Metformin Protects Against Traumatic Brain Injury by Switching M1/M2 Phenotypes and Regulating NLRP3 Inflammasome Activation

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Abstract

Background: Traumatic brain injury (TBI) always lead to inflammatory responses unregulated characterized by excessive leukocytes infiltration, microglia activation and neuronal apoptosis. Metformin, recognized as an activator of AMPK, had been reported to exert protective effects in TBI models. However, whether metformin treatment switches post-TBI-induced microglia/macrophage polarization and its mechanism has not yet been fully elucidated.

Methods: Firstly, we established a C57BL/6J mouse TBI model receiving an intraperitoneal injection of 50 mg/kg metformin daily from 2h post-injury until sacrifice to assess the leukocytes infiltration, microglial polarization and NLRP3 inflammasome signaling activation. Then, BV2 cells were pretreated with metformin for 2 hours followed by stimulated with LPS to determine the effects of metformin on microglial polarization and its mechanism.

Results: We demonstrated that post-TBI metformin administration switched microglial M1 to M2 polarization and inhibited the NLRP3 inflammasome signaling activation, thus reducing neurological deficits, brain edema, cells death and leukocytes infiltration at day 3 after TBI. Then, these findings were further confirmed in BV2 cell experiments, in which these protective effects mediated by metformin was lessened by the administration of Compound C, an AMPK inhibitor.

Conclusion: Metformin may be a potential therapeutic method to improve neurological recovery following TBI, partly by regulating microglia M1/M2 polarization and inhibiting NLPR3 inflammasome activation dependent on the activation of AMPK.

Introduction

Traumatic brain injury (TBI) is the biggest cause of death and disability in the under 40s worldwide. But there is still lack of effective therapies to promote long-term recovery in these patients. TBI mainly resulted from an extrinsic biomechanical insult to the cranium, leading to brain parenchyma structure and physiology disrupted. In terms of treatment, the primary insult is exclusively sensitive to preventive but not therapeutic interventions[1]. Following the primary injury, a secondary and delayed injury occurs in minutes, hours, months up to years characterized by peripheral immune-mediators permeation through the BBB, microglial and peripheral neutrophil activation, proinflammatory and anti-inflammatory cytokines release and immune cells recruitment. The activated/recruited inflammatory cells had the ability to eradicate cellular and molecular debris. But if this process is not properly regulated, excess cytotoxic molecules may be released eventually causing neuronal and vascular damage[2, 3].

Metformin is a drug that is widely used for the treatment of type 2 diabetes and is well-recognized as an activator of adenosine 5’-monophosphate-activated protein kinase (AMPK). The anti-inflammatory properties of metformin based on the activation of AMPK had been described on several central nervous system (CNS) diseases, such as Stroke, Spinal Cord Injury, Parkinson's Disease and even TBI [4–9]. However, there are no studies focused on the effect of metformin on the microglia/macrophage

polarization and its mechanism after TBI. Recent studies showed that enhanced AMPK phosphorylation could suppressed NLR pyrin domain containing 3 (NLRP3) inflammasome after cerebral and myocardial ischemia-reperfusion (I/R) injury[10, 11]. As the most extensively studied inflammatory complex protein in the body, NLRP3 inflammasome played an important role in initiating the inflammatory response in various central nervous system disorders. In TBI models, we had demonstrated that NLRP3 inflammasome expression was increased and was primarily observed in microglia [12]. Then, an *in vitro* study showed that inhibiting NLRP3 inflammasome expression reduced the microglial activation and promoted microglial M1 toward M2 phenotype polarization [13]. Therefore, we hypothesized that metformin-mediated AMPK activation could inhibit the NLRP3 inflammasome activation, thus shifting microglia/macrophage polarization toward anti-inflammatory M2-phenotype post-TBI.

Materials And Methods

BV2 cells

The mouse BV2 microglial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM containing 2% fetal bovine serum (2% FBS) at 37 ℃ in an atmosphere containing air supplemented with 5% CO_2[14]. To evaluate the effects of metformin on microglia polarization and its mechanisms, the BV2 cells were pretreated with 1.0mM metformin with or without Compound C (Cpd C, 5μM, Sigma-Aldrich, St. Louis, MO, USA) followed by stimulation with LPS (100ng/ml, Sigma-Aldrich, St. Louis, MO, USA). At 12h after stimulation, the treated BV2 cells were collected and prepared for immunofluorescent staining and western blot analysis.

Animals and CCI injury model

Adult male C57BL/6 mice (20-23g) obtained from the Experimental Animal Laboratories of the Academy of Military Medical Sciences were housed individually in a temperature (22℃) and humidity-controlled (60%) vivarium and maintained with free access to food and water. All procedures were approved by Nankai University Animal Ethics Committee, and were conducted in strict accordance with the ARRIVE Guidelines. In this study, a total of 75 male mice were divided into the following three groups (n = 35/group): group I, mice subjected to TBI and intraperitoneally injected with metformin, group II, mice subjected to TBI and injected with an equal volume of sterile PBS, and group III, mice subjected to sham surgery only. For each, five mice were evaluated for corner test/forelimb footfaults, flow cytometry, immunofluorescent staining, brain edema and immunoblot for selective markers.

TBI models were made using a eCCI device (eCCI Model 6.3, VCU, Richmond, VA, USA) as previously described[15, 16]. In briefly, mice were anesthetized with a single intraperitoneal injection of chloralhydrate (3.0 ml/kg) and placed in a stereotaxic frame. Then, a 2.0-mm hole was drilled on the right parietal skull (2.0 mm posterior to the bregma and 1.5 mm lateral to the sagittal suture) to expose the dura. An injury of moderate severity was induced using a single impact of 1.5-mm depth of deformation,

velocity of 4.5 m/sec and dwell time of 150 msec. Sham animals underwent the same procedure with no impact performed.

Metformin administration

Two hours after TBI, mice received daily either intraperitoneal injections of metformin (50 mg/kg/day) or injections of equal volumes of sterile PBS until sacrifice. Before the injection, the metformin or PBS was incubated at 37℃ for 30 mins[7, 8].

