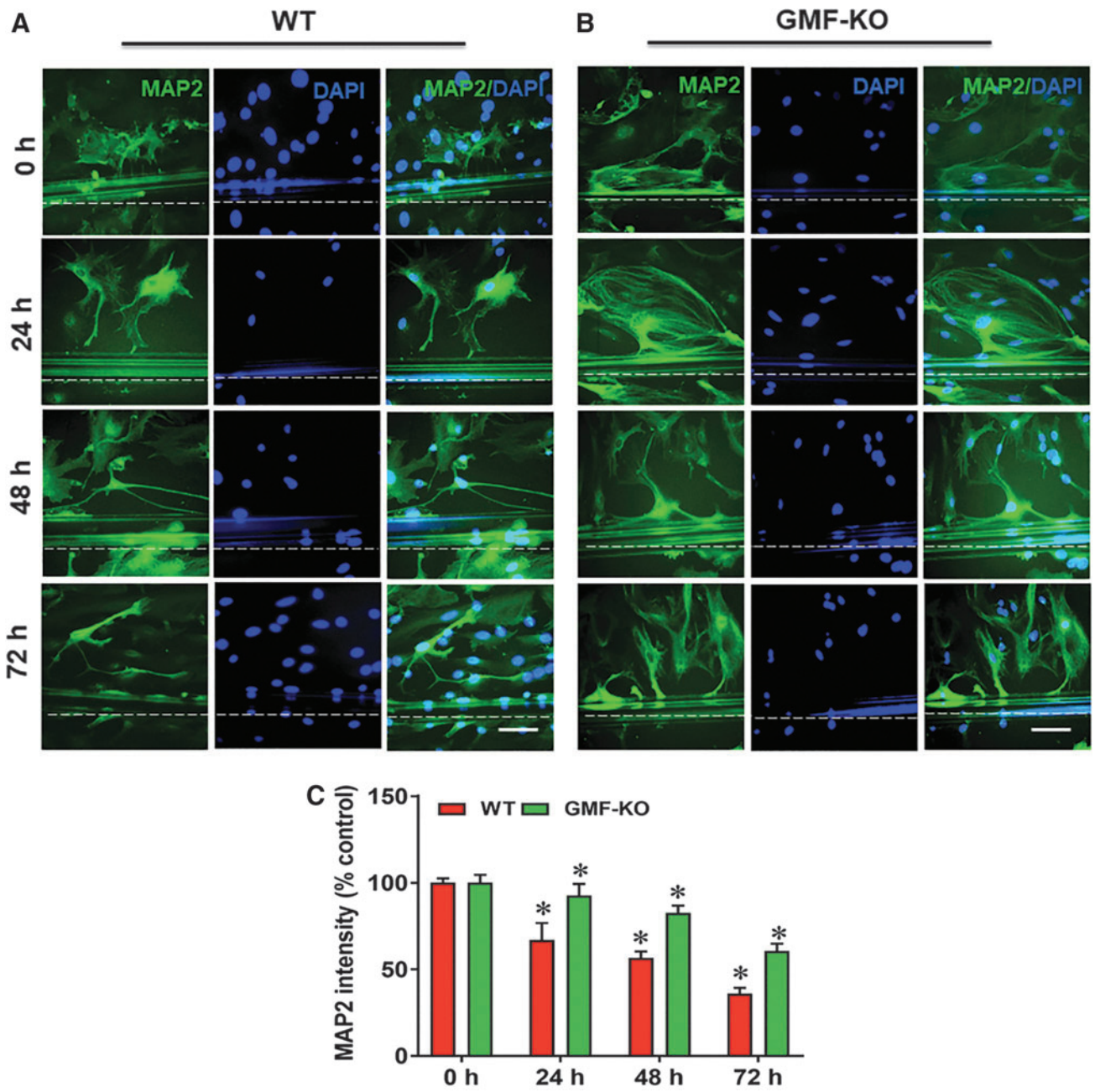

FIG. 2. The absence of glia maturation factor (GMF) promotes regeneration of neurons following neuronal injury in primary neuronal cultures. We performed immunofluorescence staining using neuronal-specific marker microtubule associated protein (MAP2), a potent and sensitive indicator of neuronal morphology and determined of neuronal pathology following injury. Subsequent to the injury to neuronal cultures obtained from wild type mice, the primary mixed neuronal cells shows poor neurons morphology with less growth cone towards the cut line over the time course 0 to 72 h **(A)** compared with primary mixed neuronal cells from GMF-deficient (GMF-KO) mice **(B).** The GMF-KO group neurons start to generate new and bigger growth cones and shows widespread MAP2 staining towards the cut line. **(C)** Quantitative analysis of MAP2 intensity towards inury line. Scale bar = 50 μm and dash lines represents the injury sites. Color image is available online.

