Resolution of Inflammation is Disturbed in Acute Ischemic Stroke With Diabetes Mellitus and Rescued By Resolvin D2 Treatment

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Research

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Abstract

Background: Inflammation plays an important role in diabetes mellitus (DM)-related acute ischemic stroke (AIS). The mechanisms of un-resolved inflammation in DM-related AIS are not fully understood. Specialized pro-resolving mediators (SPMs) are key regulators that promote resolution of inflammation. We aimed to examine resolution function in patients with AIS complicated with DM, and explore potential treatment effects of one of the SPMs, resolvin D2 (RvD2) ex vivo and in vivo.

Methods: Cultured human macrophages, which were derived from peripheral blood mononuclear cells of AIS and none-AIS patients with or without DM, were stimulated with oxidized-low density lipoprotein (ox-LDL). Levels of SPMs and inflammatory markers were analyzed and RvD2 treatment effects were evaluated in these cells. For experiments in vivo, challenges with high fat diet and low-dose streptozotocin (STZ) were used to induce DM in C57BL/6J mice. AIS model was established by permanent middle cerebral artery occlusion (pMCAO) followed by intra-cerebroventricular injection of RvD2.

Results: Compared with macrophages of AIS patients without DM, the ratios of SPMs to leukotriene B4 (LTB_4) were decreased in AIS patients with DM, accompanied by reduced expression of SPM synthesis enzyme, 15-lipoxygenase-1. Moreover, the levels of pro-inflammatory pathway markers were increased and the macrophages were skewed to M1 polarization in AIS patients with DM. In mice, treatment with RvD2 ameliorated pMCAO-induced brain injury, neurological dysfunction, and inflammatory response. Furthermore, RvD2 rescued resolution of inflammation by promoting macrophage /microglia polarization to pro-resolving M2 phenotype ex vivo and in vivo.

Conclusions: Our data demonstrate resolution of inflammation is impaired by DM in AIS patients, implicating a novel mechanism of un-resolved inflammation in DM-related AIS. Furthermore, RvD2 promotes inflammation resolution in macrophages/microglia and protects DM-related AIS, and may thus serve as a novel therapeutic target.

Introduction

Patients with diabetes mellitus (DM) have a high prevalence of acute ischemic stroke (AIS), and are more vulnerable to AIS insults, resulting in increased risks of morbidity and mortality [1, 2]. Inflammation has been shown to play crucial roles in DM pathophysiology and its complications, including AIS [3]. In AIS animal models, it has been shown that DM can trigger microglia/macrophage activation, increase levels of pro-inflammatory mediators, and activate classical pro-inflammatory signals, such as mitogen-activated protein kinase (MAPK) and nuclear factor κ-B (NFκB) pathways [4, 5].These DM-triggered pro-inflammatory activities further induce neuronal cell death, exacerbate the disruption of the blood-brain-barrier, and result in a worse outcome [4, 5]. Thus, to better understand the mechanisms of excessive inflammation in DM-associated AIS is favourable to develop novel therapeutic methods.

Advances from the studies of resolution of inflammation have discovered a mechanism of programmed pro-resolving actions. Compared with the traditional view that resolution of inflammation is passively induced after the clearance of inflammatory stimuli, there is evidence that inflammation resolution is an active and programmed process, mediated by so called specialized pro-resolving lipid mediators (SPMs) [6]. These SPMs include arachidonic acid (AA)-derived lipoxins (LXs), docosatetraenoic acid (DHA)-derived resolvins (RvDs), protectins (PDs) and maresins (MaRs), and eicosapentaenoic acid (EPA)-derived resolvins (RvEs) [6]. Upon the challenge of acute inflammation, lipoxygenases (LOXs) are activated and promote the biosynthesis of SPMs from their precursors [7–9]. Through binding to their receptors, SPMs induce pro-resolving activities and counteract pro-inflammatory processes, thus playing critical roles in promoting homeostasis of inflamed tissues.

Dysfunction of inflammation resolution may lead to excessive inflammation in certain diseases, including DM and AIS. Studies on obesity mouse models showed that SPM levels were reduced in fat tissue, and treatment with the SPMs like RvD2 counteracted local inflammation by regulating production of adipokines and monocyte function [10]. Moreover, RvD2 was also shown to be able to reduce adiposity and improve glucose tolerance in mice from diet-induced obesity [11], and rescue defective revascularization in diabetic mice [12]. These studies implicate a critical role of resolution and SPMs in DM-related disorders. It is not known how resolution of inflammation is altered in AIS complicated with DM, and whether treatment with SPMs may benefit DM-related AIS. In the present study, we aim to investigate the alterations of resolution of inflammation in AIS patients with DM, and test the treatment effects of RvD2 ex vivo and in vivo.