Behavioral testing

The corner and foot-faults tests were carried out to assess sensorimotor abnormalities at 1, 3, 7 and 14 days after TBI. With respect to corner test, we induced a corner with an angle of 30° using two cardboard pieces each with dimensions of 30 * 20 * 1 cm^3. The mice were forced to enter the corner. When the mice entered deep into the corner, the whiskers were stimulated, which caused the mice to rear and turn back to the open end. Sham mice turned either left or right, but the injured mice preferentially turned to the non-impaired (left) side. The numbers of left and right turn over ten trials were recorded[17]. For foot-faults assessment, the mice were placed and allowed walking freely on a suspended mesh grid and total 50 steps of right forelimb were recorded. With each weight-bearing step, paw might fall or slip between the wires, which was recorded as a foot-fault.

Water content

Mice were sacrificed at day 3 after TBI, a time point cerebral edema reached the peak. Immediately following sacrifice, the brain was divided along the midline and the ipsilateral tissues were weighed to obtain wet weight (WW). Then, these tissues were dried at 100°C for 24 hours, followed by weighing to obtain dry weight (DW). The water content of each sample was calculated using the following formula: (WW - DW)/WW * 100%[18].

Flow cytometry analysis

Fresh brain tissues were mechanically homogenized through a 40μm nylon cell strainer (Becton Dickinson) with PBS on ice, then cell suspensions were collected and centrifuged at 400*g for 10 mins. The pelleted brain tissues were resuspended in 5 ml of 60% Percoll (GE Healthcare Bio Science AB) and 5ml 30% Percoll was overlaid on the top of the 60% Percoll, followed by centrifuging at 500*g for 20min. Then, the cells were collected on the interface between 30% and 60% Percoll and washed twice. Next, $1*10^6$ brain cells resuspended in 100 μl PBS were stained with anti-mouse CD45, CD11b, Ly6G, CD3, CD4, CD8, NK1.1, or B220 antibodies (Biolegend, Inc.) for 30 mins to label microglia, monocyte, neutrophil, and lymphocyte subpopulations. Cells were washed and suspended in flow cytometry staining buffer. FACS analysis was performed using Accuri C6 software (BD Bio-sciences, San Jose, CA, USA)[19, 20].

To evaluate apoptotic cells, the isolated brain cells were washed with binding buffer followed by centrifugation. The pellet was then resuspended in binding buffer to a concentration of $1*10^6$ /ml. 100 μl

of cell suspension was placed into each tube for staining with 5 μl Annexin V-FITC for 10 mins at RT (room temperature) in the dark. After that, 5 μl of PI was added for another 5 mins. Then, flow cytometric analysis was performed after adding PBS to a total 500 μl volume in each tube[12, 21].

Immunofluorescent staining

On day 3 post-injury, the mice were deeply anesthetized and transcardially perfused with cold PBS and 4% paraformaldehyde. The brains further fixed with 4% paraformaldehyde and dehydrated with 15% and 30% sucrose solution overnight, followed by embedded in the O.C.T. medium (Sakura, USA) and sectioned. Then, cryosections or treated cells (cultured on coverslips) were prepared and fixed in ice cold acetone for 20 mins. Next, sections were incubated with 3% BSA for 30 mins at 37℃, followed by incubating with primary antibodies overnight as following: Iba-1(Wako) in combination with CD16/32 (BD Pharmingen), CD206 (R&D system), Arg-1 (Santa Cruz Biotechnology) or NLRP3(Adipogen Life Sciences). Then, the tissues and cells were rinsed and stained with corresponding secondary antibodies at RT for 1 hr in the dark. After counterstaining with DAPI, slice was observed using a fluorescence microscope. Omission of the primary antibody was used as a negative control.

Western blot

Total proteins of cortical contusion and BV2 cells were extracted using a commercial protein extraction kit (Beyotime Biotech, Jiangsu, China) followed by concentration detecting using BCA Protein Assay Kit (Thermo Scientific). Equal protein samples (10 μg per lane) were separated using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel and blotted onto nitrocellulose membranes. Then, relevant membranes were incubated with primary antibodies (β-actin, CST, AMPK, CST, p-AMPK, CST, Arginase-1, CST, NLRP3, Adipogen, c-caspase-1, Adipogen, IL-1β, CST, IL-10, CST, CD16, Invitrogen, TNF-α, CST and iNOS, Abcam) overnight at 4℃. Next, the membranes were washed and probed with the appropriate secondary antibodies for 1 h at RT. Then, the membranes were visualized using a Millipore ECL Western Blotting Detection System (Millipore, Billerica, MA, USA).

Statistical analysis

All data are presented as the mean ± standard deviation (SD). For comparisons among multiple groups, one or two-way analysis of variance (ANOVA), followed by post-hoc (Bonferroni) test, was used to determine significant differences. Differences between 2 groups were tested using the Student's t-test. Statistical significance was set at $p<0.05$. All analyses were performed using SPSS statistical software (version 22.0, IBM Corporation, Armonk, NY).

Results

Metformin administration improved the functional recovery following TBI.

To determine if metformin administration contributed to the neurological recovery, foot-faults and corner tests were used to assess the sensorimotor and postural asymmetries. The results showed that there was

no difference in the number of left turns between the control and metformin-treated groups on day 1 post-TBI. However, beginning from day 3 post-TBI the mice in the control group exhibited more left turns than mice treated with metformin (Fig. 1B). Then, foot-faults analysis showed that metformin treatment significantly reduced the frequency of forelimb foot-faults occurrence as compared to controls from day 3 after TBI (Fig. 1A).

Metformin administration alleviated the brain edema after TBI

Brain tissues dry-wet weight was used to evaluate the brain edema. We observed that the injury group had significantly higher brain water content than the sham. And treatment with metformin significantly reduced the brain water content compared to the controls (Fig. 1C).

Metformin administration altered immune cells populations

Flow cytometric analysis of brain cellular components revealed that metformin administration reduced the counts of microglia ($CD45^{int}CD11b^{+}$cells), neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$ cells), T cells ($CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cells), NK cells ($CD3^{-}NK1.1^{+}$ cells) in the brain at day 3 after TBI compared to the controls, whereas no changes in the invasion of B cells ($CD3^{-}B220^{+}$ cells) were observed (Fig. 2).

Metformin administration promoted microglial M1 to M2 polarization after TBI

To explore the effects of metformin on microglial polarization after TBI, we firstly co-labeled CD16/32 or CD206 with Iba-1 to determine the frequency of M1 or M2 cells and observed that TBI led to a robust increase of M1 or M2 cells. And metformin significantly reduced the counts of Iba-1/CD16/32-positive cells (Fig. 3A-B), while increased the counts of Iba-1/CD206-positive cells in the peri-contusion area as compared to the PBS-treated groups (Fig. 4A-B). Then, western blot analysis was further conducted to detect the M1-phenotype (iNOS, CD16 and TNF-α) and M2- phenotype (IL-10 and Arg-1) associated makers and results showed that TBI elevated the expression of these molecules in the ipsilateral cortices. And treatment with metformin markedly reduced the expression of iNOS, CD16 and TNF-α (Fig. 3C-D), while further increased the expression of Arg-1 and IL-10 in the ipsilateral cortices of TBI mice compared to the control on day 3 post-TBI (Fig. 4C-D).

