

## ORIGINAL ARTICLE

# A positive correlation exists between neurotrauma and TGF- $\beta$ 1-containing microglia in rats

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## ABSTRACT

**Background** Transforming growth factor-beta 1 (TGF- $\beta$ 1) regulates many processes after traumatic brain injury (TBI). Both Neuro AiD™ (MLC601) and astragaloside (AST) attenuate microglia activation in rats with TBI. The purpose of this study was to investigate whether MLC601 or AST improves outcome of TBI by affecting microglial expression of TGF- $\beta$ 1.

**Materials and methods** Adult male Sprague-Dawley rats (120 in number) were used to investigate the contribution of TGF- $\beta$ 1-containing microglia in the MLC601-mediated or the AST-mediated neuroprotection in the brain trauma condition using lateral fluid percussion injury.

**Results** Pearson correlation analysis revealed that there was a positive correlation between brain injury (evidenced by both brain contused volume and neurological severity score) and the cortical numbers of TGF- $\beta$ 1-containing microglia for the rats ( $n = 12$ ) 4 days post-TBI. MLC601 or AST significantly ( $P < 0.05$ ) attenuated TBI-induced brain contused volume ( $119 \pm 14 \text{ mm}^3$  or  $108 \pm 11 \text{ mm}^3$  vs.  $160 \pm 21 \text{ mm}^3$ ), neurological severity score ( $7.8 \pm 0.3$  or  $8.1 \pm 0.4$  vs.  $10.2 \pm 0.5$ ) and numbers of TGF- $\beta$ 1-containing microglia ( $6\% \pm 2\%$  or  $11\% \pm 3\%$  vs.  $79\% \pm 7\%$ ) for the rats 4 days post-TBI.

**Conclusions** There was a positive correlation between TBI and cortical numbers of TGF- $\beta$ 1-containing microglia which could be significantly attenuated by astragaloside or NeuroAiD™ (MLC601) in rats.

**Keywords** Astragaloside, neuro AiD™ (MLC601), transforming growth factor- $\beta$ 1, traumatic brain injury.

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## Introduction

Microglia, the resident macrophages of the brain, shift to activated states in response to traumatic brain injury (TBI): this changes their morphology, migrates to injury sites and releases tumour necrosis factor-alpha (TNF- $\alpha$ ) [1–4], transforming growth factor-beta (TGF- $\beta$ ) [5,6] and others. TGF- $\beta$ 1 a versatile cytokine regulates many processes after TBI, including cell survival, gliosis, inflammation and cell proliferation [7,8]. Recently, cerebral contusion caused by TBI was associated with neurological motor deficits, brain apoptosis and activated microglia (e.g. microgliosis, amoeboid microglia and microglial overexpression of TNF- $\alpha$ ) in rats [4,9]. However, it is not known whether microglial activation of TGF- $\beta$ 1 can be induced by TBI.

Recently, we have demonstrated both Neuro AiD™ (MLC601) [4] and astragaloside (AST) [9] two traditional Chinese medicine attenuate cerebral contusion, neurological motor deficits and activated microglia (e.g. microgliosis, amoeboid microglia and microglial overexpression of TNF- $\alpha$ ) in rats with

TBI. Again, it is unknown whether the proposed TBI-induced overexpression of TGF- $\beta$  in the contused brain regions including the frontal cortex, parietal cortex, hippocampus and striatum can be affected by MLC601 and/or AST.

In this study, using a rat model, we have shown that TBI causes cerebral contusion, neurological motor deficits and microglial overexpression of TGF- $\beta$  in the ipsilateral cortex, which all are significantly attenuated by MLC601 or AST therapy. There was a positive correlation between neurotrauma and TGF- $\beta$ 1 containing microglia. Our data suggest that MLC601 or AST improves outcomes of TBI in rats by reducing microglial overexpression of TGF- $\beta$ .

