TGR5 activation attenuates neuroinflammation via Pellino3 inhibition of Caspase-8/NLRP3 after middle cerebral artery occlusion in rats

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Abstract

Background NLRP3 (nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3) plays an important role in mediating inflammatory responses during ischemic stroke. Bile acid receptor TGR5 has been identified as an important component in regulating inflammatory response in brain. In this study, we investigated the mechanism of TGR5 on alleviating neuroinflammation after middle cerebral artery occlusion (MCAO).

Methods—Sprague-Dawley rats were subjected to MCAO and INT777 was administered intranasally 1 hour after MCAO. Small interfering RNA for TGR5 and Pellino3 were administered through intracerebroventricular injection 48 hours before MCAO. Infarct volumes, neurological scores, ELISA,flow cytometry immunofluorescence staining, Western blot and co-immunoprecipitation were evaluated.

Results— Endogenous TGR5 and Pellino3 expressions were increased after MCAO.

TGR5 activation by INT777 significantly decreased pro-inflammatory cytokines levels, reduced cleaved caspase-8 and NLRP3 expressions, thereby reducing brain infarction, improving both short- and long-term neurobehavioral assessment. Ischemic damage induced interaction of TGR5 with Pellino3. Knockdown of either TGR5 or Pellino3 increased expressions of cleaved caspase-8 and NLRP3, aggravated brain impairments, abolished the anti-inflammatory effects of INT777 after MCAO.

Conclusions—TGR5 activation attenuated brain injury by inhibiting neuroinflammation after MCAO, which may be mediated by Pellino3 inhibition of Caspase-8/NLRP3.

Background

Stroke is a leading cause of death and disability worldwide, affecting millions of lives every year [1]. Accumulating evidence suggests that innate immunity and inflammatory responses are involved in ischemic brain injury[2, 3].Recent findings demonstrate that the nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome, which is abundantly expressed in the brain, plays an important role in detecting cellular damage and mediating inflammatory responses to aseptic tissue injury during ischemic stroke[4–6]. Research has also shown that pharmacological targeting of the NLRP3-mediated inflammatory response may help to develop therapeutic strategies for preventing the deterioration of cerebral function[4].

TGR5 is a plasma membrane-bound G protein-coupled bile acid receptor, which has varied levels of expression in different tissues[7, 8].Previous studies showed that activation of TGR5 suppresses proinflammatory cytokine production and phagocytosis by monocytes/macrophages[7, 9].Recent research has demonstrated that TGR5 activation blocked NLRP3 inflammasome-dependent inflammation via the TGR5-cAMP-PKA axis, including lipopolysaccharide induced systemic inflammation, alum-induced peritoneal inflammation, and type-2 diabetes-related inflammation[10]. In central nervous system research, studies identified that TGR5 activation alleviated neuroinflammation and improved outcomes in

a model of experimental autoimmune encephalomyelitis and hepatic encephalopathy[11, 12]. Our previous research showed that TGR5 activation could alleviate brain injury following middle cerebral artery occlusion (MCAO)[13].However, the effects of TGR5 on neuroinflammation after ischemic stroke have not been investigated.

Pellino3 is a E3 ubiquitin ligase protein with anti-inflammatory properties[14, 15].Pellino3 reduced caspase-8 cleavage and inhibited tumor necrosis factor-α-induced cell death[16]. Several research supported that caspase-8 was upstream and could activate NLRP3 in human monocytes, intraocular pressure-induced retinal ischemia, and after chemotherapeutic treatment[17–19]. Recent research indicated that caspase-8 inhibition decreased the neuropathological consequences of cerebral or retinal infarction and TGR5 inhibited caspase-8 activation after liver ischemia/reperfusion injury[20, 21].

In the present study, we hypothesized that (1) TGR5 activation could attenuate neuroinflammation after MCAO; (2) the anti-inflammation mechanism of TGR5 activation may be mediated by Pellino3 inhibition of Caspase-8/NLRP3.

Materials And Methods

All protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and Zhejiang University. All animal care and use were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). All experiments are reported in compliance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines. A total of 383 Sprague-Dawley male rats (2–3 months, weight 250–300 g) were used (Supplemental Table 1) and the investigators are blinded to group assignments during outcome assessments.

MCAO Model

Transient MCAO was induced as previously described[22], with some modifications. Briefly, anesthesia was induced intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg). Atropine was then administered (0.1mg/kg, subcutaneous). The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed. The ECA was ligated and 4-0 nylon suture with silicon was inserted into the ICA through the ECA stump to occlude the MCA. The suture was removed 2 hours after occlusion. Sham operated rats underwent the same surgical procedures except that the MCA was not occluded. After closing the skin incision, rats were kept at approximately 37°C on an electric heating blanket until completely recovered from anesthetic.

Experimental Design

The experimental design is shown in supplemental figure I.

Experiment 1

To determine the time course of endogenous TGR5 and Pellino3 expression after MCAO by Western blot, 30 rats were divided into 5 groups: Sham, MCAO after different time point (6,12,24 and 72 hours). The additional 20 rats were divided into sham (n=10) and MCAO (24 hours) groups (n=10): 8 rats for test the localization of TGR5 in the brain with immunofluorescence staining and 12 rats for evaluate the expression of TGR5 in activation of microglia or infiltrated of macrophage with flow cytometry.

Experiment 2

A total of 96 rats were divided into 5 groups: sham (n=24), MCAO+vehicle (n=30), MCAO+INT777 (0.16 mg/kg，n=6), MCAO+INT777 (0.48mg/kg，n=30), MCAO+INT777 (1.44 mg/kg，n=6). Based on neurological tests and brain edema at 24 hours after MCAO, middle dosage of INT777 was chosen for ELISA, Western blot at 24 hours, neurobehavior at 72 hours after MCAO, and long-time neurobehavior.

Experiment 3

18. rats were divided into 3 groups for exploring the association between TGR5 and Pellino3 by co-immunoprecipitation: sham (n=6), MCAO+vehicle (n=6), MCAO+INT777 (48mg/kg，n=6). The immunofluorescence samples for co-labelingof TGR5 with pellino3 were shared with experiment 1 while the rats for exploring the effect of INT777 on expression of TGR5 and Pellino3 in totalprotein were shared with experiment 3.

Experiment 4

168 rats were randomly assigned to 10 groups for mechanism study: sham (n=36), MCAO+vehicle (n=36), MCAO+scramble siRNA (n=12), MCAO+TGR5 siRNA (n=12), MCAO+Pellino3 siRNA (n=12), MCAO+Z-IETD-FMK (n=12), MCAO+INT777 (0.48mg/kg, n=12), MCAO+INT777(0.48mg/kg)+scramble siRNA (n=12), MCAO+INT777(0.48mg/kg)+TGR5 siRNA (n=12), MCAO+INT777(0.48mg/kg)+Pellino3 siRNA (n=12).Neurobehavioral scores, brain infarction, and Western blot were examined at 24 hours after MCAO.

Drug Administration

The method of intranasal administration of INT777 was performed 1 hour after MCAO as previously described[13,23], with some modifications; rats were administered either saline(vehicle), INT777 (0.16mg/kg), INT777 (0.48mg/kg), or INT777 (1.44 mg/kg) as nose drops (5 μL/drop) over a period of 20 minutes, alternating drops every 2 minutes between the left and right nares. The total volume delivered was 50μL.

Z- IETD-FMK, caspase-8 inhibitor, was dissolved in sterile DMSO and was delivered at a dose of 1 mg/kg via intravenous (tail vein) injection immediately after MCAO[20].

Intracerebroventricular siRNA Injection

Three different formats of TGR5-siRNA or Pellino3-siRNA (OriGene Technologies) were diluted with transfection reagent (entranser^{TM},Engreen Biosystem)and were injected 48 hours before MCAO by intracerebroventricular injection (ICV) as previously described[24,25].The TGR5-siRNA, Pellino3-siRNA scramble-siRNA mixture (100 pmol in 5 μL) was delivered into the ipsilateral ventricle using a Hamilton microsyringe under the guidance of a stereotaxy instrument. The stereotactic ICV injection site was relative to bregma: posterior 1 mm, right lateral 1.5 mm, depth 3.5 mm. The injection was administered over 5 minutes and the needle was left in place for an additional 5 minutes after injection to prevent possible leakage and was then slowly withdrawn over 4 minutes. After the needle was removed, the burr hole was sealed with bone wax. The incision was closed with sutures and the rat was allowed to recover.

Neurological Scores

A neurological examination was performed by a blinded investigator at 24 hours or 72 hours after MCAO as previously described[26].The assessment consisted of 7 tests covering spontaneous activity, symmetry in limb movement, symmetry of forelimb outstretching, climbing, body proprioception, and response to vibrissae touch, and beam walking). The neurological scoring ranged from 3 (most severe deficits) to 21 (normal).

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Infarction volume was determined by staining with TTC(Sigma) after MCAO as previously described[27].The possible interference of brain edema on infarct volume was corrected by standard methods (whole contralateral hemisphere volume–nonischemic ipsilateral hemisphere volume), and the infarct volume was expressed as a ratio of the whole brain volume[28].

Immunofluorescent Staining

Double and triple immunofluorescence staining for brain was performed on fixed frozen ultrathin sections as previously described[13,29].Sequential coronal slices (10μm) were obtained with a cryostat (CM3050S; Leica Microsystems, Wetzlar, Germany) and permeabilized with 0.3% Triton X-100 in PBS for 30 min. Sections were blocked with 5% donkey serum for 1 hour and incubated at 4°C overnight with primary antibodies including: rabbit polyclonal anti-TGR5 (1:100,ab72608,abcam), mouse monoclonal anti-Pellino3 (1:100,sc-376466,Santa Cruz Biotechnology),mice polyclonal anti-NeuN (1:200, ab104224, Abcam), goat polyclonal anti-GFAP (1:200,ab53554,abcam) , goat polyclonal anti-Iba-1 (1:200,ab107159,abcam).It was then incubated with the appropriate fluorescence dye-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) in the dark room for 2 h at room temperature. The slices were visualized with a fluorescence microscope (DMi8; Leica Microsystems, Germany) or confocal LSM 710 microscope and fluorescence intensity was quantified using ImageJ. 3 sections were chosen from each brain with each section containing 3 microscopic fields from the ischemic boundary zone.

Assessment of Long-Term Neurobehavior

From day 21 to day 27 after MCAO, Morris water maze was performed as previously described[30].An accelerating rotarod test provides an index of fore and hindlimb motorcoordination and balance[31].

Evaluation of TNF-α, IL-1β and IL-18 level

Twenty-four hours after MCAO, rats were sacrificed and brain tissue homogenates were obtained from the infarct cerebral hemisphere. The levels of TNF-α, IL-1β and IL-18 were measured in brain tissue homogenates using specific ELISA kits according to the manufacturers' instructions and quantified by a microplate reader at 450 nm[32].