Methods

Study population and clinical data collection

Patients were enrolled from Department of Neurology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and were divided into four groups: non-diabetic patients with acute ischemic stroke (nonDM+AIS group), diabetic patients with acute ischemic stroke (DM+AIS group), diabetic patients (DM group) and control individuals with no DM nor neurological problems (Control group). AIS was confirmed by computer tomography (CT) or magnetic resonance imaging (MRI). DM patients were diagnosed according to type 2 DM criteria of American Association of Clinical Endocrinologists [13]. Exclusion criteria includes coma, severe cardiac problems (atrial fibrillation, heart failure or acute myocardial infarction), hepatic and renal dysfunction, history of endocrine diseases except DM, malignant tumor, hematological diseases, autoimmune disease, or stroke patients receiving thrombolysis or thrombectomy. Patients with acute systematic inflammatory disease within 1 month were also excluded from the study. The authors declare that the supporting data were available within the article. The study was approved by the ethical committee and got informed consent.

Clinical characteristics of participants were collected covering age, gender, medical history, smoking and drinking history. General laboratory indicators were determined by the Dept of Laboratory Medicine,

Shanghai Jiao Tong University Affiliated Sixth People's Hospital, following routine procedures, and included glycated hemoglobin (HbA1c), fasting blood glucose (FBG), triglycerides, cholesterol, low-density lipoprotein (LDL), counts of total white blood cells, neutrophils and monocytes. The study was approved by the ethical committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and written consent was obtained from all participants or their authorized relatives.

Human macrophage culture and stimulation

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll separation as previously described [14]. Briefly, about 10ml periphery venous blood was collected from the enrolled patients with a fasting condition. All patients with AIS were sampled within the first 72 hours from disease onset. The samples were then diluted with phosphate-buffered saline (PBS) (Dulbecco) (1:1), added to Ficoll separation liquid (GE Healthcare), and centrifuged for 20 min at 600xg. Derived PBMC were cultured in 6-well plate with 1640 culture medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) for 2 h. The plates were then rinsed with PBS, and the adherent cells were collected as monocytes. Finally, monocytes of $1 \subseteq 10^6$ cells/well were differentiated into mature macrophage by 7-day culture in 1640 culture medium containing 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF), 10% FBS and 1% penicillin/streptomycin. The culture medium was exchanged every 2-3 days. On day 7, the macrophages were derived and stimulated with 10 μg/ml ox-LDL (Thermo Fisher Scientific) for 24 h in FBS/M-CSF-free 1640 culture medium. For RvD2 treatment study, macrophages from DM+AIS patients were treated with 10ug/ml ox-LDL together with RvD2 (10nmol/ml) or vehicle for 24 hours. The culture medium and macrophages were collected and stored at -80℃ until further processing.

Enzyme immunoassay analysis

The levels of RvD1, RvD2, MaR1, LXA_4, and LTB_4 in the culture medium were determined by enzyme immunoassay (EIA) kits (Cayman Chemical) according to the manufacturer's instructions.

Animals

Male C57BL/6J mice were housed in condition of controlled dark cycle (a 12-h light), temperature (21±2°C), and humidity (60–70%), with free access to standard rodent diet and tap water. All animal procedures were performed according to guidelines of the Medical Experimental Animal Administrative Committee of Shanghai, and have been approved by the ethical committee on animal well-fairs of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong Unviersity. The DM model were induced in accordance with previously reported [15]. Briefly, male C57BL/6J mice, 8–12 weeks old, weighing 23–25 g, were fed with high fat diet (Research Diets, D12492, 60% fat, 20% carbohydrate and 20% protein) for 6 weeks, then injected intraperitoneally with streptozotocin (Sigma) at a dose of 50 mg/kg for five consecutive days. After 2 weeks, tail vein blood sampling were collected and blood glucose levels were measured. Establishment of DM model was confirmed as blood glucose levels >250 mg/dl.