## Methods

### Animals

Male Sprague-Dawley rats ( $252 \pm 16 \text{ g}$ ) from the animal resource center of the Taiwan Department of National Science and Technology (TNOSAT) were used for all experiments. The

TNOSAT's policies on the care and use of laboratory animals were followed. The institutional review board of Chi Mei Medical Center approved the experiments (protocol number: 100120701). All efforts were made to minimize animal suffering and reduce the number of rats used. The animals were housed under controlled laboratory conditions with a 12-h light/dark (cycle), a temperature of  $22 \pm 2$  °C and a humidity of 60%–70% for at least 1 week before drug treatment or surgery. They had free access to a standard rodent diet and unlimited fresh drinking water.

### Induction of traumatic brain injury

Traumatic brain injury was induced using a fluid percussion injury device (Virginia Commonwealth University Biochemical Engineering, Richmond, VA, USA) (amplitude: 2.2 atm) on rats placed in a Kopf stereotaxic frame [10] as detailed previously [4].

### Experimental design

The rats were divided into four groups: (i) sham group: the rats were subjected to sham operation ( $n = 12$ ); (ii) TBI + vehicle (TBI + V): the rat were subjected to TBI and injected with normal saline (1 mL/kg, i.p.) 1 h post-TBI ( $n = 12$ ); (iii) TBI + astrogaloside (TBI + AST): the rats were subjected to TBI and injected with AST (40 mg/kg; i.p., Fusol Material Co., Ltd., Tainan, Taiwan) 1 h post-TBI ( $n = 12$ ) [9]; and (iv) TBI + MLC601: the rats were subjected to TBI and injected with MLC601 (0.4 mg/kg, i.p., Moleac Pte Ltd., Singapore) 1 h post-TBI ( $n = 12$ ) and then with one injection per day for consecutive 3 days [4,11]. The researchers who did the TBI surgery were blinded to the treatment code.

### Neurological severity scores

Acute neurological injury was assessed in all rats the day before and 4 days after surgery using a neurological severity score, a composite of the motor, sensory and reflex test scores [12]. One point was given for failure to perform a task. The higher the score (maximum: 14 points), the more severe the injury.

### Cerebral contusion assay

The triphenyl tetrazolium chloride (TTC) staining procedures were used for determining cerebral contusion extent caused TBI as detailed previously [13].

### Immunohistochemical determination

Serial 50-µm sections corresponding to coronal coordinates 0.8–5.3 mm posterior to the bregma were obtained in 2 M HCl for 30 min, rinsed in 0.1 M boric acid (pH 8.5) for 3 min at room temperature and then incubated with primary antibodies in PBS containing 0.5% normal bovine serum at 4 °C, overnight; secondary antibodies for 1 h at room temperature. The

antibodies sequentially were mouse monoclonal anti-Iba1 antibody (Abcam, 1 : 200), rabbit monoclonal anti-TGF-β antibody (Abcam, UK, 1 : 2000), Alexa Fluro 568-conjugated donkey anti-mouse IgG antibody (Invitrogen, 1 : 400) and DyLight 488-conjugated donkey anti-goat antibody (Abcam, 1 : 400). After they had been incubated, the sections were washed with PBS, their nuclei were costained with 4,6-diamidino-2-phenylindole (DAPI) using DAPI-containing mounting medium (Vectashield R; Vector Laboratories, Burlingame, CA, USA) and they were subsequently analysed using a fluorescence microscope (Zeiss GmbH, Göttingen, Germany) equipped with a digital camera (AxioCam 512 colour; Zeiss). The labelled cells were calculated in five coronal sections from each rat and expressed as the mean number of cells per section. For negative control sections, all the procedures were carried out without the primary antibodies. In each image, immune-positive cells showing staining with a cellular morphology and above background were manually and exhaustively counted using the Axiovision Image Analysis Software (Zeiss, GmbH). All cell counts were performed by an investigator (C.P.C.) blinded to the treatment status.