Cell isolation and flow cytometry

24 hours after stroke, the rat brain was isolated and subjected to mechanical and enzymatic dissociation using tissue dissociation kit(Miltenyi Biotec),as previous described[33,34]. Single suspension of cells was mixed with the Percoll solution and centrifuged for 30 min, 950 rcf at room temperature. Cells were resuspended in PBS containing 2% BSA and were incubated with the respective antibodies at 4 ℃: CD45(#202207, Biolegend), CD11b (#201805,Biolegend)、TGR5(ab72608,abcam). Cell Quest software was used to determine immune subpopulations. Cells were then gated for CD45 high and CD45 intermediate populations. Alexa Fluor®488-labeled donkey anti-rabbit IgG secondary antibody were used for final detection. Data were analyzed using Flow Jo 7.6.1 software.

Western Blots Analysis

Brain samples were collected 24 hours after MCAO. Western blot was performed as described previously[35].Primary antibodies were TGR5 (1:1000, ab72608,abcam), Pellino3 (1:1000, Santa Cruz Biotechnology), caspase-8 (1:1000, ab25901,abcam), NLRP3 (1:500, NBP2-12446、NOVUS Biologicals), caspase-1 (1:1000, NBP1-45433、 NOVUS), IL-1β (1:1000, ab2105,abcam). β-actin was used as an internal loading control. The secondary antibodies were all from Santa Cruz Biotechnology.

Co-Immunoprecipitation (Co-IP)

Co-IP was performed as previously described[23,36].500 μg protein was first pre-treated with TGR5 polyclonal antibody (1:50) or Pellino3 (1:50) with agitation on a rotator. Protein A/G agarose (20 μL; Sigma) was added to each sample and incubated overnight at 4 °C. Then the mixture was precipitated by high-speed freezing centrifugation at 12000 rpm for 10 seconds. To remove non-specifically bound proteins, the sediment was washed three times with NP-40 buffer. Agarose-bound immunocomplexes were then released by denaturing solution in loading buffer. TGR5 and Pellino3 proteins in immunocomplex denaturing solution and total protein solution (for comparison) were analyzed by Western blot as above.

Statistical Analyses

The analysis of the data was performed using SigmaPlot 11.0 and GraphPad Prism 6 (GraphPad software, San Diego, CA). The data are presented as the mean± standard deviation (SD). Data from different groups were compared using 1-way ANOVA followed by post hoc Tukey tests. Non-parametric

data (neurological scores, beam walking) were analyzed with the Kruskal–Wallis test followed by Dunn's post-hoc. No further adjustment was made for multiple comparison for the overall number of tests. In all statistical analysis, a value of $P<0.05$ represents statistical significance.

Results

Mortality and exclusion

A total of 383 rats were used and 301 rats underwent MCAO induction. The mortality of MCAO rats was 13.6% (41 of 301) and no rats died in the other groups (Supplemental table 1). 10 animals were excluded if rats didn't show signs of neurobehavioral deficits when waking up from MCAO (body twisting when lifted by the tail and walking in circles) or if subarachnoid hemorrhage was observed during euthanasia.

Endogenous TGR5 receptor were increased after MCAO

As shown in Fig. 1a, TGR5 expression was significantly increased at 12 hours and reached peak at 24 hours but declined at 72 hours after MCAO group ($P<0.05$ versus Sham). Pellino3 level was elevated at 12 hours after MCAO, reached its highest level at 24 hours, but then significantly decreased at 72 hours after MCAO (Fig.1a; $P<0.05$ versus Sham).

Double immunofluorescence staining showed that TGR5 expression was upregulated in microglia and neurons in the penumbra area 24 hours after MCAO (Fig. 1b–c). There was no significant difference in astrocyte between sham group and MCAO group（Fig. 2a,b）.After stroke, the expression of TGR5 was increased in $CD11b^{+}CD45^{intermediate}$ microglia or $CD11b^{+}CD45^{high}$ macrophages (Fig.2 c,d).

TGR5 receptor agonist treatment reduced brain infarction and improved neurological function at 24hours and 72hoursafter MCAO

Compared with MCAO+vehicle group, treatment with the middle (0.48mg/kg) or high doses (1.44 mg/kg) of INT777 significantly reduced infarct volume, improved neurological scores respectively at 24 hours after MCAO (Fig.3a,c,d; $P<0.05$). The middle dosage of INT777 also decreased cerebral infarction and improved neurological function at 72 hours after injury (Fig. 3b,e,f); $P<0.05$versus MCAO+Vehicle). Therefore, we chose this dosage for the long-term and mechanism studies. There was no difference in respiratory parameters, blood pressure, body temperature (Supplemental Table 2).

Activating TGR5 improved long-term neurobehavioral outcome after MCAO

We performed Morris water maze and rotarod tests on days 21-27 after MCAO. The three groups exhibited a similar latency to escape onto the visible platform and had similar swimming distances during the first day of visible platform tests (Fig.4a,c; $P>0.05$). For the hidden platform trials and the probe trial, the results showed that the animals in the vehicle group required more time to reach the platform (Fig.4b; $P<0.05$), traveled significantly longer distance (Fig.4c; $P<0.05$), and spent less time in the target probe quadrants (Fig.4d; $P<0.05$) when compared with sham group. INT777 decreased the latency to find the

platform, reduced travel distance, and lead to animals spending remarkably more time in the target quadrant (Fig.4b-d; *P*<0.05 versus MCAO+vehicle).

In the rotarod test, INT777 treatment significantly improved motor coordination on the 5 RPM and 10 RPM tests (Fig.4e; *P* < 0.05 versus MCAO+vehicle).

INT777 suppressed neuroinflammation induced by MCAO

At 24 hours after MCAO, the expressions of cleaved caspase-8, NLRP3 were dramatically increased (*P*< 0.05 versus Sham) and INT777 treatment lead to lower expressions(Fig.5a-c; *P*<0.05 versus MCAO+vehicle). The levels of TNF-α, IL-1β and IL-18 were enhanced in the MCAO group compared to the sham group (*P* < 0.05). In INT777 treatment group, the levels of cytokines in the brain tissue were reduced significantly (*P* < 0.05 versus MCAO+vehicle, Fig.5d).

MCAO induced interaction of TGR5 with Pellino3

In the sham group, double immunofluorescence staining showed that TGR5 and pellino3 co-localized together in the brain. After MCAO, co-labeling of TGR5 with pellino3 increased in the penumbra area (Fig. 6a). Triple-fluorescence staining showed that TGR5 and pellino3 co-localized in microglia (Supplemental Fig.2a).

Western blot analysis showed that both TGR5 and Pellino3 expression increased after MCAO (Fig.6b; *P*<0.05 versus sham). CO-IP showed that TGR5-Pellino3 interaction was found in theischemic hemisphere (Fig.6b).

Pellino3 siRNA increased expressions of cleaved caspase-8 and NLRP3 after MCAO

Firstly, the relationship between caspase-8 and NLRP3 was explored. Administration of caspase-8 inhibitor Z-IETD-FMK inhibited the expression of NLRP3. Pellino3 siRNA significantly reduced Pellino-3 expression at 24 hours after MCAO. Western blot results showed that pellino3 knockdown markedly increased the expression of cleaved caspase-8 and NLRP3 (supplemental Fig 2b,c; *P*<0.05 versus MCAO+vehicle). Pellino3 siRNA significantly increased infarct volume and aggravated neurological scores, while Z-IETD-FMK improved neurological damage (Supplemental Fig.2d; *P*<0.05 versus MCAO+vehicle).

TGR5 or Pellino3 knockdown prevents the anti-inflammation of INT777 after MCAO

Compared with the vehicle group, TGR5 knockdown dramatically inhibited the expressions of TGR5 and Pellino3 while increasing cleaved caspase-8 and NLRP3 expressions (Fig.6a,b; *P*<0.05). TGR5 siRNA significantly aggravated brain infarct volume and neurological impairments (Fig.7c; *P*<0.05 versus scramble siRNA group).

TGR5 siRNA reversed the effect of INT777 on the expression of pellino3, cleaved caspase-8, and NLRP3 (Fig.8a,b; *P*<0.05 versus MCAO+INT777+scramble siRNA). Western blot showed that Pellino3 siRNA also

significantly reversed the effect of INT777 on cleaved caspase-8 and NLRP3, as well as cleaved caspase-1 and IL-1β when compared with scramble siRNA at 24 hours after MCAO (Fig. 8a, b; $P<0.05$).

Administration of TGR5 siRNA or Pellino3 siRNA significantly abolished the neurological improvements causing by treatment with INT777 at 24 hours after MCAO (Fig.8c; $P<0.05$ versus MCAO+INT777+scramble siRNA).

Discussion

In this study, we investigated the role of TGR5 in anti-inflammation after MCAO. This study demonstrated that TGR5 and Pellino3 were upregulated in the injured hemisphere after MCAO. INT777 improved both short- and long-term neurofunction after MCAO which were accompanied by increasing Pellino3 expression , inhibiting expressions of pro-inflammatory cytokines and microglial activation , reducing the expressions of cleaved caspase-8 and NLRP3. In contrast, silencing of endogenous TGR5 or Pellino3 by siRNA increased cleaved caspase-8 and NLRP3 expressions, exacerbated brain injury. Furthermore, knockdown TGR5 or Pellino3 abolished the *anti-neuroinflammatory* effects of INT777. Taken together, our study suggests that TGR5 may be involved in inhibiting neuroinflammation in the brain via Pellino3 inhibition of caspase-8/NLRP3 after MCAO in rats.

Previous studies have suggested a protective role for TGR5 agonists in inflammation. Research shows that TGR5 may suppress gastric, liver and renal inflammation[37-39]. Furthermore, Yang et al found that TGR5 inhibited expression of inflammatory cytokines in liver ischemia[21]. Upregulation of TGR5 was observed in the cortex of mice of hepatic encephalopathy and central infusion of the TGR5 agonist delayed neurological decline[12]. It has been reported that TGR5 was constitutively expressed in microglia in vivo and in vitro[40]. Bile acid *TUDCA* binding to TGR5 causes an increase in intracellular cAMP levels in microglia leading to production of anti-inflammatory markers, while reducing pro-inflammatory cytokines[40]. In the study, We observed that INT777 significantly decreased the expression of the NLRP3 and improved neurobehavioral functions after MCAO. In addition, TGR5 siRNA aggravated brain impairments, abolished the neuroprotective effects of INT777.

Although the exact mechanisms of TGR5–mediated neuroprotection are not well clarified, Pellino3 may play a critical role in the TGR5-mediated signaling pathway. Pellino3 belongs to the mammalian Pellino family of E3 ubiquitin ligases that play important roles in innate immunity[41,42]. Pellino3-deficient mice showed heightened diet-induced inflammation and IL-1β expression that exacerbated insulin resistance[15]. Smith et al found that Pellino3 decreased TLR2-mediated NF-κB activity and proinflammatory gene induction in response to H. pylori LPS in epithelial cells[43]. In the present study, we observed that endogenous Pellino3 expression was increased at 24 hours after MCAO and INT777 further augmented Pellino3 expression. Double immunofluorescence staining demonstrated that co-localization of TGR5 with Pellino3 increased after MCAO. CO-IP also showed an interaction between TGR5 and Pellino3 after MCAO. Furthermore, we observed that silencing TGR5 inhibited the upregulation of Pellino3 by INT777. Taken together, these findings support that TGR5 can act upstream to increase

Pellino3 expression, thereby alleviating neuroinflammation. Yang et al found that loss of Pellino3 in mice led to high levels of caspase-8 and hepatoxicity and lethality in response to TNF in vivo[15]. We found that cleaved caspase-8 was increased after MCAO, which was consistent with previous observations[44,45]. Pellino3 siRNA resulted in a significant increase in cleaved caspase-8 expression and abolished the effects of INT777 on cleaved caspase-8 expression.