Permanent MCAO Model

NonDM mice and DM mice underwent permanent middle cerebral artery occlusion (pMCAO) surgery as described previously [16]. NonDM and DM mice were grouped to following three groups respectively: (1) sham group; (2) pMCAO group: mice underwent pMCAO procedure and injected with PBS; (3) RvD2-treated group: mice underwent pMCAO procedure and treated with RvD2 (Cayman Chemical Company, USA, CAS. No. 10007279). Briefly, mice were anesthesized with 1% pentobarbital sodium (10 mg/kg) and midline incision was made on the neck. A monofilament nylon filament (with a diameter of 0.16 mm, 25mm in length, Beijing Cinontech Biotech Co. Ltd) coated with silicon hardener mixture (a diameter of 0.20±0.01 mm) was inserted from a small incision of the left external carotid artery and gently entered into internal carotid artery to occlude middle cerebral artery, maintaining body temperature at 37°C throughout the procedure. The establishment of occlusion was confirmed as MCA cortical blood flow reducing to 20% of the baseline monitored by a laser Doppler flowmetry (Moor Instruments, Devon, England). Sham group underwent the same procedure without inserting the monofilament nylon filament. In the RvD2-treated group, animals were treated with a single injection of 1nmol RvD2 into the left lateral ventricle (AP=-0.2mm, ML=-1mm, DV=-2.5mm) immediately before pMCAO surgery. The control were stereotaxically injected with equivalent PBS as a vehicle.

Neurobehavioral Tests

Behavioural assessment by modified neurological severity score (mNSS) and Grip test were performed 3 days before treatment for training and at 24h after pMCAO.

Each mouse was calculated by averaging scores of three independent evaluations. The mNSS system consists of test scores including motor skills (flexion of limbs and walking on the floor), balance (beam walking), and reflex (pinna and cornea reflexion). Scores range from 1 to 14 (normal: 0, severe deficit: 14). Grip test was carried out with a grip-force measurement system. Briefly, the tail was gently and steadily pulled to measure the maximum force. When the mouse released the wire mesh screen, the grip strength meter software collected the peak grip force [17].

Immunohistochemistry and Immunofluorescence

The brain infarct volume were evaluated by cresyl violet staining. A series of 20μm-thick coronal sections with an interval of 200μm between each section were cut and mounted on slides. Following fixation, sections were stained using 1% cresyl violet (Sigma) for 30 min at room temperature, and then processed by a microscope (Olympus) and assessed by Image J software. Infarct volume was calculated by subtracting the volume of intact area in the ipsilateral hemisphere from the whole volume of the contralateral hemisphere. For immunofluorescent staining of microglia and neuronal markers, the slides were blocked with 10% bovine serum albumin (BSA), and incubated with primary antibodies at the following dilutions: Iba-1(goat, 1:200, Abcam) and Neuron (rabbit, 1:500, Abcam). Nuclei staining with DAPI was applied for counterstaining. The immunofluorescent staining was visualized with a fluorescence microscope (Olympus IX53) and analyzed by Image J.

Quantibody Cytokine Array

The cytokine levels in the brain tissue of nonDM and DM mice were measured quantitatively using the Quantibody Mouse Cytokine Array 1 (RayBiotech, QAM-CYT-1) according to the manufacturer's instructions. Data extraction was done using GenePix Pro 5.1 software. Cytokine levels were normalized to milligram of tissue.

Western blot analysis

Macrophages or brain tissue of the ipsilateral hemispheres of pMCAO were extracted with RIPA buffer supplemented with inhibitors for protease and phosphatase. Protein concentration was quantified using a BCA kit (Thermo Scientific). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% BSA and incubated overnight with primary antibodies against 5-LOX 1:200 (Cayman Chemical); 15-LOX 1:1000 and CD206 1:1000 (both from Abcam); inducible nitric oxide synthase (iNOS) 1:1000 (ProteinTech); p38 1:1000; phosphorylated p38 (p-p38) 1:1000; p65 1:1000 and phosphorylated p65 (p-p65) 1:1000 (all from Cell Signaling Technology) at 4°C overnight. The membranes were washed and then incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) secondary antibodies for 1 h at room temperature. Finally, the blots were detected by enhanced chemiluminescence (ECL). Digital images were processed by a CCD camera (Bio-rad) and quantified using Image J software.

Statistical analysis

All statistical analyses were performed using the IBM SPSS software (version 22, IBM Company). Differences between groups were analysed by Mann-Whitney U test, or analysis of variance (ANOVA) followed by Tukey's post hoc test. Categorical variables were analysed by $\chi 2$ test. $P < 0.05$ was considered as statistically significant in all analyses.

Results

Clinical characteristics

Patients enrolled in this study included 13 control individuals (Control group), 14 patients in the DM group, 16 patients in nonDM+AIS group and 14 patients in the DM+AIS group. Clinical characteristics are shown in Table 1. The rates of smoking and drinking were higher in the nonDM+AIS group than in the Control group. There was no significant difference in age, history of hypertension and coronary heart disease between the four groups. The NIHSS score between nonDM+AIS and DM+AIS was not significantly different. The plasma concentrations of HbA1c and FBG were higher in the diabetic group than in the non-diabetic group (Table 1). There was no significant difference between the four groups in the laboratory test results for LDL, triglycerides, cholesterol, white blood cells count, neutrophils and monocytes (Table 1).