### Statistical analysis

The data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Fisher's post hoc test. Analyses for behavioural variables used Student's t-test to compare variables between groups. Bonferroni's analysis was then performed when appropriate to determine *post hoc* significance at individual time point. Data were analysed using statistical Software, and, in all cases, statistical significance was set at  $P < 0.05$ . Relations between brain injury (evidenced by both brain contused volume and neurological severity score) and numbers of TGF-β<sub>1</sub>-containing microglia were analysed by calculating Pearson product-moment correlation coefficient.

## Results

### MLC601 or AST attenuates TBI-induced cerebral contusion and neurological deficits

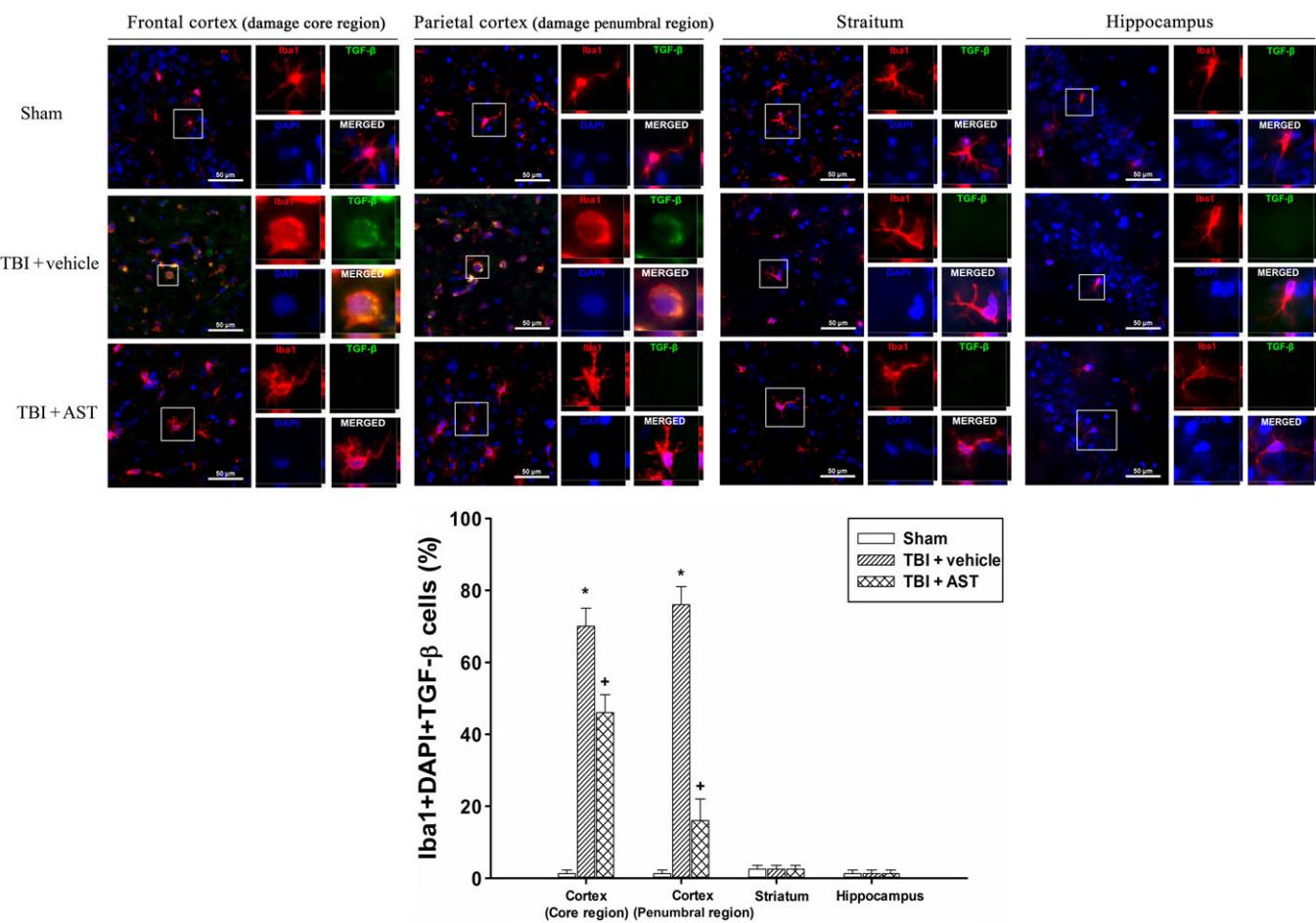
As demonstrated in our previous studies, intraperitoneal administration of MLC601 at a dose of 0.4 mg/kg 1 h post-TBI and repeated for consecutive 2 days [9] or AST at a dose of 40 mg/kg once 1 h postinjury [9] significantly attenuated TBI-induced cerebral contusion and neurological deficits in rats evaluated 4 days postinjury. In the present results, contusion volume values for the sham group rats, TBI rats treated with vehicle, TBI rats treated with MLC601 and TBI rats treated with AST were  $2 \pm 1$  mm<sup>3</sup> ( $n = 12$ ),  $160 \pm 21$  mm<sup>3</sup> ( $n = 12$ ),  $119 \pm 14$  mm<sup>3</sup> ( $n = 12$ ) and  $108 \pm 11$  mm<sup>3</sup> ( $n = 12$ ), respectively. The neurological severity scores for the sham group rats,