Several lines of evidence have confirmed that caspase-8 functions as a regulatory molecule for microglia pro-inflammatory activation[44,46]. However, the role of caspase-8 as a regulator of the NLRP3 inflammasome remains controversial. Several studies suggested that caspase-8 could act upstream of NLRP3 activation. For example, when inhibitor of apoptosis was absent, caspase-8 was essential for TLR-mediated, NLRP3-induced, caspase-1 processing[47]. Chi et al found that Caspase-8 promoted NLRP3 inflammasome activation and IL-1β production in acute glaucoma[18]. In contrast, studies of caspase-8 conditional knockout dendritic cells (caspase-8-cKO DCs) implicated caspase-8 as an inhibitor of RIPK3-mediated NLRP3 activation[48].[49] In our study, we found that inhibition of caspase-8 activation significantly down-regulated the expression of NLRP3, which agreed with the role of caspase-8 in intraocular pressure-induced retinal ischemia[17]. Furthermore, our data demonstrated that TGR5 siRNA or Pellino3 siRNA significantly reversed the effect of INT777 on cleaved caspase-8 and NLRP3 expressions, and abolished the neuro-protection of INT777.Our findings support the Pellino3/caspase-8/NLRP3 signaling pathway as part of underlying neuroprotection mechanism of TGR5 activation after MCAO.

This study has a few limitations. Firstly, TGR5 has been shown to interact with other membrane receptors[49] and therefore there may be additional mechanisms or interactions which may play a role in TGR5's neuroprotection. Secondly, there may be cross-talk between the downstream signaling molecules which may also play a role in the protection observed and an early time point to observe the downstream and upstream relationship should also be addressed in future studies. Thirdly, the protective effects of INT777 may be direct (*i.e.* via TGR5 signaling) but may also include indirect effects through the reduction of the infarction. It is very likely that reducing infarction will reduce pro-inflammation. Fourthly, INT777 is protective in the research and we couldn't exclude the possibility that other TGR5 agonist may extending the *time window* for treatment. Finally, research demonstrated that activating TGR5 markedly attenuated hypoxia/reoxygenation induced hepatocellular apoptosis[20]. We cannot exclude the possibility of TGR5 activation in decreasing infarct volume through reducing neuron death and further study should be explored.

Conclusions

In summary, the present study showed that TGR5 elevation occurred after MCAO, and an exogenous TGR5 agonist inhibited neuroinflammation and improved neurological outcome, which may be via Pellino3 inhibition of caspase-8/NLRP3 after MCAO in rats. This observation supports that TGR5 may be an attractive candidate for anti- neuroinflammation treatment after MCAO.

Abbreviations

CCA :common carotid artery ; Co-IP: Co-Immunoprecipitation; ECA:external carotid artery ; ICA :internal carotid artery ;MCAO: middle cerebral artery occlusion; NLRP3:nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3;TTC:Triphenyltetrazolium Chloride

Declarations

Acknowledgements

Not applicable

Authors' contributions

H.L. was involved in research design, experimental performances, animal surgery, Western blot, and immunohistochemistry, drafting the manuscript except neurobehavioral tests, and data analysis. N.M. gave technical assistant and manuscript preparation. D.W.M. discussed the results and edited part of manuscript. Y.X.and Z.Z. carried out intracerebroventricular injection, co-immunoprecipitation, behavioral tests and data analysis. J.T. participated in research design. B.L. and J.H.Z. are the corresponding authors; they took care of all aspects including research design, data analysis and manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experimental protocols were approved by the Loma Linda University and Zhejiang University institutional animal care and use committee in accordance with the Helsinki Declaration of 1975.

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interest. All the authors listed have approved the manuscript.

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Figures

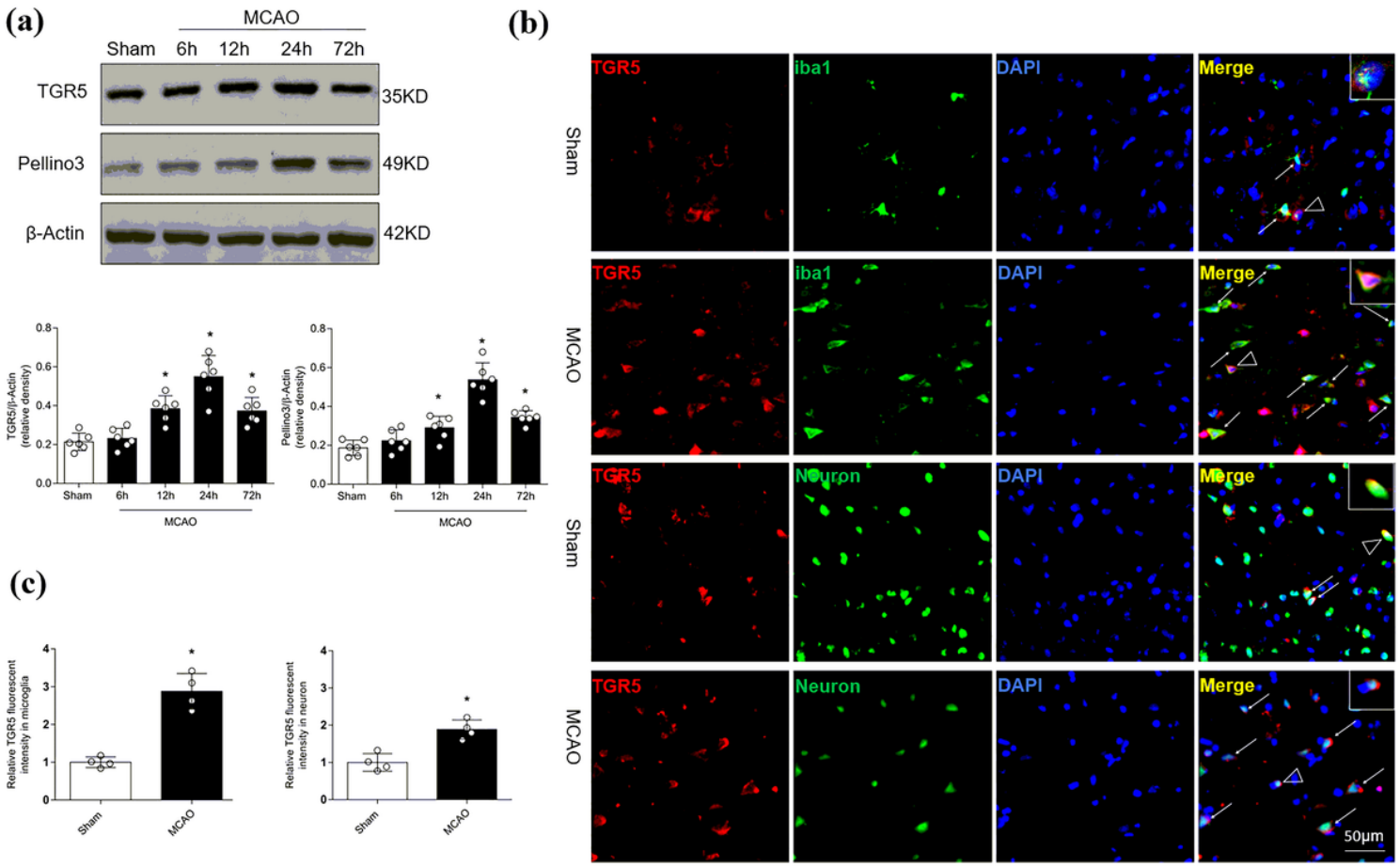
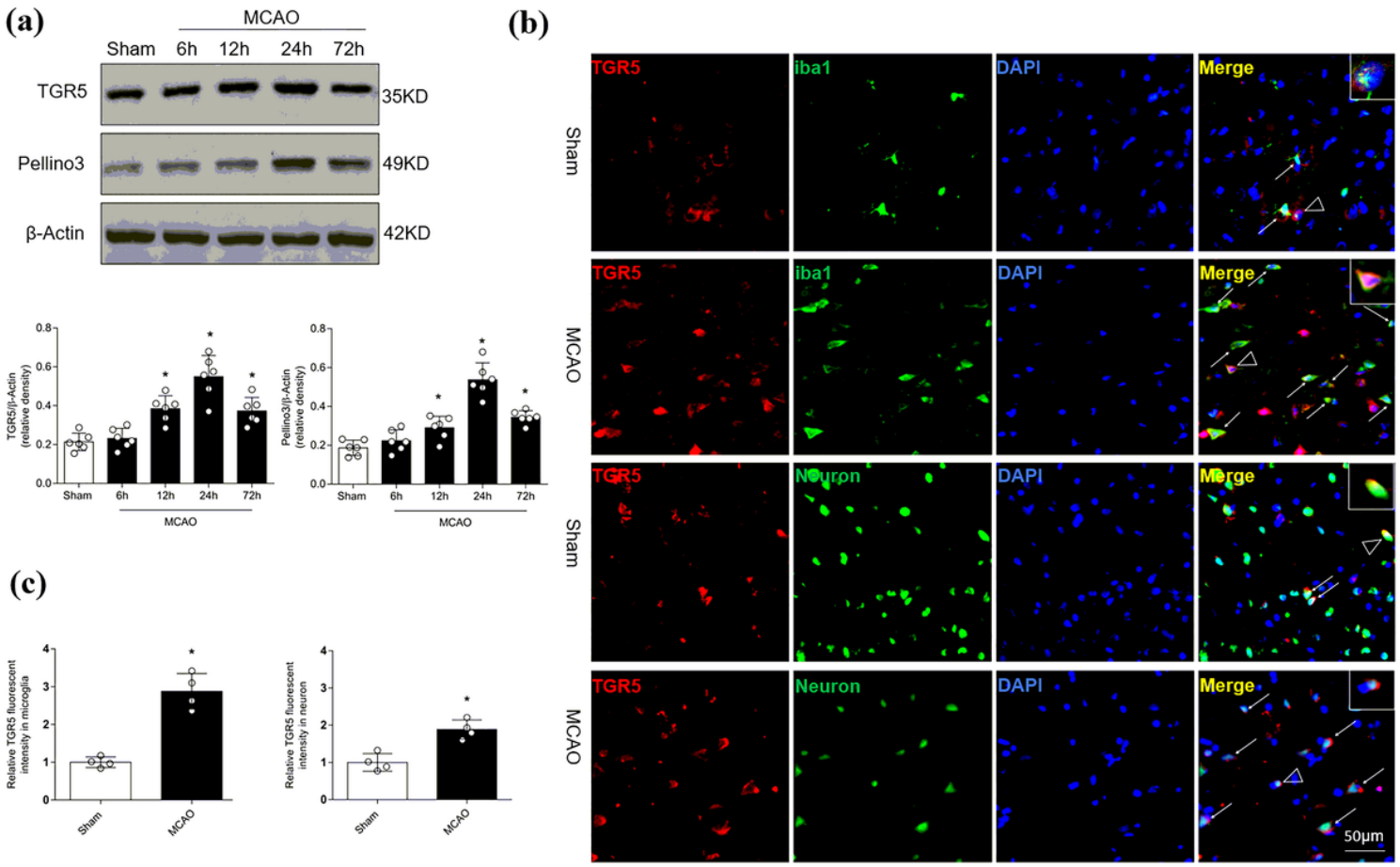


Figure 1

Expressions of TGR5 and Pellino3 after MCAO. a Representative Western blot images and quantitative analyses of TGR5 and Pellino3 time course from right hemisphere after MCAO. n=6 per group, *P<0.05 vs sham group. b Double immunofluorescence staining for TGR5 (red) in microglia (Iba-1, green), neuron (green) in the penumbra following MCAO. n=4 per group. c The relative fluorescent intensity of TGR5 in microglia and neuron, n=4 for each group,*P<0.05 vs sham. Bars represent mean±SD. Scale bar, 50 μm. Iba-1 indicates ionized calcium binding adaptor molecule 1.