that were subjected to mechanical injury and supernatants were collected from WT and GMF-KO cell culture medium. Those supernatants were used to determine oxidative stress markers such as GPx, GSH, LDH, PGE2, and NO release into the culture medium over different time-points (0 h, 24 h, 48 h, and 72 h). The results obtained are shown in Figure 6A-E. Traumatized primary neuronal cells release of LDH was quantified to assess cell injury. Results show that traumatized primary neuronal cells released more LDH compared with control cells ($p < 0.05$). NO is a major secretory product that initiates host defense and accumulates in the brain after injury. PGE2 is known to be a potent vasodilator and increases vascular permeability. Traumatized primary neuronal cells shows higher production of NO and PGE2 compared with control cells ($p < 0.05$). Further, we found that scratch-induced WT primary neuronal cells show decreased levels of GSH and GPx activity compared with GMF-KO primary neuronal cells. These

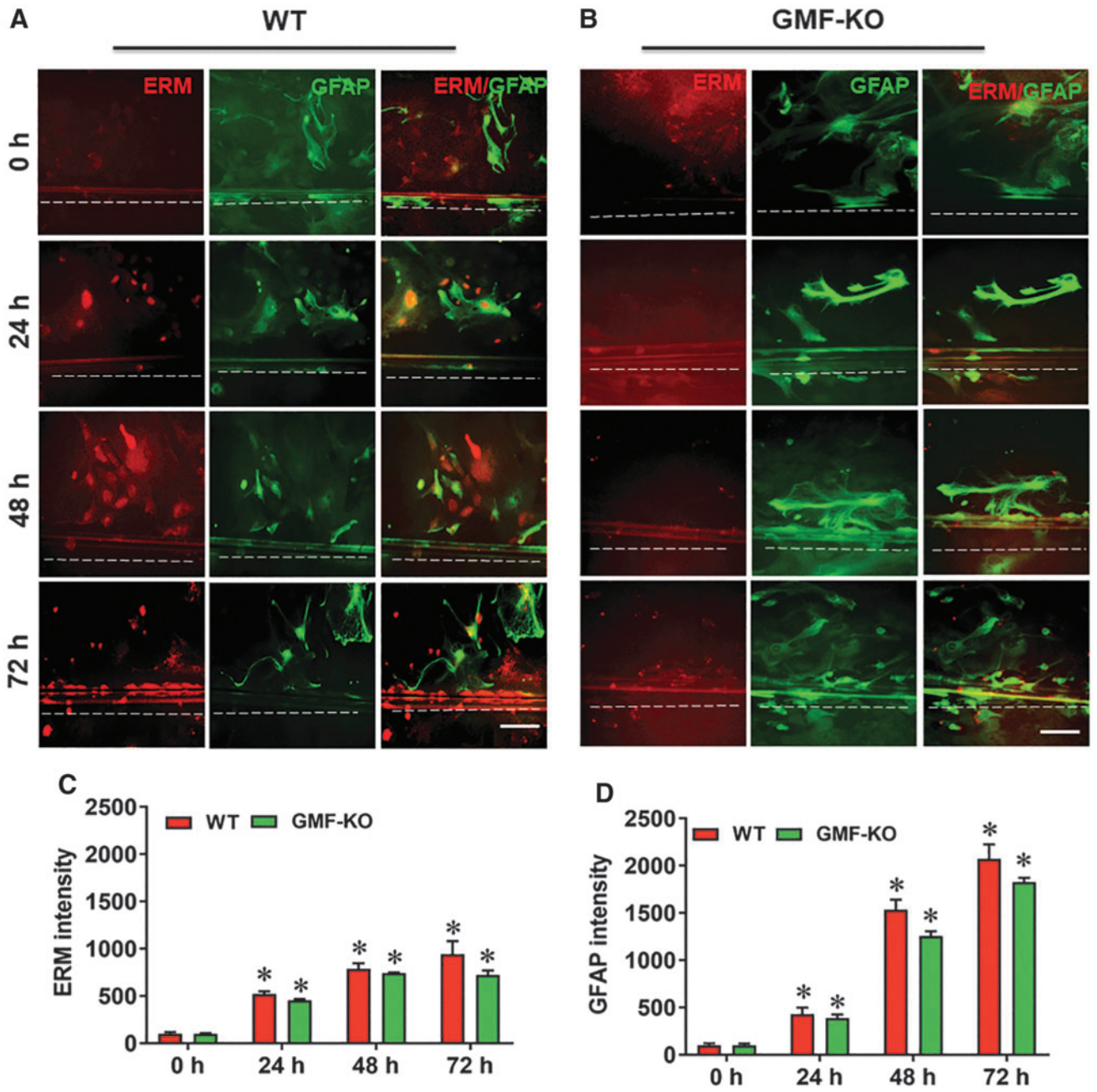

FIG. 3. The absence of glia maturation factor (GMF) reduces the expression of Ezrin/Radixin/Moesin (ERM) proteins in astrocytes following cell injury to primary neurons and glial cultures. Representative immunofluorescence staining of mixed neurons and glial cells cultures for a marker of ERM (red) with glial fibrillary acidic protein (GFAP; green) over the different time course study (0, 24, 48 and 72 h following injury). Mixed primary neurons and glial cells from GMF-deficient (GMF-KO) mice shows gradually decreased expression of ERM and GFAP compared with wild type mixed primary neurons and glial cells **(A, B).** Scale bar = 50 μm. **(C, D)** Fluorescent intensity was quantified using ImageJ analysis software and expressed as percentage change versus respective 0 h control group. The dash line represents the injury site. Data are presented as mean ± standard error of the mean ($n = 3$). *$p < 0.05$ versus 0 h control group. Color image is available online.

results demonstrate that absence of GMF could reduce the oxidative flux via improvement in antioxidant status, leading to changes in cell viability in the primary neuronal cells.

Discussion

To investigate the effect GMF on cellular and inflammatory changes following injury, we used an *in vitro* scratch injury model of TBI. This model is highly reproducible and distinct injury makes it possible to compare the effects on cells surrounding the injury. In the present study, we demonstrated that scratch injury induced the upregulation of free radicals and inflammatory mediators. Reactive astrocytes and activated microglia are one of the key components of the secondary cellular response following brain injury and responsible for producing inflammatory mediators and promoting a variety of cellular responses, including axonal degeneration and delayed