TBI rats treated with vehicle, TBI rats treated with MLC601 and TBI rats treated with AST were  $0, 10.0 \pm 0.5, 7.8 \pm 0.4$  and  $8.0 \pm 0.4$ , respectively ( $n = 12$  for each group). Compared to those of TBI + vehicle group rats, TBI + MLC group rats or TBI + AST group rats had significantly lower values of both contusion volume and neurological severity score ( $P < 0.05$ ).

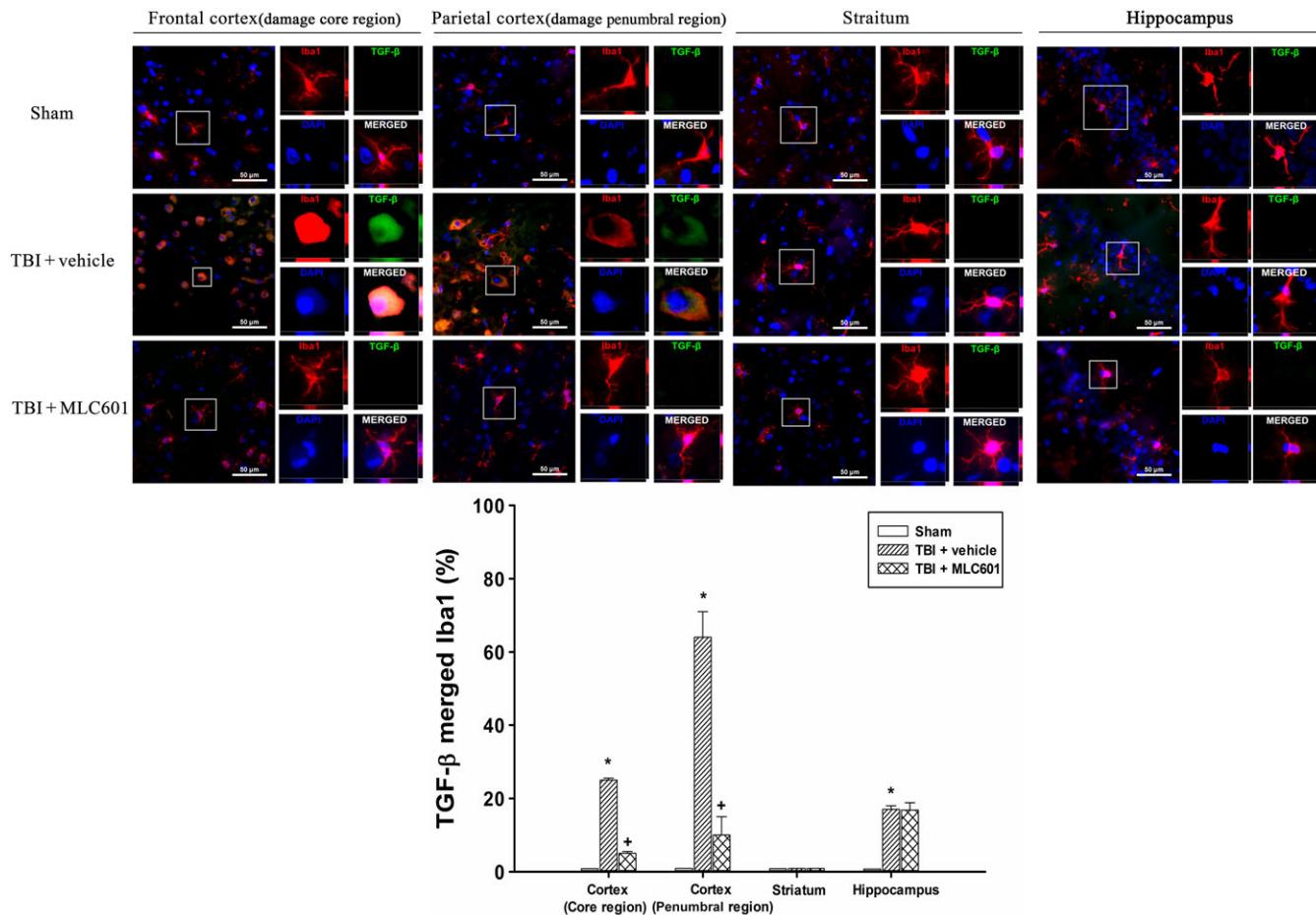
### MLC601 or AST reduces TBI-induced increased number of TGF- $\beta_1$ -containing microglia in contused cortical regions