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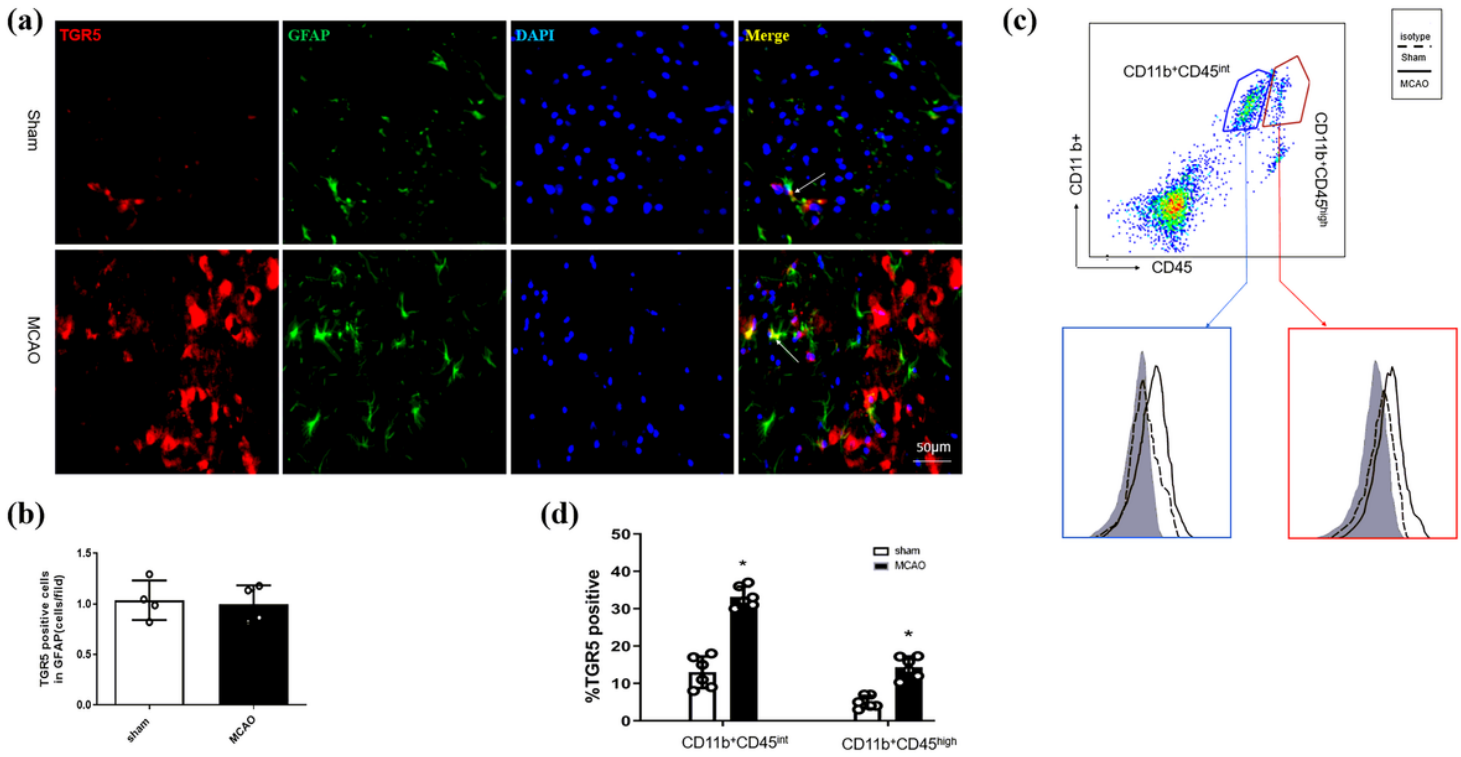

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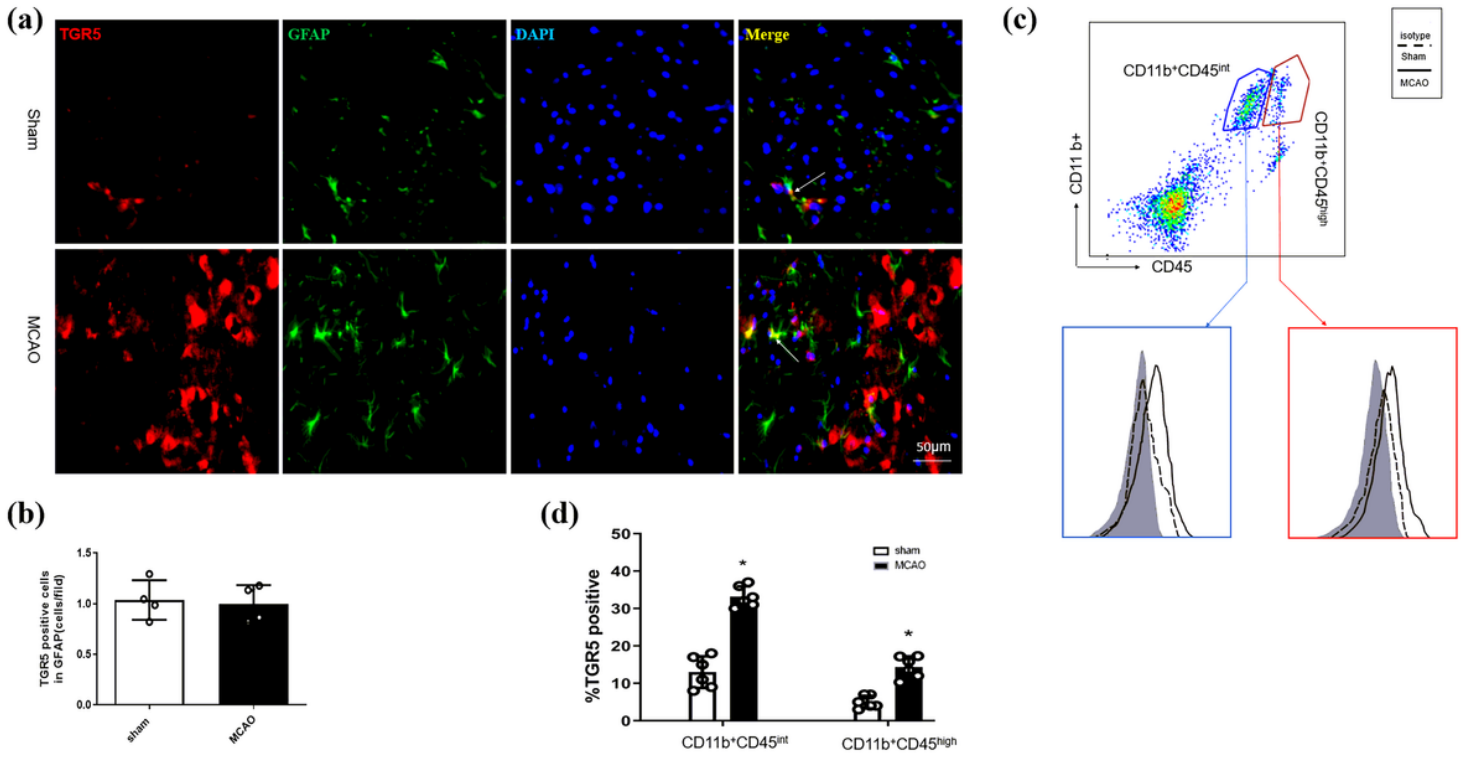

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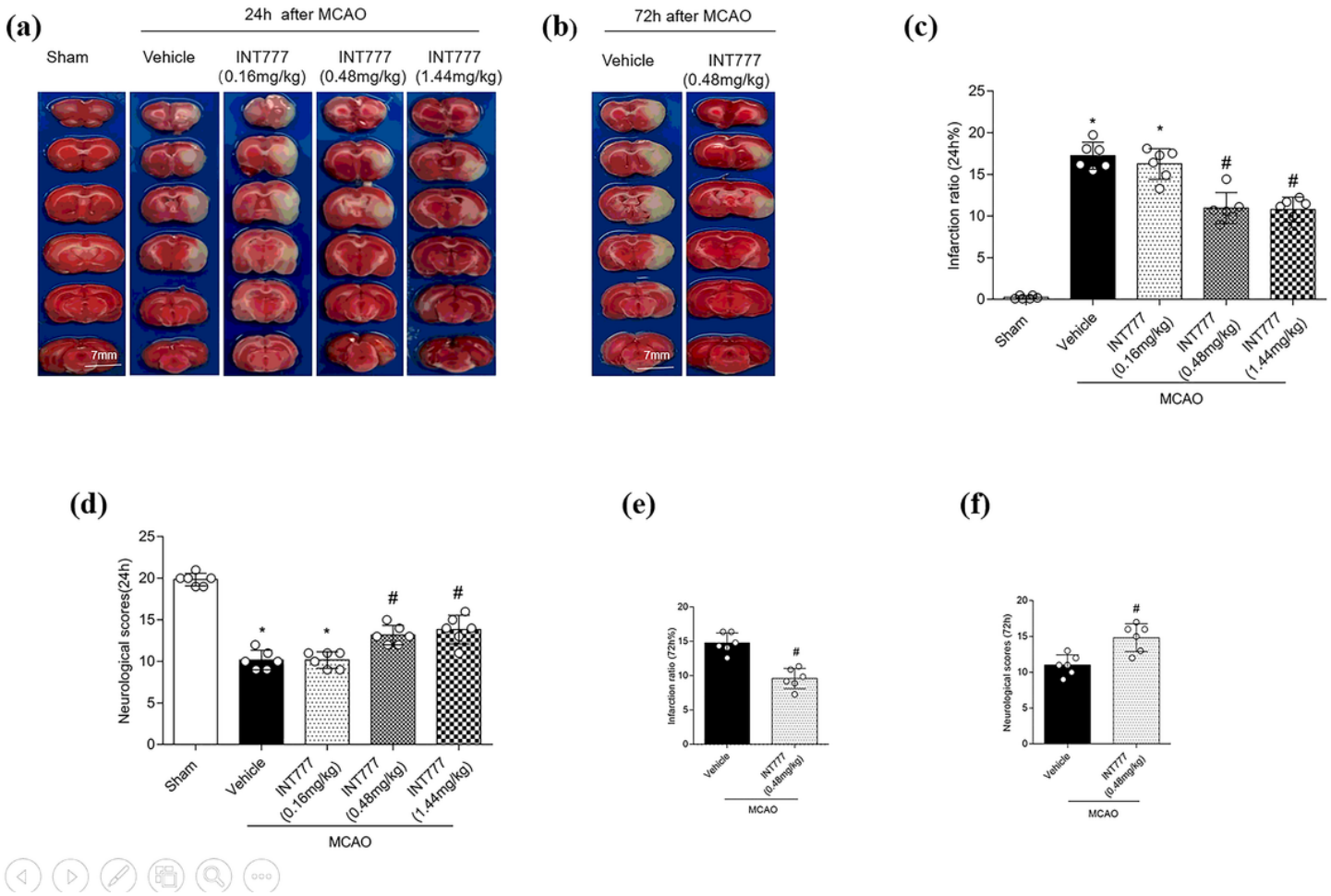