Metformin administration inhibited the NLRP3 inflammasome signaling after TBI

Our previous study showed that NLRP3 inflammasome expression was primarily observed in microglia after TBI. So we co-labeled Iba-1with NLRP3 via immunofluorescent staining to evaluate the NLRP3 level at day 3 post-TBI. The results showed that the intensity of Iba-1/NLRP3-positive cells was decreased in the metformin group as compared to the controls (Fig. 5A-B). Then, western blot analysis was also used to detect the expression of NLRP3 inflammasome associated molecules at day 3 post-TBI. And similar results were obtained that metformin treatment inhibited the TBI-induced upregulation of NLRP3, c-caspase-1 and IL-1β in the ipsilateral cortices (Fig. 5D, F).

Metformin facilitated the phosphorylation of AMPK, and phosphorylated AMPK could mediate the activation of NLRP3 inflammasome. So we also evaluated the AMPK phosphorylation by western blot, and the results showed that there was a moderate increase in AMPK phosphorylation in ipsilateral brain tissue at day 3 post-TBI, while metformin treatment further augmented TBI-induced AMPK phosphorylation (Fig. 5 C, E).

Metformin administration decreased the cell death after TBI

Flow cytometry was used to measure the cell apoptosis. Briefly, isolated brain cells were stained with Annexin V-fluorescein isothiocyanate/propidium iodide followed by flow cytometric analysis. The results showed that reduced cell apoptosis was observed after metformin treatment versus the controls (Fig. 5G-H).

Metformin promoted microglial M1 to M2 polarization and inhibited NLRP3 inflammasome activation *in vitro*

In vitro experiment, we firstly administered BV2 cells with three different doses of metformin (0.1mM, 0.5mM and 1.0mM), and results showed that 1.0 mM metformin markedly reduced the TLR4, NF-κB and IL-1β expression compared to other groups (Fig. S1). We, therefore, selected this dose as the optimal concentration for the follow-up *in vitro* experiments**.** Immunofluorescent staining was conducted to explore whether metformin switched microglial M1 to M2 polarization, and results showed that pretreatment with metformin significantly reduced the intensity of $CD16/32^{+}$/$Iba\text{-}1^{+}$ staining whereas enhanced the intensity of $Arg\text{-}1^{+}$/$Iba\text{-}1^{+}$ staining. But when Compound C given together with metformin, the effects were abrogated (Fig. 6B-E). Additionally, western blot analysis also confirmed that metformin suppressed the M1 markers (CD16 and iNOS) and enhanced the M2 markers (IL-10), while Compound C blocked this M2 microglia polarization (Fig. 6F-G).

Then, we measured the protein levels of NLRP3, c-caspase-1 and IL-1β in BV2 cells by western blot. As shown in Figure 6J, the elevated levels of NLRP3, c-caspase-1 and IL-1β significantly decreased by pretreatment with metformin, while the effect caused by metformin was lessened by the administration of Cpd C (Fig. 6J-K). Additionally, we also detected the upregulated phosphorylation of AMPK in metformin treated BV2 cells. But when metformin given together with CC, the phosphorylation of AMPK was attenuated (Fig. 6H-I).

Discussion

In this study, we enriched the evidence that metformin administration could improve the neurological recovery after TBI characterized by reduced brain edema and leucocytes infiltration. Then, we used cell and TBI models to demonstrate that enhancement of AMPK phosphorylation mediated by metformin had the ability to switch microglial M1 to M2 polarization partly by inhibiting the NLRP3 inflammasome activation. Therefore, post-TBI metformin administration may be an easy and effective treatment to improve long-term neurological recovery.

AMPK served as an energy sensing metabolic switch, which played a central role in the regulation of glucose and lipid metabolism in mammalian cells[22, 23]. Several studies supported the anti-inflammatory actions of AMPK. Sag et al. observed inhibition of AMPK-α expression by RNA interference in LPS-stimulated macrophages significantly increased the mRNA levels of TNF-α, IL-6, and COX-2[24]. Nath et al. showed that Ampk-α1$^{-/-}$ mice exacerbated EAE disease severity, and spleen monocytes isolated from Ampk-α1$^{-/-}$ mice secreted more IFN-γ than those from wild-type mice [25]. Therefore, AMPK loss favors a pro-inflammatory state. And enhanced AMPK phosphorylation by metformin had been showed protective effects in CNS diseases such as Experimental Stoke, Intracerebral Hemorrhage, Traumatic Brain Injury et al[7–9].

As the major cellular component of the innate immune system and the first line of defense in CNS, whenever injury or disease occurs, microglia plays essential roles in restoration of the normal brain microenvironment by eradicating cellular and molecular debris [26–30]. But if overactivated, the microglia would produce high levels of pro-inflammatory and cytotoxic mediators that hinder CNS repair and contribute to neuronal dysfunction[31]. These activated microglia are divided into two major activated subsets: classically activated (M1) and alternatively activated (M2) macrophages [32]. The M1 phenotype favors production and release of pro-inflammatory cytokines that can exacerbate neural injury, while the M2 is associated with release of neurotrophic factors that promote tissue repair [33–35]. Microglia could shift from one phenotype to another phenotype in some specific microenvironment, and M2-polarized microglia could alleviate the progression of TBI disease [36–38]. In present study, we used *in vivo and in vitro* approaches to demonstrated that metformin significantly reduced the expression of M1-associated markers and increased the expression of M2-associated markers. However, when AMPK inhibitor Compound C given together with metformin, the effect of metformin on M2 polarization was abolished. These data suggested that metformin effectively promoted microglial M1 to M2 polarization by activating AMPK.