Four days after the rats had been subjected to TBI, immunofluorescence staining showed that in the contused frontal cortex (or injured core region) and parietal cortex (or

injured penumbral region), but not in the striatum or hippocampus, of the vehicle-treated TBI rats, activated microglia changed their morphology by assuming an amoeboid shape (Figs 1 and 2). The amoeboid microglia in both the frontal and parietal cortical regions caused by TBI were significantly attenuated by MLC601 (Fig. 1) or AST (Fig. 2). It was also found that TBI + vehicle group rats had a significant increase in the numbers of TGF- $\beta_1$ -containing microglia (or colocalization of Iba1 + TGF- $\beta_1$  + DAPI specific stained cells) in the contused frontal and parietal cortical regions compared with the sham controls (60%–70% vs. 2%–4%,  $P < 0.01$ , Figs 1 and 2). However, the number of TGF- $\beta_1$ -containing microglia in the contused striatal or hippocampal regions of the TBI + vehicle rats



**Figure 1** AST decreased TBI-induced increase in the number of colocalization of microglia (or Iba1, red colour), TGF- $\beta$  (green colour) and nuclei (or DAPI, blue colour) specific cells in contused brain regions. Representative panels staining 4 days after sham operation or TBI, respectively, for a sham operation rat, a TBI rat treated with vehicle and a TBI rat treated with AST (40 mg/kg). \*The TBI + vehicle group (▨) showed a significant increase in the number of colocalization of microglia and TGF- $\beta$  specific marker cells in contused cortical regions, but not in contused striatal or hippocampal regions 4 days after TBI compared with the sham controls (□) ( $P < 0.01$ ). †The TBI + AST group showed a significant decrease in the number of colocalizations of microglia and TGF- $\beta$  specific marker cells in contused cortical regions (▨) ( $P < 0.01$ ). Each column and bar is the mean  $\pm$  SD of 12 rats per group.