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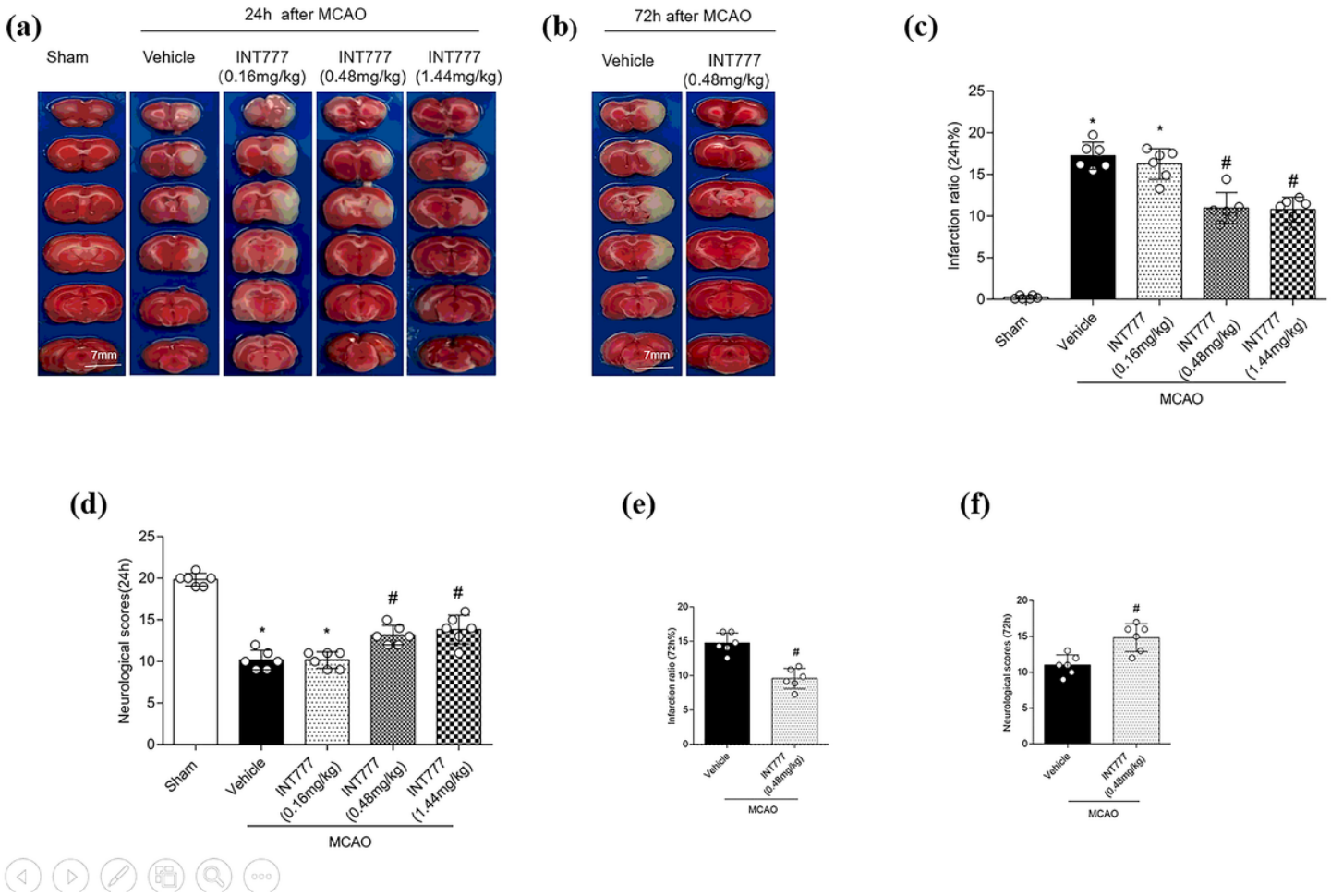
The protective role of TGR5 in MCAO. INT777 ameliorated brain injuries both at 24 and 72 hours after MCAO. Representative TTC staining images of coronal sections (a, b). Quantified infarct ratio, neurological scores (c-f). n=6 for each group. *P<0.05 vs sham, #P<0.05 vs MCAO+vehicle. Bars represent mean±SD.



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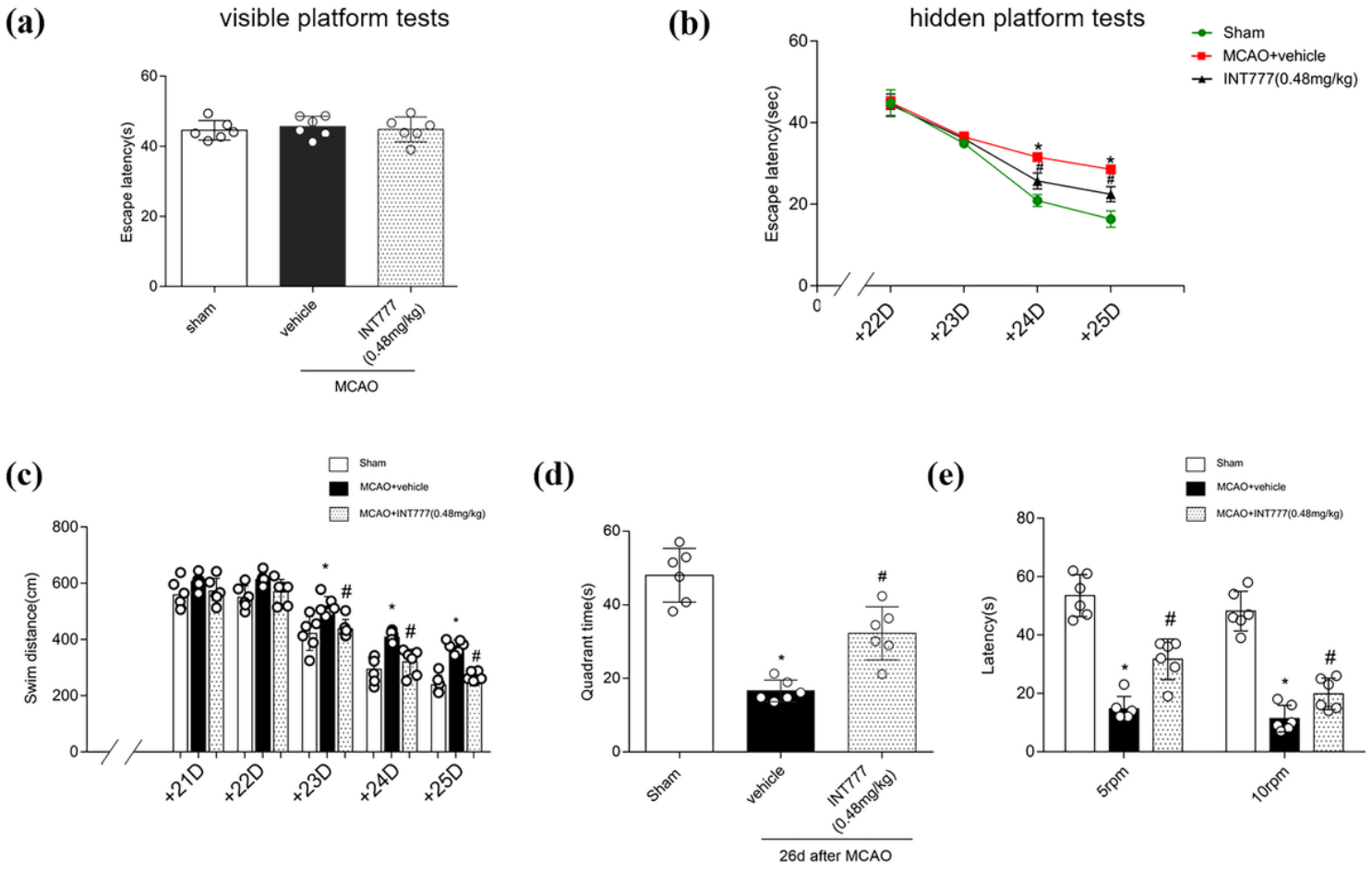
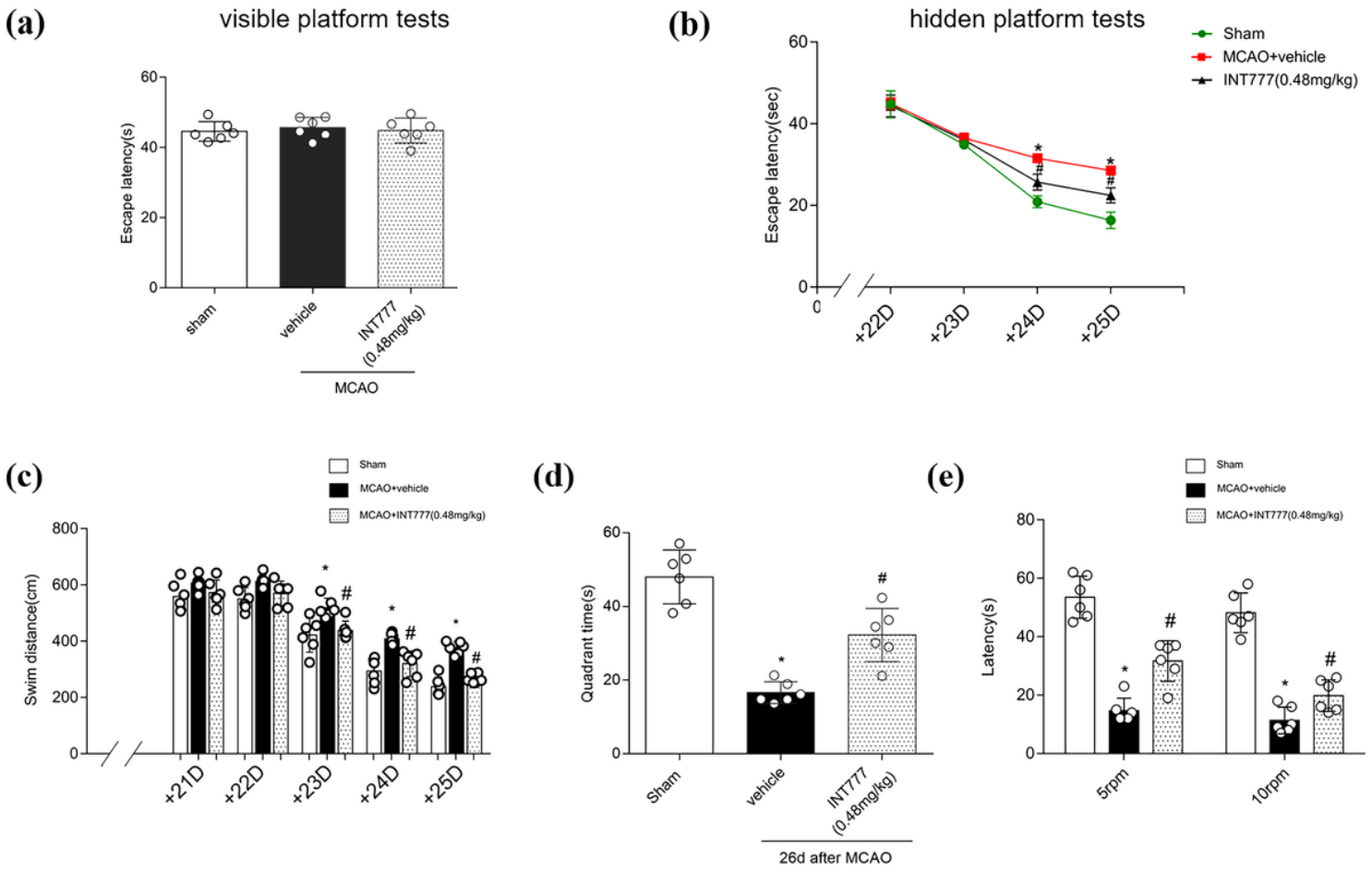