NLRP3 inflammasome, as the most extensively studied inflammasome mainly localized in immune cells, is composed of NLRP3, oligomers apoptosis-associated speck-like (ASC) adapter protein and caspase-1. When NLRP3 is activated, the inflammasome oligomerizes and recruits ASC protein, whose domain subsequently transformed the caspase 1 into active cleaved-caspase-1. The cleaved-caspase-1 afterwards facilitates the maturation and secretion of proinflammatory IL-1β and IL-18, as main effectors having an important role in inflammation after TBI [39, 40]. Our previous study showed that NLRP3 was primarily observed in microglia[12]. An *in vitro* microglia experiments showed NLRP3 protein expression in macrophages was upregulated by LPS stimulation in M1 macrophages but not in M2 phenotype [41], and inhibition of the NLRP3 inflammasome converts LPS-stimulated M1 macrophages toward an M2 phenotype [13]. Moreover, NLRP3 inflammasome-associated components were increased in brains after injury and played a critical role in post-traumatic neuroinflammation in experimental fluid percussion injury (FPI), CCI, cold, weight-dropping or blast-induced TBI models and in humans suffering from moderate/severe TBI. Blocking or inhibiting the activation of the NLRP3 inflammasome had the ability to attenuate tissue damage induced by TBI[12, 42–47]. Therefore, suppressing the NLRP3 inflammasome

activities not only may promote the microglia to an M2 phenotype, but also could reduce the neuroinflammation and improve the recovery. It had been reported that upregulation of phosphorylated AMPK inhibited the activation of NLRP3 inflammasome in in cerebral or myocardial I/R injury [10, 11]. In this study, we used *in vivo* and *in vitro* approaches to showed that the increased phosphorylation of AMPK caused by metformin could inhibited the expression of NLRP3 inflammasome-associated components after TBI.

Then, in the CNS, peripheral leukocytes are rarely found in the brain parenchyma due to the existence of blood-brain barrier (BBB) [48, 49]. Following TBI, there was a substantial increase of infiltrating leukocytes, and the counts were positively correlated with the severity of brain injury [50]. Among the leukocytes, neutrophils are regarded as the first ones to infiltrate into the tissue[51, 52]. Infiltrated neutrophils could release many molecules associated with neuroinflammation, such as IL-1β, TNF-α, ROS, and MMP-9 et al[53–55]. Depletion of neutrophils or inhibition of neutrophils recruitment exerted some protective effects on brain injury[56–59]. In this study, treatment with metformin significantly reduced the infiltration of neutrophils following TBI.

As a key component of the innate immune system, NK cells also play an important role in the pathological process following brain injury. In mice that subjected to MCAO, NK cells in the periphery decreased rapidly after brain ischemia and then returned to basal numbers, while plenty of NK cells appeared in the brain during early stages of ischemia, then decreased afterward, suggesting NK cells infiltrated across the blood-brain barrier after ischemia[60]. When subjected to MCAO, $Rag2^{-/-}\gamma c^{-/-}$ (lacking T, NKT, B, and NK cells) mice had smaller infarct areas and less neurological deficits than $Rag2^{-/-}$ (lacking T, NKT, and B cells) mice, suggesting that NK cells might favor cerebral infarction independently of T, NKT, and B cells[61]. Furthermore, Kong et al. also showed TBI induced a reduction in the number of NK cells in peripheral blood of TBI patients, and the magnitude of the reduction appears to parallel the severity of TBI[62]. In this study, reduced infiltration of NK cells was observed in the brain of TBI mice that receiving metformin treatment versus vehicle controls.

Additionally, lymphocytes were involved or recruited in the injury cortex within the first 24h after MCAO or TBI. Hurn et al. observed a reduction in histologic injury and peripheral immune activation after MCAO in SCID mice (lacking T and B cells) versus wild-type mice, indicating that lymphocytes are involved in the evolution of brain ischemia/reperfusion [63]. Several studies stated that T lymphocytes are increasingly recognized as key modulators of detrimental inflammatory cascades in acute ischaemic stroke[64, 65]. And transgenic mice or antibody-mediated depletion of T cells could lead to smaller infarcts and better functional outcome[66–68]. There are still many studies about the detrimental role of non-regulatory T cells on acute ischemic stroke, while conflicting results about the role of B cells were showed in different studies. For instance, Doyle et al. reported that genetic deficiency and pharmacologic ablation of B-lymphocytes prevents the appearance of delayed cognitive deficits in mice suffering stroke[69]. Then, a report by Ruxandra et al. showed intraparenchymal application of mature B lymphocytes improved structural and functional outcome after TBI[70]. However, Yilmaz et al. induced MCAO in mice deficient in lymphocytes ($Rag1^{-/-}$), $CD4^+$ T cells, $CD8^+$ T cells, B cells or IFN-γ, and demonstrated that $CD4^+$ and $CD8^+$

T lymphocytes, but not B lymphocytes, contribute to the inflammatory responses and neurological deficit[71]. Schuhmann et al. also showed that pharmacologic depletion of B cells and reconstitution of $Rag1^{-/-}$ mice with B cells in experimental stroke during the acute phase did not influence lesion volume and functional outcome[72]. Therefore, exact role of B lymphocytes on the evolution after TBI requires further study in future experiments. In this study, we showed that treatment with metformin significantly reduced the infiltration of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells, but not B cells after TBI.

In conclusion, we showed that metformin administration significantly reduced the brain edema, leucocytes invasion and neurological deficits following TBI. Then, using *in vivo* and *in vitro* approaches, we further demonstrated that post-TBI metformin treatment inhibited the NLRP3 inflammasome activation dependent on the activation of AMPK, thus shifting microglia/macrophage polarization toward anti-inflammatory M2-phenotype.

Abbreviations

AMPK: adenosine 5'-monophosphate-activated protein kinase, Arg-1: arginase-1, BBB: blood-brain barrier, BSA: Bovine Serum Albumin, CD: cluster of differentiation, CNS: central nervous system, COX: cyclooxyganese, Cpd C: Compound C, DAPI: 4',6-diamidino-2-phenyl-indole, DMEM: dulbecco's modified Eagle's medium, eCCI: electromagnetic controlled cortical impact, FACS: Fluorescence Activated Cell Sorter, FITC: fluorescein isothiocyanate, Iba-1: ionized calcium-binding adapter molecule-1, IFN: interferon, iNOS: inducible nitric oxide synthase, IL: interleukin, I/R: ischemia-reperfusion, LPS: Lipopolysaccharide, MCAO: middle cerebral artery occlusion, NF-κB: Nuclear factor-kappa B, NK: Natural Killer, NLRP3: NLR pyrin domain containing 3, PBS: Phosphate Buffer Saline, PI: Prodium Iodide, RT: room temperature, SD: standard deviation, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide, TBI: Traumatic brain injury, TLR4: toll-like receptor 4, TNF: tumor necrosis factor.