**Figure 2** MLC 601 decreased TBI-induced increase in the number of colocalization of microglia (red), TGF- $\beta$  (green) and DAPI (blue) specific cells in contused brain regions. Representative panels staining 4 days after sham operation of TBI, respectively, for a sham operation rat, a TBI rat treated with vehicle and a TBI rat treated with MLC 601 (0.4 mg/kg). \*The TBI + vehicle group (▨) showed a significant increase in the number of colocalization of microglia and TGF- $\beta$  specific marker cells in contused cortical regions but not in contused striatal or hippocampal regions 4 days after TBI compared with the sham controls (□) ( $P < 0.01$ ). †The TBI + MLC601 group showed a significant decrease in the number of colocalization of microglia and TGF- $\beta$  specific marker cells in contused cortical regions (▨) ( $P < 0.01$ ). Each column and bar is mean  $\pm$  SD of 12 rats per group.

was not significantly different from the sham controls (Figs 1 and 2;  $P > 0.05$ ). Furthermore, compared to those of TBI + vehicle group rats, TBI + MLC601 group rats (2%–4%; Fig. 1) or TBI + AST group rats (10%–42%; Fig. 2) had significantly lower numbers of TGF- $\beta_1$ -containing microglia in the contused cortical regions of TBI + vehicle group rats ( $P < 0.05$ ,  $n = 12$  for each group).

#### Positive correlation between cerebral contused volume or neurological severity score and the cortical numbers of TGF- $\beta$ -containing microglia

Pearson correlation analysis revealed that there was a positive correlation between cerebral contused volume or neurological

severity score and the cortical numbers of TGF- $\beta$ -containing microglia for the rats 4 days post-TBI (Table 1).

#### Discussion

There are three isoforms of TGF- $\beta$ -1,2 and 3 in the brain [14], whereas TGF- $\beta_1$  is increased acutely in response to injury and/or ageing [14–17], trauma, infection, ischaemia, encephalitis, autoimmune diseases and neurodegenerative disease [18]. TGF- $\beta$  has been shown to have both beneficial and harmful effects in neuroprotection [14,19–22]. Several studies show that chronic overexpression or infusion of TGF- $\beta_1$  to uninjured rodents reduces hippocampal and subventricular zone (SVZ) neurogenesis [23–25]. In adult neural stem and progenitor cell

**Table 1** Pearson correlation analysis between cerebral contused volume and neurological severity score and cortical numbers of TGF- $\beta$ -containing microglia (or Iba1-TGF- $\beta$ -DAPI specific stained cells) in rats ( $n = 12$ ) 4 days post-TBI

Brain contused volume	Neurological severity scores
No. of TGF- $\beta$ -containing microglia	
R	0.724*
P	0.000

\* $P < 0.05$ ; † $P < 0.05$ .

cultures and after intracerebroventricular infusion, TGF- $\beta_1$  induced a long-lasting inhibition of neural stem and progenitor cell proliferation and a reduction in neurogenesis [23]. Chronically increased TGF- $\beta_1$  strongly inhibited hippocampal neurogenesis in aged mice [24]. In an animal model of Huntington disease, stem cell quiescence in the hippocampal neurogenic niche is associated with elevated TGF- $\beta_1$  signalling [25]. Inhibition of TGF- $\beta$  attenuates brain injury and neurological deficits in a rat model of germinal matrix haemorrhage [26]. Conversely, other publications have indicated that during certain inflammatory or ischaemic injuries, TGF- $\beta_1$  can increase NSC proliferation and neurogenesis [27–29]. Neurogenic niche modulation by activated microglia in adult dentate gyrus can be increased by TGF- $\beta$  [27]. Intranasal delivery of TGF- $\beta_1$  in mice after stroke reduces infarct volume and increases neurogenesis in the subventricular zone [28]. Prenatal inflammation impairs adult neurogenesis and memory-related behaviour through persistent hippocampal TGF- $\beta_1$  downregulation [29].