Figure 4

INT777 improved long-term neurobehavior after MCAO. a,b Escape latency during the visible platform test and hidden platform tests. c Swim distance of water maze test. d Probe quadrant duration of water maze test. e Rotarod test of 5RPM and 10RPM. n=6 for each group. *P<0.05 vs sham, #P<0.05 vs MCAO+vehicle. Bars represent mean±SD.



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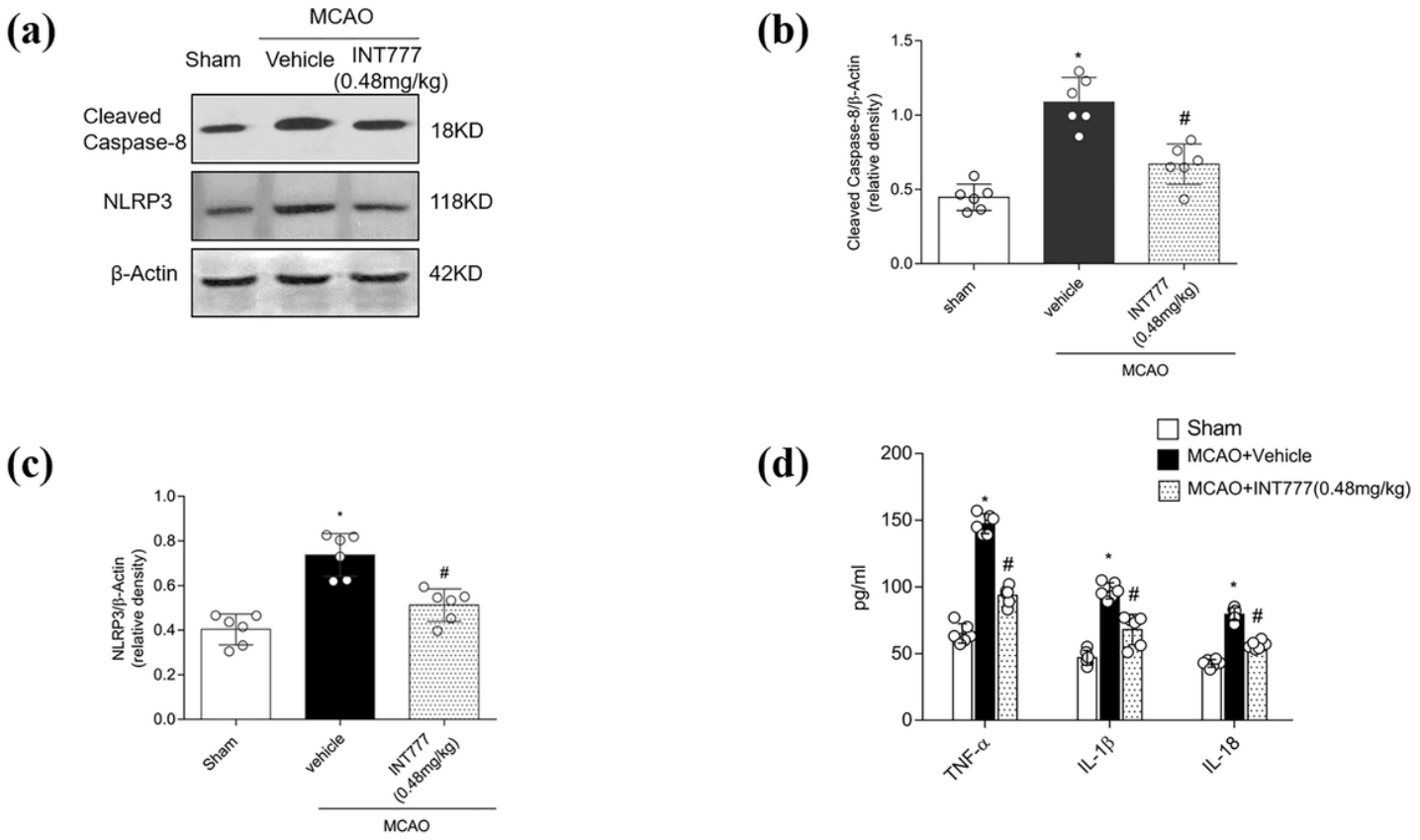

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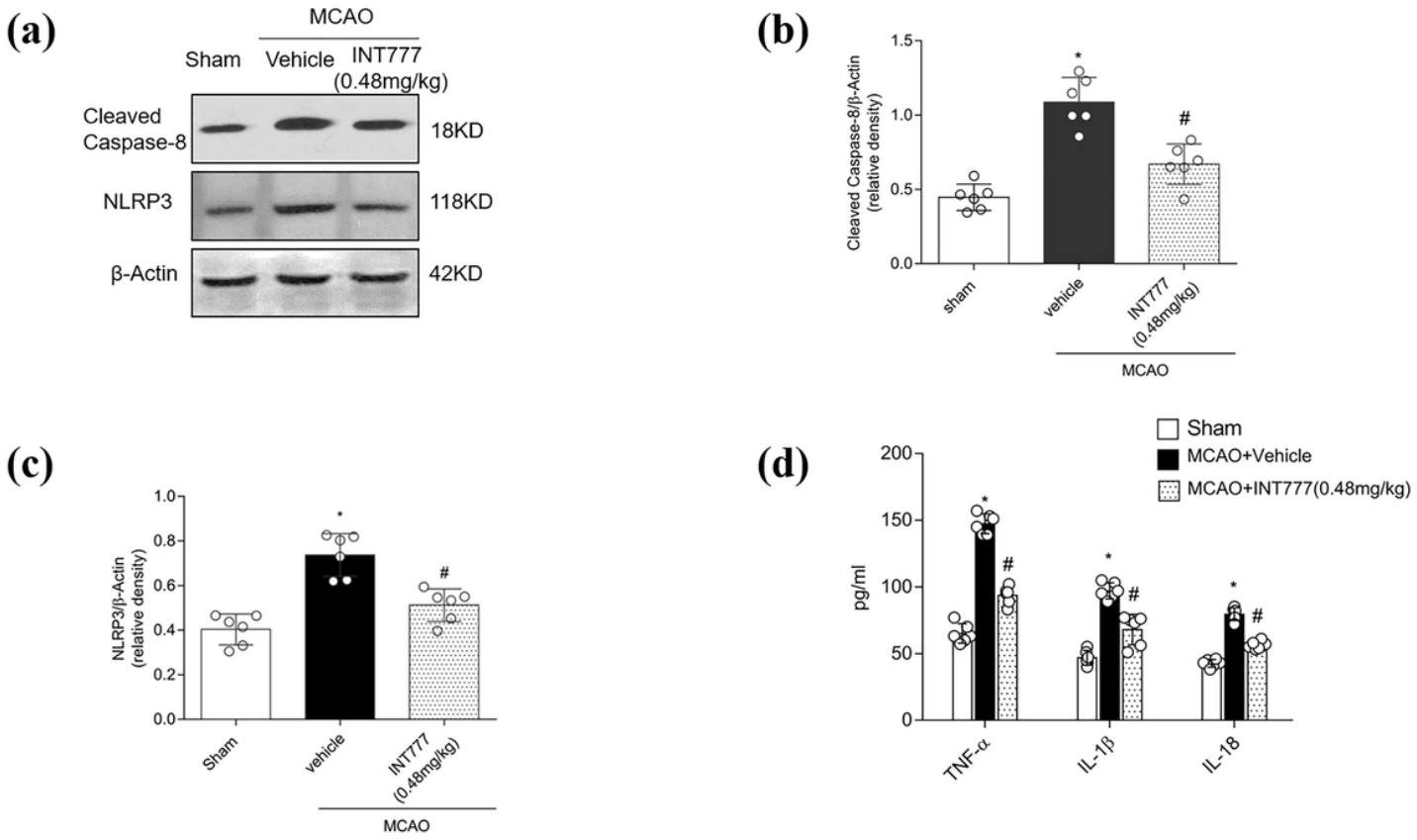