Table 1
Characteristics of the patient groups as stratified by diabetes and acute ischemic stroke.

	Control (n = 13)	DM (n = 14)	nonDM+AIS (n = 16)	DM+AIS (n = 14)	P value
Age	65 ± 6	63 ± 8	68 ± 0	67 ± 6	0.33
Gender (male/female)	4/9	9/5	11/5‡	12/2§	0.03
Hypertension (%)	8(61.5)	11(78.6)	14(87.5)	12(85.7)	0.33
NIHSS	/	/	3±3	3±3	0.65
HbA1c	6.01 ± 0.45	7.98 ± 1.30†	5.85 ± 0.48	8.43 ± 1.79§	<0.01
FBG (mmol/L)	4.84 ± 0.42	7.57 ± 2.61†	5.22 ± 0.78	8.09 ± 2.13§	<0.01
TG (mmol/L)	1.72 ± 0.76	2.24 ± 1.82	1.61 ± 1.12	1.47 ± 0.54	0.34
TC (mmol/L)	4.66 ± 0.79	4.58 ± 1.42	4.77 ± 0.80	4.77 ± 0.94	0.82
LDL (mmol/L)	2.56 ± 0.67	2.64 ± 0.88	2.80 ± 0.68	3.09 ± 0.71	0.25
WBC $\times 10^9$/L	6.45 ± 1.21	6.64 ± 1.43	7.63 ± 2.82	7.21 ± 1.80	0.36
Neutrophils $\times 10^9$/L	4.00 ± 1.06	4.08 ± 1.14	4.99 ± 2.26	4.93 ± 1.80	0.24
Monocytes $\times 10^9$/L	0.48 ± 0.18	0.46 ± 0.18	0.57 ± 0.21	0.49 ± 0.15	0.40

Data are presented as mean ± SD; P value shows comparison by ANOVA analysis among the four groups. †p < 0.05 Control *vs.* DM; ‡p < 0.05 AIS *vs.* DM;§p < 0.05 AIS *vs.* DM+AIS. AIS, acute ischemic stroke; DM, diabetes mellitus; FBG, fasting blood glucose; LDL, low-density lipoprotein; NIHSS, National Institute of Health Stroke Score; TC, total cholesterol; TG, triglyceride; WBC, white blood cell.

DM impaired resolution function in macrophages of AIS patients

We explored changes in lipid mediators (LMs) produced by macrophages stimulated with ox-LDL. The ratio between SPMs and LTB_4 has been widely used as a marker reflecting the balance of resolution and inflammation [18–20]. We calculated the ratios between each SPM and LTB_4 to evaluate the function of resolution in macrophages from the different patient groups. The ratios of RvD1/LTB_4, RvD2/LTB_4 and MaR1/LTB_4 were significantly lower in AIS compared to nonAIS groups (Fig. 1A). The LXA_4/LTB_4 ratio in AIS groups was not statistically different compared to nonAIS groups, although there was a trend of decrease (Fig. 1A). We further explored the influence of DM on resolution function of macrophages stimulated with ox-LDL. Interestingly, the results revealed that the ratios of RvD1/LTB_4, RvD2/LTB_4 and MaR1/LTB_4 were lower in DM+AIS group than that in nonDM+AIS group (Fig. 1A). There was no significant difference between DM and the Control group (Fig. 1A). On the other hand, there was a

significant decrease of SPMs/LTB_4 ratio when comparing DM+AIS group with DM group, but there was no difference when comparing nonDM+AIS group with control individuals (Fig. 1A).

To further explore the dysfunction of inflammation resolution, we assessed the levels of key SPM synthases (including 5-LOX and 15-LOX-1) by Western blot. Consistent with the levels of SPM, the levels of 15-LOX-1 were lower in DM+AIS group than nonDM+AIS group, but there was no significant difference between control and DM group (Fig. 1B). There was no significant difference in the levels of 5-LOX among the four patient groups (Fig. 1B).

DM exacerbated inflammation in macrophages of AIS patients

Next, we analysed the inflammatory markers in these macrophages. There was no significant difference in the levels of the pro-inflammatory M1 marker iNOS between DM+AIS and nonDM+AIS groups, while the levels of M2 marker CD206 was lower in DM+AIS group compared to nonDM+AIS group (Fig. 2A). To further evaluate the M2/M1 polarization, we calculated the ratio of CD206/iNOS, which was lower in DM+AIS patients than that in nonDM+AIS patients (Fig. 2A). There was no difference between the DM and Control group with regards to M1 and M2 markers (Fig. 2A). The analysis of representative MAPK pathway markers p38 and NFκB pathway markers p65 exhibited similar results. There was no difference in the total levels of p38 and p65 between the four groups (Fig. 2B). However, the levels of their phosphorylated forms (p-p38 and p-p65) and the ratios to their total levels (p-p38/p38 and p-p65/p65) were higher in DM+AIS group than that in nonDM+AIS group (Fig. 2B). There was no difference between Control and DM with regard to these inflammatory markers.