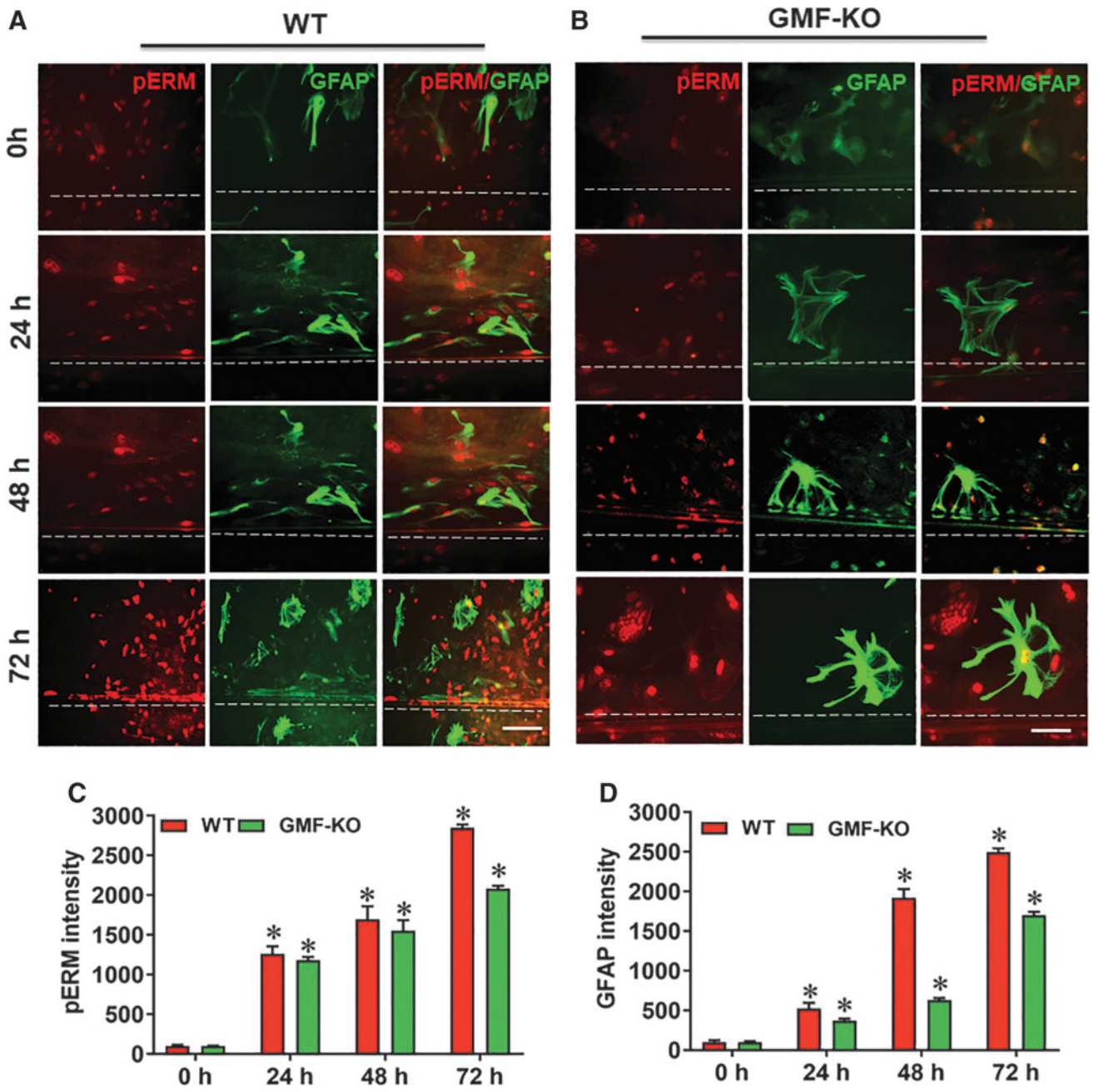

FIG. 4. The absence of glia maturation factor (GMF) greatly reduced the phosphorylation of Ezrin/Radixin/Moesin proteins after injury in mice primary mixed neurons and glial cells. Representative immunofluorescence staining of phosphorylated Ezrin/Radixin/Moesin (pERM; red) with glial fibrillary acidic protein (GFAP; green) in primary mixed neurons and glial cells from wild type (WT) and GMF-deficient (GMF-KO) mice subjected to scratch injury. There is a gradual reduction in the expression of pERM and GFAP in the GMF-KO group compared with the WT group **(A, B).** Dash line represent injury site, scale bar = 50 μm. Fluorescent intensity was quantified using ImageJ software and expressed as a percentage change in intensity versus 0 h control group **(C, D).** Data are represented as mean ± standard error of the mean (n = 3-6) in each group). *$p < 0.05$ versus 0 h control group. Color image is available online.

regenerative process.[27,28] The mixed primary cultures isolated from WT mice along the scratch boundary showed upregulated GFAP and Iba1 expression along the injured line demonstrated with mixed primary cultures compared with cultures isolated from GMF-KO mice. Using the scratch injury model, we can show that GFAP and Iba1 expression could be downregulated with the absence of GMF.

Quantitative analysis of immunoreactive proteins in the injured area demonstrates that the directional migration and expression of astroglial cells towards the injury site were reduced significantly in the GMF-KO cells proving that GMF functions as a key protein involved in the secondary impact pathogenesis following TBI. MAP2 is a main component of the microtubule family proteins, which may have several important functions, such as the mitotic movement of the chromosome, neurotransmitter release at synaptic region, structural growth, and stabilization of neuritic process.[29] The destruction of cytoskeletal protein MAP2 has been shown in the early stages of TBI and as a novel indicator of the diffuse brain injury severity and early mortality after TBI.[30–33] In the present