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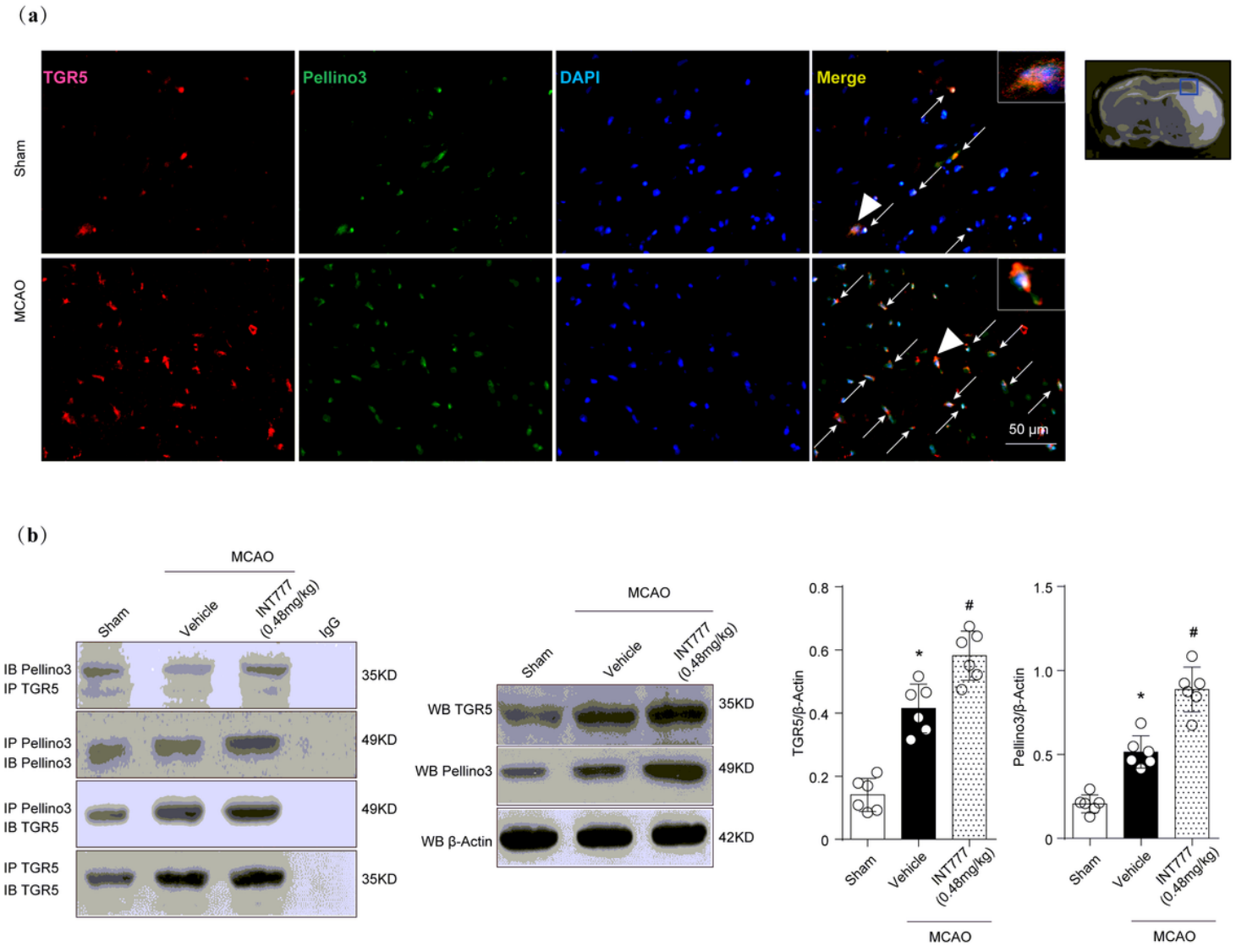

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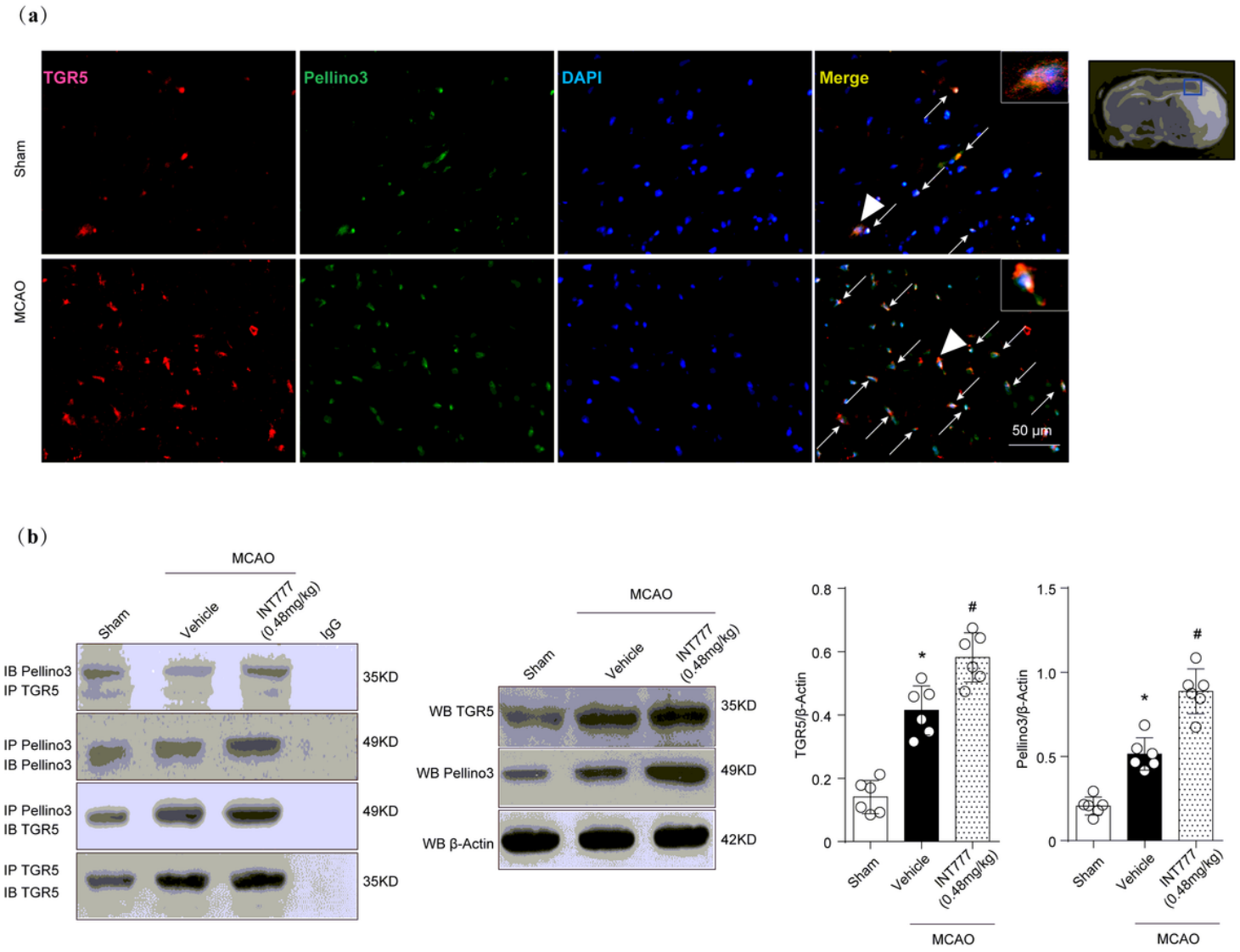
TGR5 interacted with Pellino3 after MCAO. a Double immunofluorescence staining showed that co-localization of TGR5 (red) and Pellino3(green) was increased in penumbra 24 hours after MCAO. n=4 per group. b Representative CO-IP bands showed that interactions of TGR5 with Pellino3 occurred at 24 hours after MCAO. Protein bands of TGR5 and Pellino3 in total protein solution were detected by Western blot and relative OD ratios were reported in right panel. n=6 for each group. *P<0.05 vs sham, #P<0.05 vs MCAO+ vehicle. Bars represent mean±SD.



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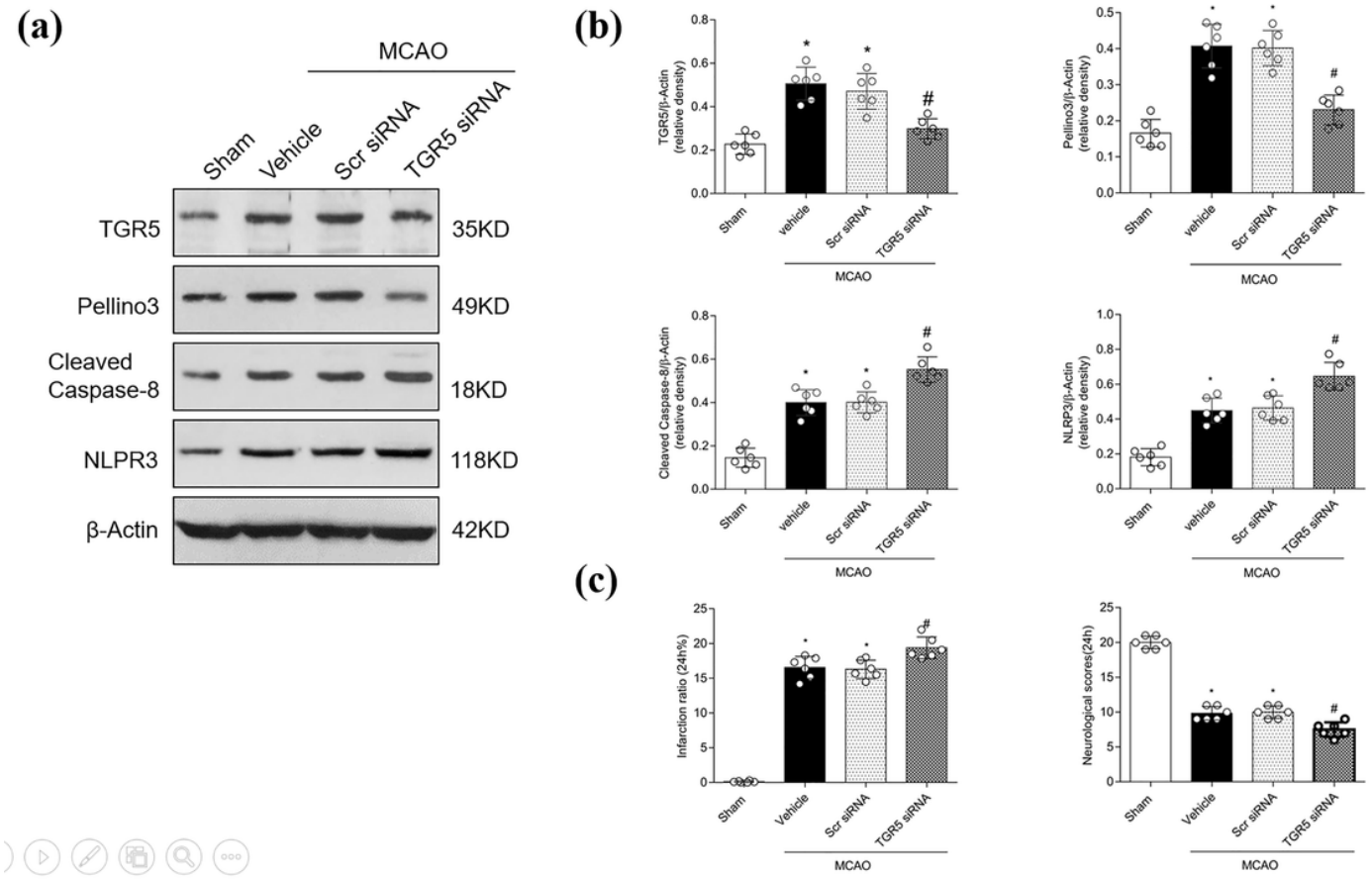