Longan *et al.* [8] subjected adult mice to controlled cortical impact (CCI) injury and isolated RNA from the SVZ and the dentate gyrus (DG) at different time points. qPCR array analysis showed that cortical injury caused significant alterations in the mRNA expression of components and targets of the TGF- $\beta$ , the bone morphogenetic proteins (BMPs) and activin signalling pathways in the SVZ and the DG after injury, suggesting that these pathways could regulate postinjury neurogenesis. In the present study, we subjected adult rats to lateral fluid percussion injury and performed serial sections corresponding to coronal coordinates 0.8–5.3 mm posterior to the bregma from the brains at 4 days postinjury. Immunohistochemical analysis revealed that four days following lateral fluid percussion TBI, massive contusion occurred in the frontal and parietal cortical regions, and in both striatum and hippocampus of rat brain. Immunofluorescence staining analysis showed that the numbers of Iba1 + DAPI + TGF- $\beta_1$  specific stained cells in the frontal and parietal cortical regions were significantly increased following TBI. However, the numbers of Iba1 + TGF- $\beta_1$  + DAPI specific stained cells in adult neurogenic regions such as hippocampus were insignificantly affected by TBI. Our present results indicate that microglia overexpression of TGF- $\beta_1$

in the cortical regions may be associated with the occurrence of TBI in rats.

Astragaloside, the main component of astragalus, is traditionally prescribed in treatment of cerebrovascular diseases, ageing and immune disorders [30–33]. A mixture of AST is shown to reduce brain infarction in rodents following focal cerebral ischaemia [31,34]. MLC601 (NeuroAid), a Traditional Chinese Medicine, is currently used for stroke patients [35–40] or stroke rodents [11,41]. Traumatic brain injury causes increased release of several mediators from injured and dead cells and elicits microglial activation [42]. Activated microglia change their morphology, migrate to injury sites and release TNF- $\alpha$  and others. In previous studies, we used a controlled fluid percussion injury model of TBI in the rat and found that early treatment with AST [9] or MLC601 [4] had better effects in reducing TBI-induced cerebral contusion. Cerebral contusion caused by TBI was associated with neurological motor deficits, brain apoptosis and activated microglia (e.g. microgliosis, amoeboid microglia and microglial overexpression of TNF- $\alpha$ ), which all were significantly attenuated by AST [9] or MLC601 [4] therapy. The present data further showed that microglial overexpression of TGF- $\beta_1$  caused by TBI in rats was significantly attenuated by AST or MLC601. Our present results suggest that AST or MLC601 may improve outcomes of TBI in rats by reducing microglial activation of TGF- $\beta_1$ . Thus, it can be derived from the foregoing statements that TBI-induced cerebral contusion was associated with microglial overexpression of both TGF- $\beta_1$  and TNF- $\alpha$ , which could be attenuated by AST or MLC601. In the present study, Iba1 stain was chosen as a marker of activated microglia. AST may improve outcomes of TBI by limiting macrophage recruitment from periphery and resident microglia in the central nervous system [43].

The transforming growth factor-beta-activated kinase-1 (TAK1), a member of the mitogen-activated protein kinase family, is characterized as a key regulator in inflammatory and apoptosis signalling pathways [44]. TAK1 can be activated by TGF- $\beta$  [45]. It was found that the TAK1 pathway is activated after experimental TBI and the inhibition of TAK1 affords significant neuroprotection and amelioration of neurobehavioural deficits after experimental TBI in rats [44]. It is likely that the TGF- $\beta_1$ -TAK1 pathway is activated (70%–80% increase in microglia TGF- $\beta_1$  specific cells) after experimental TBI and inhibition of microglial expression of TGF- $\beta_1$  (10%–18% increase) by AST or MLC601 might improve outcomes of TBI via inhibiting TGF- $\beta_1$ -TAK1 pathway. This is confirmed by the fact that the inhibition of TAK1 activation decreased infarct volume after focal cerebral ischaemia [46].

Finally, it should be mentioned that in Fig. 1, all injured groups had few positive cells of TGF- $\beta_1$  staining in hippocampus whereas in Fig. 2, injured groups had around 20% positive cells in hippocampus. Although the injured rats were

randomly assigned to each group, the reason for the discrepancy between the two groups of results is not apparent now.

In conclusion, in this study, we provide first evidence to indicate that there is a positive correlation between TBI and the cortical numbers of TGF- $\beta_1$ -containing microglia which can be significantly attenuated by AST or MLC601 in rats.

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