Declarations

Author Contributions

WG: Contributed to the design and analysis of the study and wrote the manuscript. WJ and XX: performed the TBI model and flow cytometry. SZ and DY: performed histological examination and western blot. FL and ZZ: Performed the cell culture and rt-PCR. JZ: Evaluate the neurological deficits and brain edema. All authors approved the final version of the 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 care and experimental procedures were approved by Nankai University Animal Ethics Committee. The participants working with the animal model received training abiding by the rules of Institutional Animal Care and Use Committee Guidebook (IACUC).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

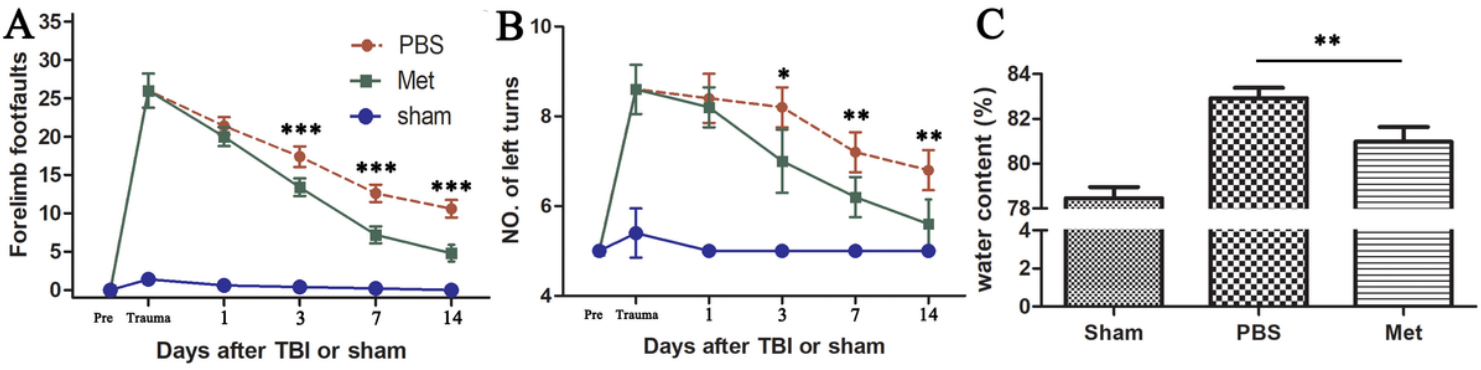

Figure 1

Effects of metformin on neurological function recovery and brain edema. (A-B) Neurological function recovery was evaluated by forelimb footfaults and corner turn test. (C) Brain edema was evaluated by

brain water content of the ipsilateral hemisphere using wet-dry weighting method at day 3 post-TBI. Data were presented as means ± SD, *p < 0.05, **p < 0.01, and ***p < 0.001 according to ANOVA.

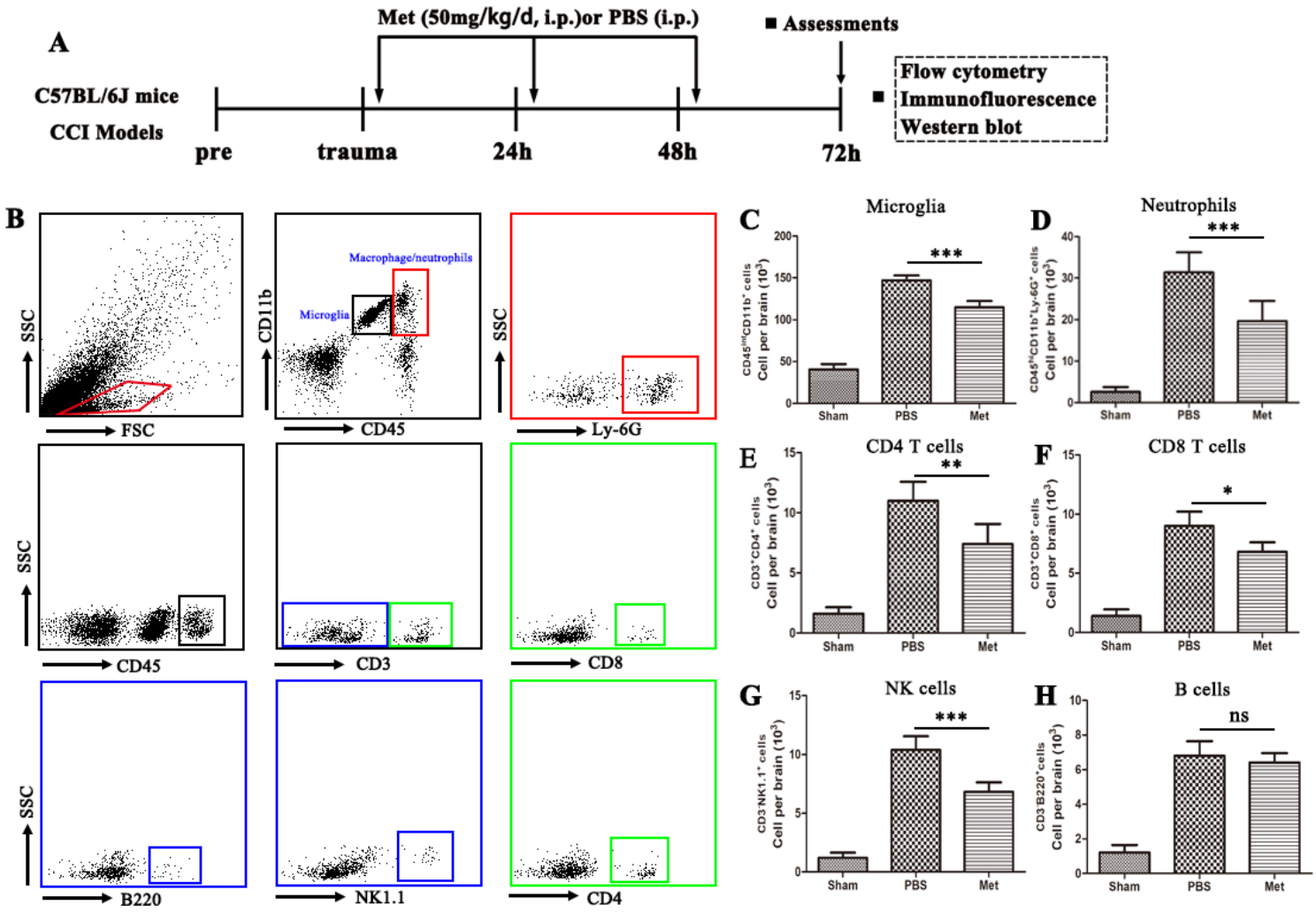

Figure 2

Effects of metformin treatment on the immune cell populations isolated from the brain tissues at day 3 post-TBI. (A-G) Representative flow cytometry plots and quantitative analysis of microglia (CD45intCD11b+), neutrophils (CD45hiCD11b+Ly-6G+), NK cells (CD3-NK1.1+), B cells (CD3-B220+), CD4+T cells (CD3+CD4+) and CD8+T cells (CD3+CD8+). Data were presented as means ± SD, *p < 0.05, **p < 0.01, and ***p < 0.001 according to ANOVA.