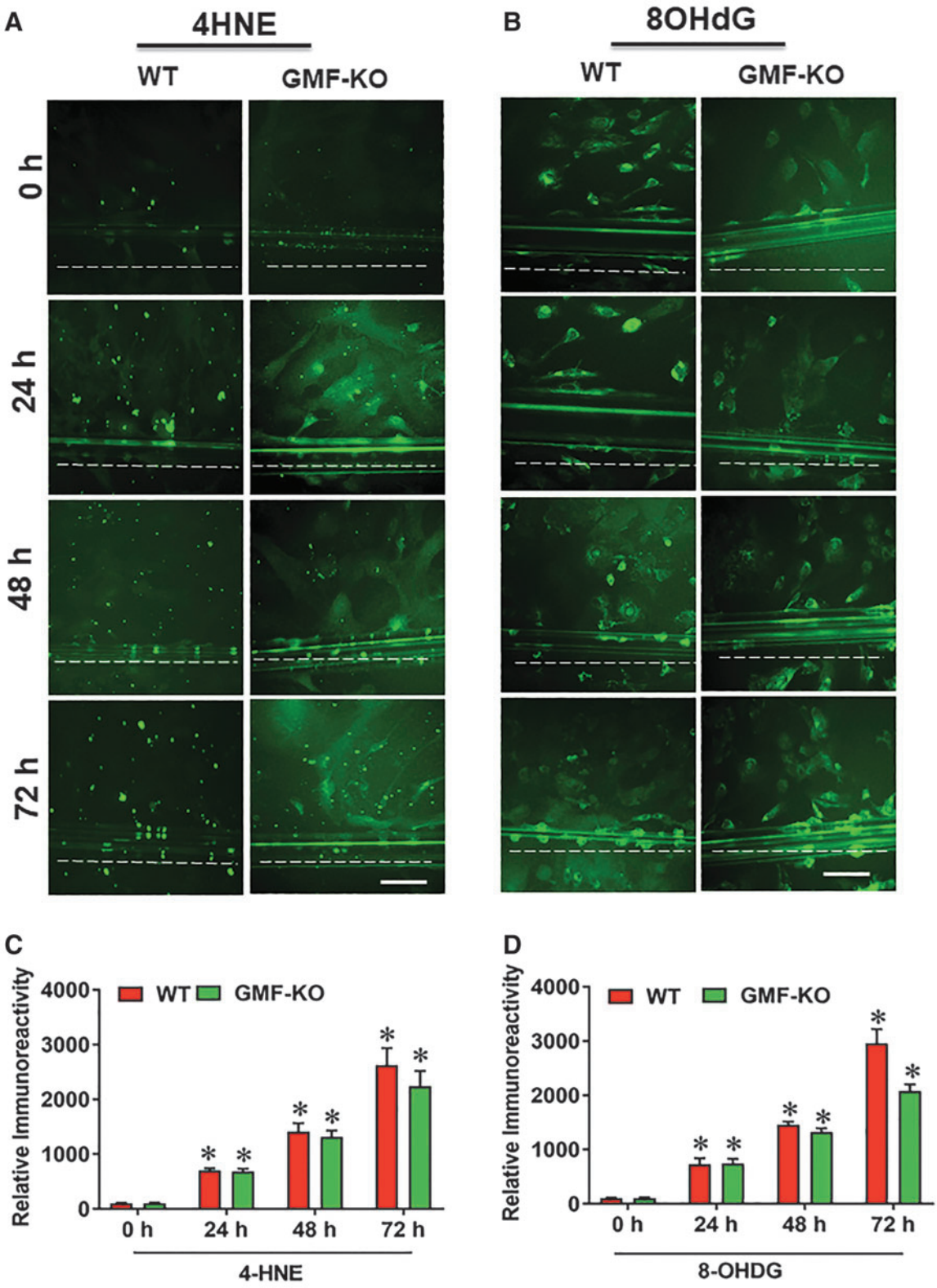

FIG. 5. The absence of glia maturation factor (GMF) limits oxidative damage in scratch mediated neuronal injury. Representative immunofluorescence staining for the markers of oxidative damage 4-HNE for lipid peroxidation (4-HNE) and 8-oxo-2′-deoxyguanosine (8-OhdG) for DNA damage. **(A, B)** Comparative staining for 4-HNE and 8-OHdG over the time course of, 0, 24, 48, and 72 h following the injury of primary neuronal cells from wild type (WT) and GMF-deficient (GMF-KO) mice. The GMF-KO group shows gradually decreased 4-HNE and 8-OHdG levels compared with the WT group. The dashed line represents the boundary of the injury site. Scale bar 50 μm. Fluorescent intensities were quantified using ImageJ analysis software and expressed as percentage change over respective 0 h control group **(C, D).** Data are presented as mean ± standard error of the mean ($n = 3$). *$p < 0.05$ versus 0 h control group. Color image is available online.