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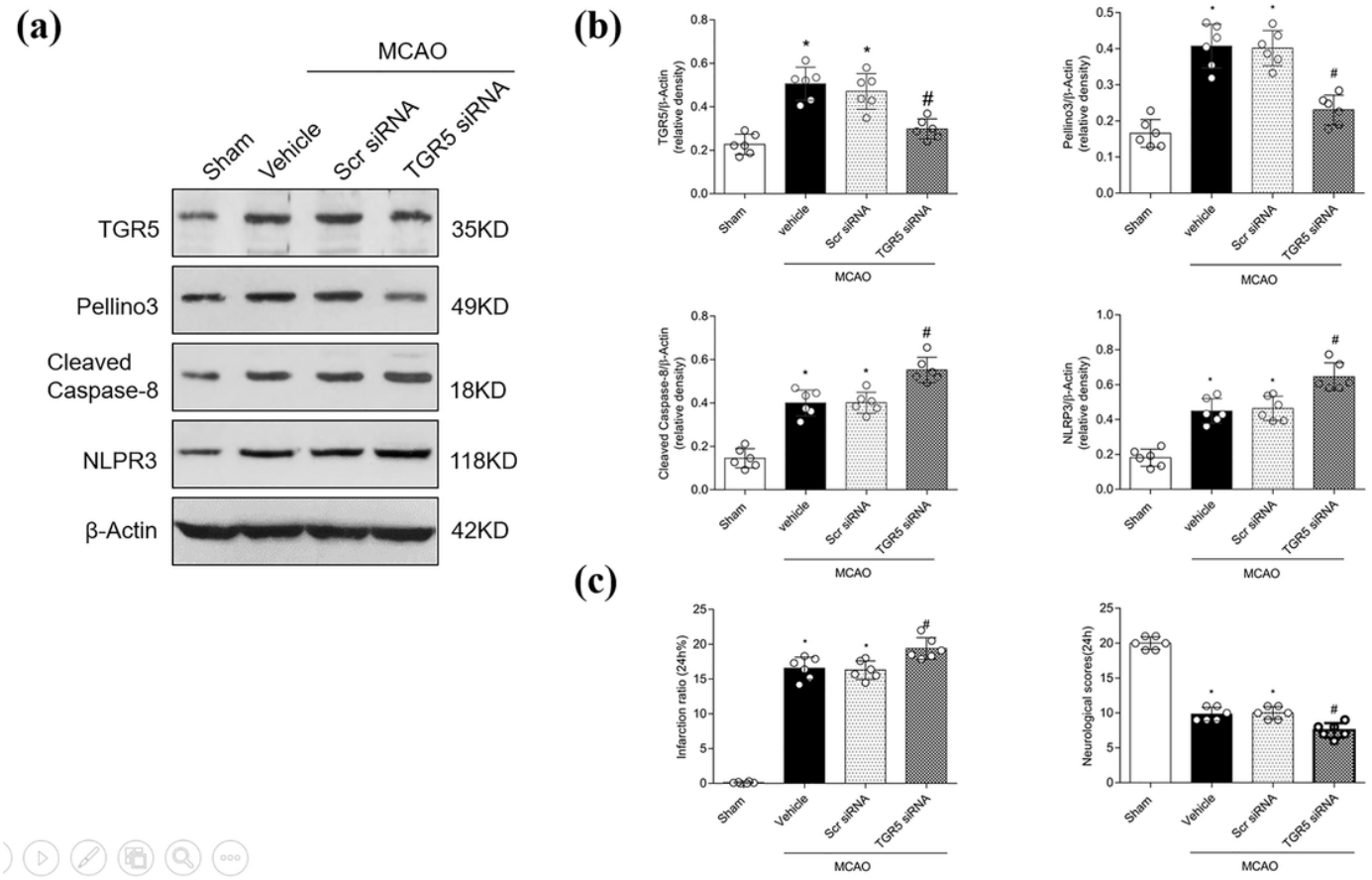
The effect of knockdown TGR5 on pellino3, cleaved caspase-8 and NLRP3 expression and brain damage after MCAO. The band of Western blot analysis(a) and the relative density (b) of TGR5,Pellino3, cleaved caspase-8 and NLRP3. c TGR5 siRNA increased infarct volume, worsen neurobehavioral deficits. n=6 per group. *P<0.05 vs sham, #P<0.05 vs MCAO+ Scr siRNA. Bars represent mean±SEM. Scr siRNA, scramble siRNA.



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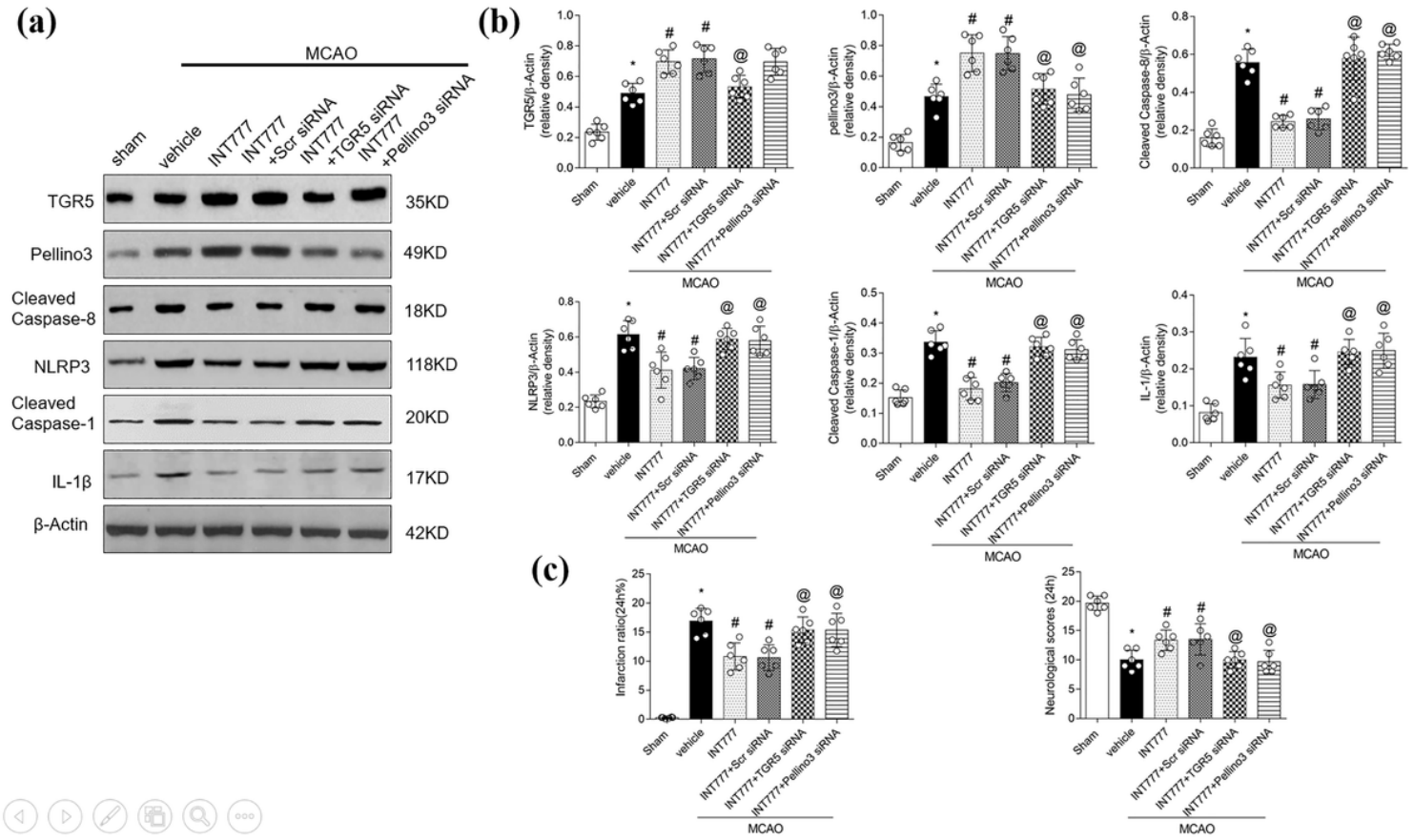

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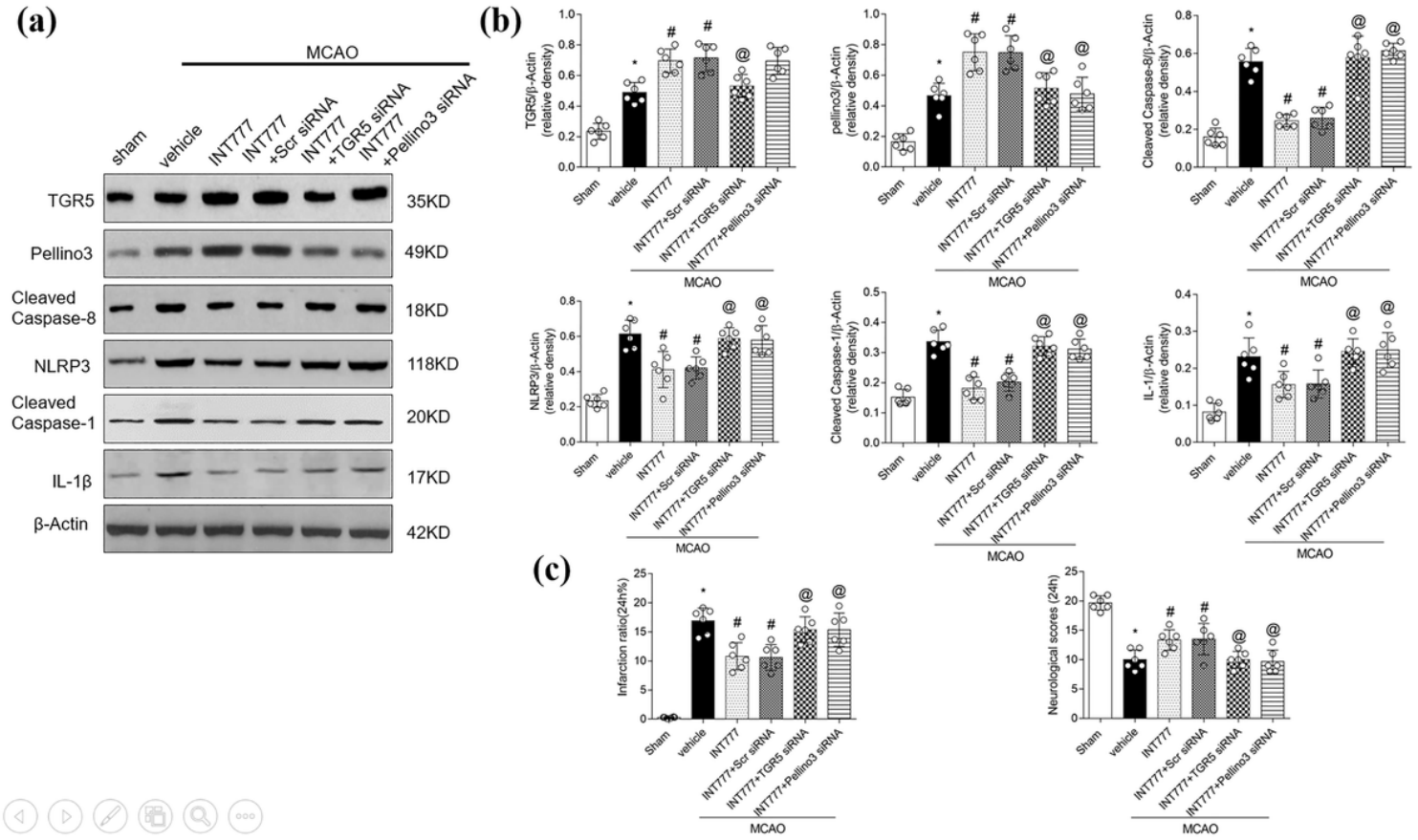

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