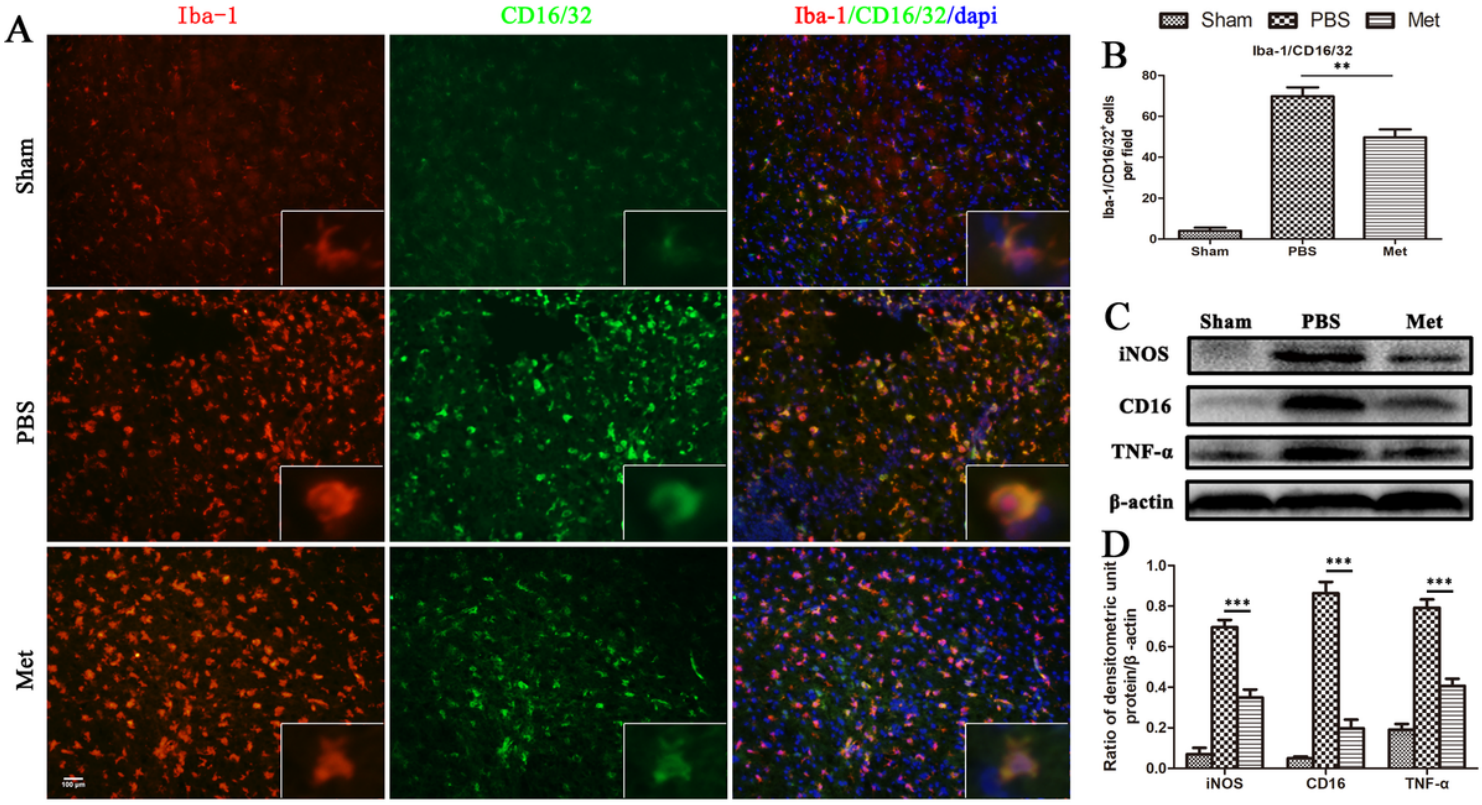

Figure 3

Effects of metformin treatment on the expression of M1-phenotype associated makers at day 3 after TBI. (A-B) Representative picture of immunofluorescence and quantitative analysis showing co-localization of CD16/32 (green) and Iba-1 (red) in the contusion area to determine M1 microglia. (C-D) Representative western blotting bands and densitometric quantifications of M1-phenotype makers (Arg-1 and IL-10) at day 3 after TBI. Data are presented as the mean ± SD, Scale bar: 100 μm. **p < 0.01 and ***p < 0.01 according to ANOVA.