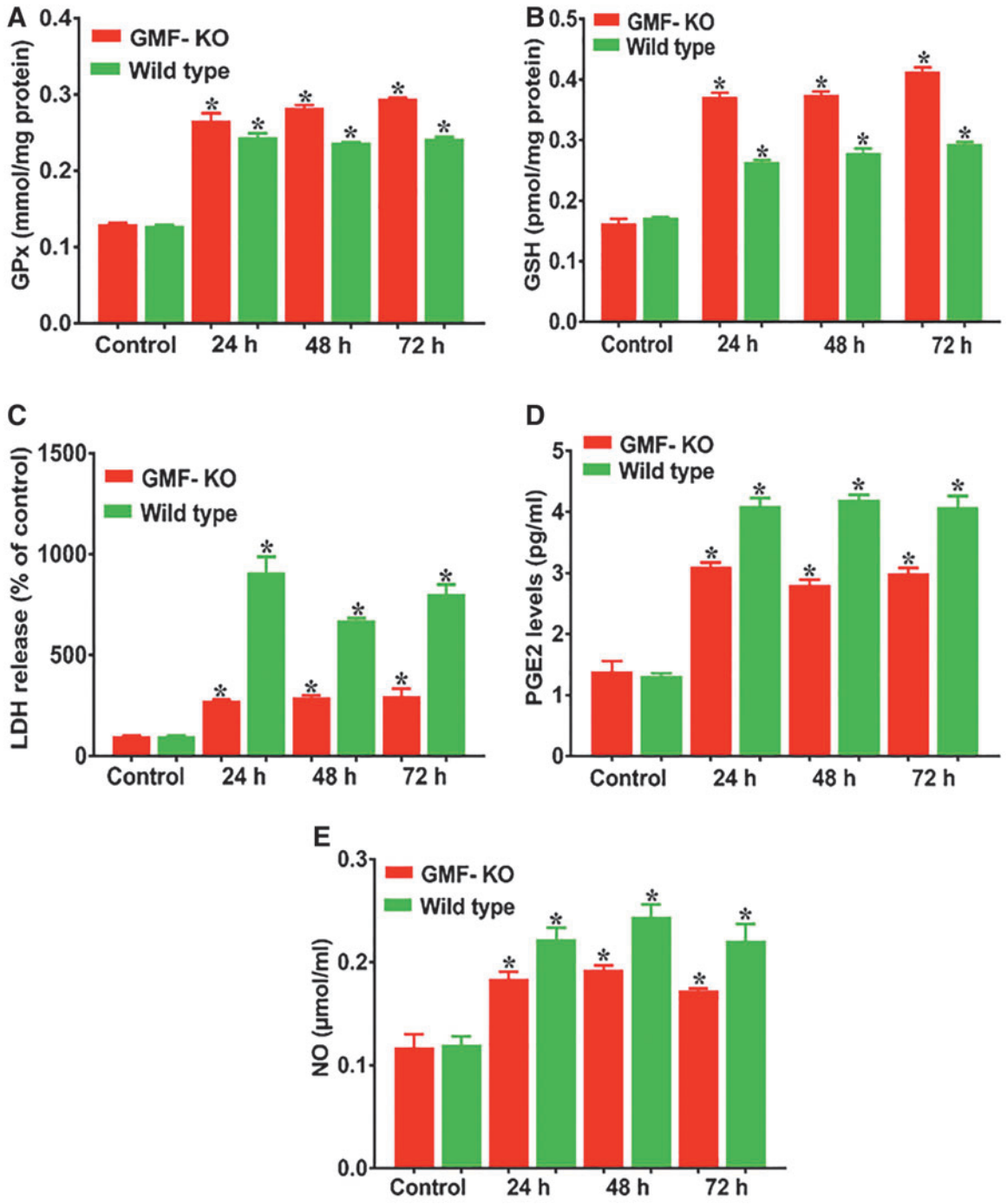

FIG. 6. The absence of glia maturation factor (GMF) reduces oxidative stress–mediated neuronal injury of injured primary neuronal cells. Primary neuronal cells from wild type (WT) and GMF-deficient (GMF-KO) were subjected to mechanical injury and supernatants were collected from WT and GMF-KO cells. Those supernatants were used to determine oxidative stress markers such as **(A)** glutathione peroxidase (GPx), **(B)** glutathione (GSH), **(C)** lactate dehydrogenase (LDH), **(D)** prostaglandin E2 (PGE2), and **(E)** nitric oxide (NO) release into the culture medium at different time-points (24, 48 and 72 h) after the injury. The absence of GMF significantly decreased oxidative stress markers such as LDH, NO, GSH and PGE2, and improved antioxidant enzymes such as GSH and GPx compared with control cells. The values are given as the mean ± standard error of the mean of four experiments in each group. $^*p < 0.05$ compared with WT and GMF-KO control. Color image is available online.

study, we have shown that the MAP2 expression was gradually reduced in WT primary cells, while GMF-KO cells show the improved expression of MAP2. The absence of GMF in primary neuronal cells prevents cytoskeletal protein degradation and maintains neurites regeneration along the injury line as seen in the 24 to 72 h immunofluorescence staining of MAP2 following scratch induced injury–specific traumatic brain injury.

ERM proteins belong to a family of highly homologous proteins that are known as key organizers of specialized membrane domain and are known to connect the actin cytoskeleton with the cell

membrane.[34] Previous studies have shown that migrating neuroblasts in the rostral migratory system also highly express ERM molecules, such as radixin, after TBI,[35,36] raising the possibility that involvement of ERM in the regulation of neuroblast migration into the injured brain region. In the present study, time-lapse double immunofluorescence staining demonstrates that ERM, pERM with GFAP expression were increased in mixed primary neuronal cells and may be due to the involvement of ERM on the neuroblast migration. Representative immunofluorescence staining using specific antibody of ERM and pERM showed higher expression in astrocytic cells in WT following time-lapse 24 to 72 h post injury. However, we noticed comparable reduction of ERM and its activated product pERM in GMF-KO astrocytes.

Oxidative stress plays a major role in secondary brain injury following TBI, due to excessive production of reactive oxygen species (ROS) and exhaustion of the endogenous antioxidant system.[37,38] Several oxidants and their derivatives are detected following TBI that enhance the production of ROS and the exhaustion of the antioxidant defense system such as GPX and GSH.[39] Lipid peroxidation, which is a measure of the oxidative degradation of lipids, increased the membrane permeability, leading to the cells damage. 4-HNE has been utilized as a marker of lipid peroxidation. Oxidative DNA damage occurs from ROS in cells following TBI that creates break in DNA strands, causing DNA damage. 8-OHdG has been used as an index for the oxidative DNA damage in present study. Interestingly we observed that knocking down of GMF markedly improved antioxidant defense system and attenuated 4-HNE and 8-OHdG over the time course immunofluorescence staining 0, 24, 48, and 72 h following scratch injury in primary mixed neuronal culture of GMF-KO mice compared with WT mice primary mixed neuronal culture. These results strongly suggest that the role GMF in oxidative stress damage and its absence in the antioxidant defense system.

NO is known as a universal mediator of biological effects in the brain, implicated in the pathophysiological process following TBI.[40,41] Prostaglandins and thromboxanes (PGE2) are metabolic products of arachidonic acid release and aggravate the injury following TBI. Excessive PGE2 synthesis is regulated by expression of COX-2, and play a major role in inflammation following injury.[42,43] Our biochemical analysis of PGE2 and NO release in culture media following scratch injury showed marked induction of PGE2 and NO during the time course study 0, 24, 48 and 72 h in WT mixed neuronal culture media following TBI. However, the knocking down of GMF showed gradually marked reduction in the release of PGE2 and NO over the time course (0, 24, 48, and, 72 h) in cell culture media following injury. From our observations, we can suggest that the knocking down of GMF downregulates NO and PGE2, which might have contributed to a decrease in physiologic variables following injury.

Conclusions

The results of our present study demonstrate the role of scratch injury specific proteins in the regulation of cell migration and neurite regeneration. Using the *in vitro* scratch injury as a model of TBI, we show that the absence of GMF in the neuronal population can contribute significantly to reduces glial activation mediated neuro inflammation, ROS generation, oxidative stress damage, and neuronal cell death following scratch injury. Taken together, results from our current study demonstrate that the downregulation of GMF should have clinical benefits after TBI.

Acknowledgments

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Asgar Zaheer, PhD
Department of Neurology
Center for Translational Neuroscience
Medical Science Building, M741A
University of Missouri School of Medicine
1 Hospital Drive
Columbia, MO 65212
USA

E-mail: zaheera@health.missouri.edu