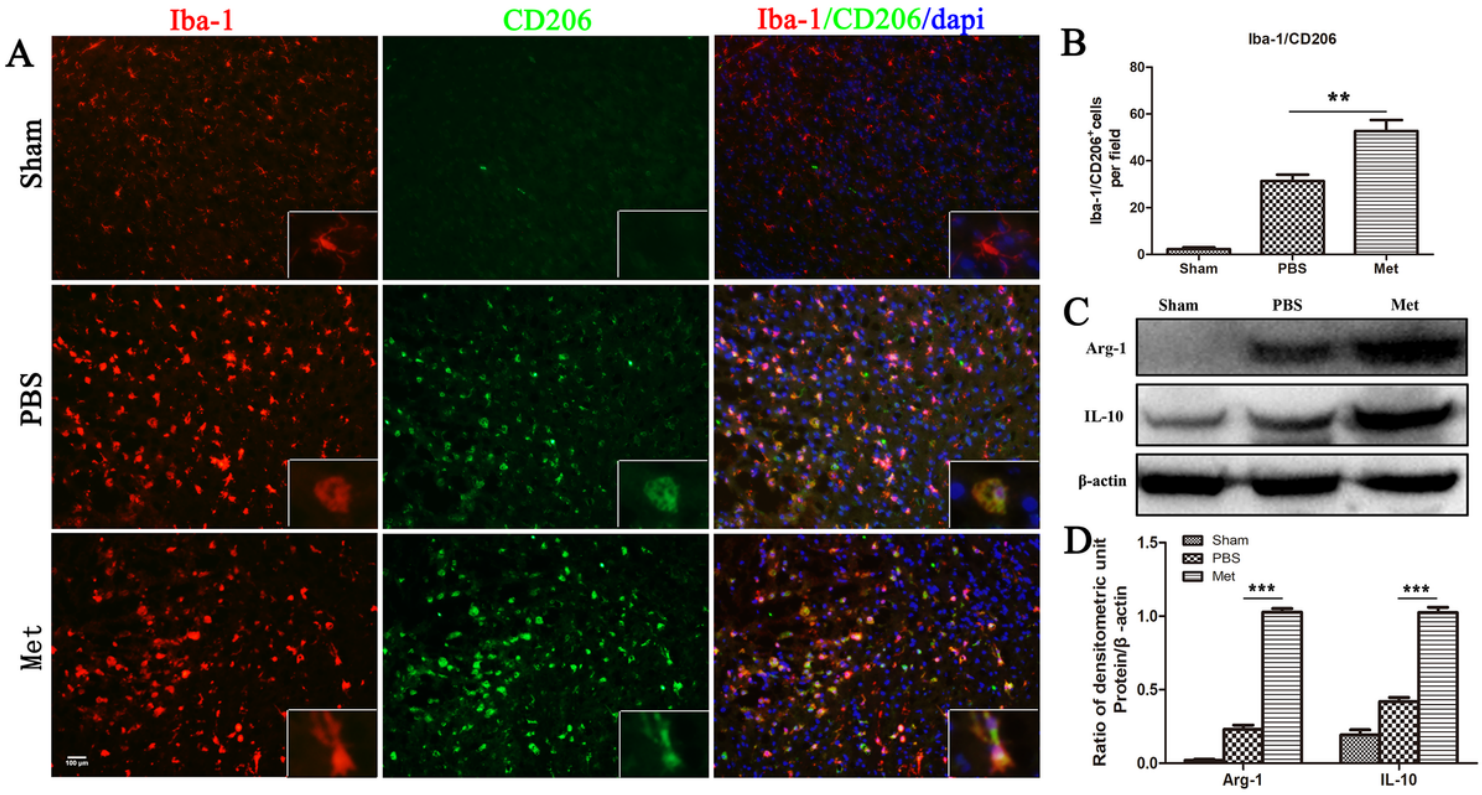

Figure 4

Effects of metformin treatment on the expression of M2-phenotype associated makers at day 3 after TBI. (A-B) Representative picture of immunofluorescence and quantitative analysis showing co-localization of CD206 (green) and Iba-1 (red) in the contusion area to determine M2 microglia. (C-D) Representative western blotting bands and densitometric quantifications of M2-phenotype makers (Arg-1 and IL-10) at day 3 after TBI. Data are presented as the mean ± SD, **p < 0.01 and ***p < 0.01 according to ANOVA.

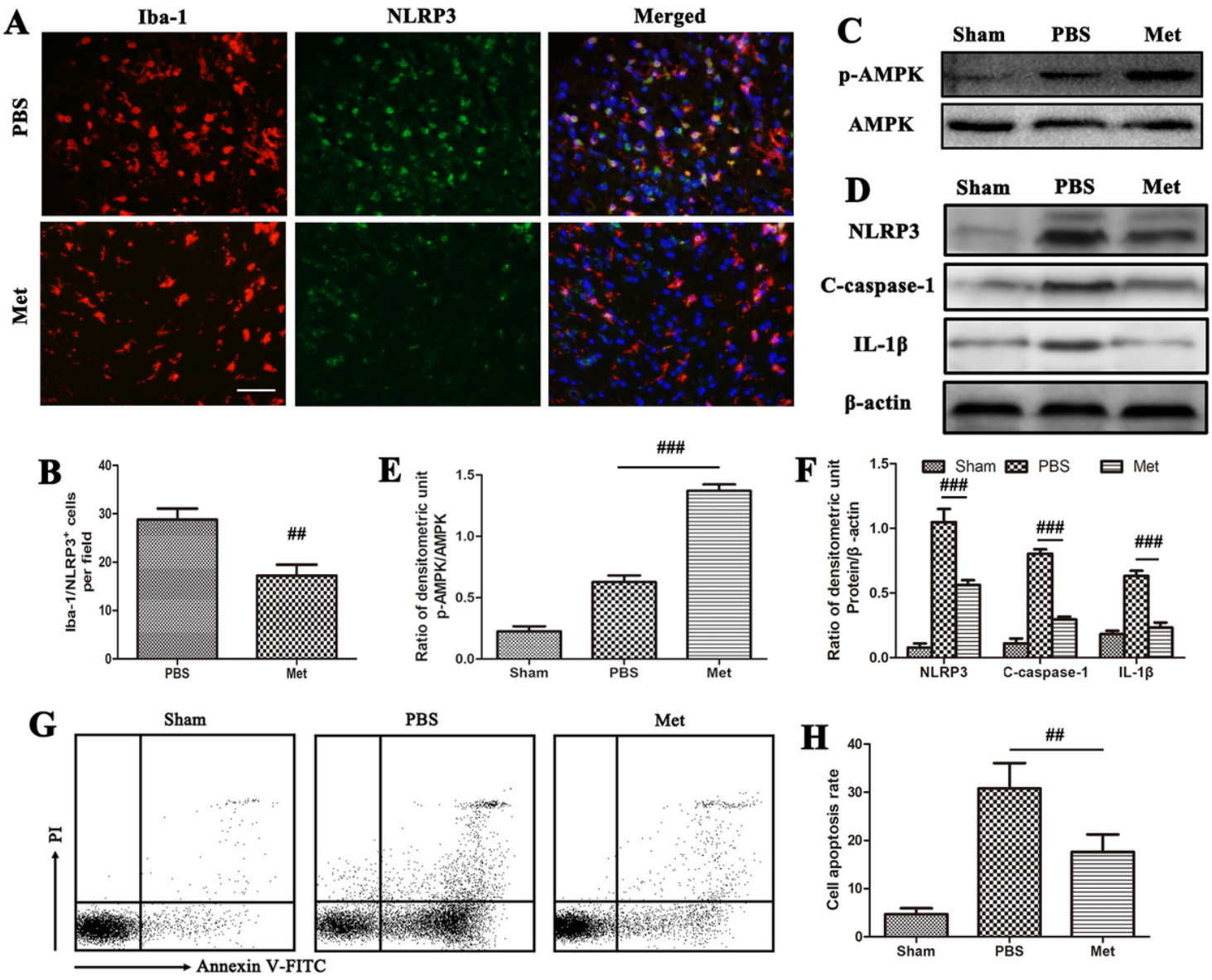

Figure 5

Effects of metformin administration on NLRP3 inflammasome signaling activation and cell apoptosis in the brain after TBI. (A-B) Representative images and statistical analysis of Iba-1/NLRP3 double-labeled cells in the contusion area of PBS-, and metformin-treated mice at day 3 after TBI. (C-F) Representative western blotting bands and densitometric quantifications of p-AMPK/AMPK, NLRP3, c-caspase-1 and IL-1β expression the ipsilateral cortices of sham-, PBS-, and metformin-treated groups. (G-H) Representative flow cytometry plots and quantitative analysis of cell apoptosis in the sham-, PBS-, and metformin-treated groups at day 3 after TBI. Data are presented as the mean ± SD, Scale bar: 100 μm. ##p < 0.01 and ###p < 0.001 according to ANOVA.

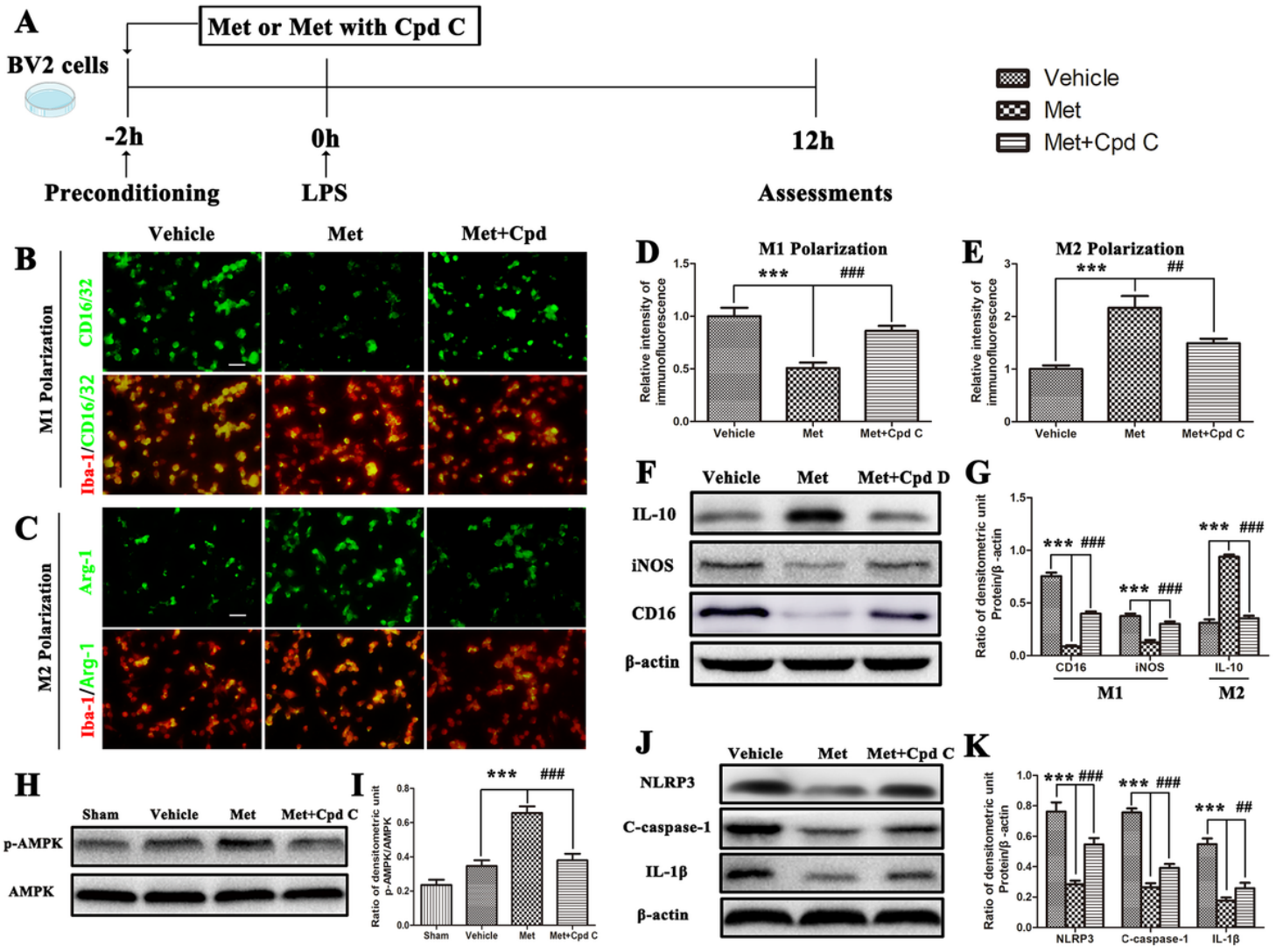

Figure 6

Effects of metformin on BV2 microglial polarization and NLRP3 inflammasome signaling activation in LPS stimulated BV2 cells. (A) Schematic diagram of the experimental design. (B-E) Representative images of double immunostaining and quantitative analysis of BV2 microglial polarization. M1-phenotype: Iba-1+ (red) and CD16/32+ (green), M2-phenotype: Iba-1+ (red) and Arg-1+ (green). Scale bar = 100μm. (F-G) Representative western blotting bands and densitometric quantifications of M1-phenotype (CD16 and iNOS) or M2-phenotype (IL-10) related molecules in Vehicle-, Met- and Met+Cpd C groups. (H-I) Representative immunoblots and densitometric quantifications are shown for phosphorylation of AMPK in different groups. (J-K) Representative immunoblots and densitometric quantifications are shown for NLRP3, c-caspase-1 and IL-1β expression in different groups. Data were presented as means ± SD, *p < 0.05, **p < 0.01, and ***p < 0.001, #p < 0.05, ##p < 0.01, and ###p < 0.001 according to ANOVA.

Supplementary Files

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