RvD2 inhibited ox-LDL-induced inflammation in macrophages of AIS patients with DM

The above results demonstrated impaired resolution and excessive inflammation in macrophages of AIS patients with DM. Next, we explored the effect of RvD2 in these macrophages. Ox-LDL stimulation significantly reduced the ratio of CD206/iNOS in macrophages from DM+AIS patients, indicating a polarization towards a pro-inflammatory phenotype. Treatment with RvD2 markedly reduced this polarization as shown by an increased CD206/iNOS ratio (Fig. 3A). Moreover, the MAPK pathway markers p-p38 and ratio of p-p38/p-38, as well as NFκB pathway markers p-p65 and ratio of p-p65/p65, were all increased by ox-LDL stimulation (Fig. 3B), and treatment with RvD2 significantly downregulated these markers (Fig. 3B).

RvD2 rescued DM-exaggerated brain injury in pMCAO mice

To test the treatment effects of RvD2 in DM-related AIS in vivo, we established stroke model by pMCAO in mice with DM induced by high fat diet and STZ. The grip test results showed that, compared with nonDM+pMCAO mice, the grip test performance was significantly worse in DM+pMCAO mice. Treatment with RvD2 partially rescued the behavioural impairment (Fig. 4A). Consistent with the results of grip test,

the score of mNSS was significantly higher in DM+pMCAO mice than nonDM+pMCAO mice, and RvD2 treatment ameliorated the neurological defict as shown by decreased score of mNSS in DM+pMCAO mice (Fig. 4A).

We also evaluated RvD2 effects on pathological changes by immunohistochemistry. Triphenyltetrazolium chloride (TTC) staining showed that infarct volume was increased by DM in mice with pMCAO, while RvD2 treatment significantly decreased the infarct volume (Fig. 4B). The protective effect of RvD2 was further confirmed by immunostaining of neurons. Compared with nonDM+pMCAO mice, the number of surviving neurons in the infarct area of DM+pMCAO mice was significantly decreased (Fig. 4C). RvD2 treatment increased the density of neurons in DM+pMCAO mice, indicating a protective effect of RvD2 in DM+pMCAO mice (Fig. 4C).

RvD2 inhibited pMCAO-induced inflammation in diabetic mice

Next, we further tested whether RvD2 protect DM-related AIS via regulating inflammation. As shown by Western blot, the levels of MAPK pathway markers p-p38 and NFκB pathway markers p-p65, as well as their ratios to total protein levels (p-p38/p38 and p-p65/p65), were significantly higher in DM mice compared with nonDM mice with pMCAO. RvD2 down-regulated these pro-inflammatory pathway maker in DM pMCAO mice (Fig. 5A-B). Besides, DM increased the expression of GM-CSF, IL-2, IL-5, IL-17 and TNF-α (Fig. 6A) and decreased the expression of IL-4 and IL-10 (Fig. 6B) in mice after pMCAO. RvD2 treatment inhibited the expression of these pro-inflammatory factors, while enhanced the expression of anti-inflammatory cytokines.

Immunofluorescent study showed that there were more Iba-1-positive microglial cells in the infarct area of DM than nonDM mice after pMCAO, and RvD2 treatment decreased the number of Iba-1-positive microglial cells in DM+pMCAO mice (Fig. 7A). Moreover, analysis of microglia M2/M1 makers illustrated that ratio of CD206/iNOS was decreased in DM pMCAO mice as compared with nonDM+pMCAO mice, and RvD2 treatment significantly elevated this ratio, indicating that RvD2 promoted the switch of microglia from M1 phenotype to M2 (Fig. 7B).

Discussion

In the present study, we investigated the function of resolution in macrophages from diabetic patients with acute ischemic stroke, and found that SPMs secretion was downregulated upon stimulated with ox-LDL, thus might lead to uncontrolled inflammation. Moreover, therapeutic administration of RvD2 enhanced inflammation resolution in these macrophages ex vivo. We also identified that RvD2 could protect against cerebral ischemia injury in DM mice with pMCAO and promote inflammation resolution in vivo. These findings uncovered new knowledge about uncontrolled inflammation in DM-related AIS, and provided evidence of potential novel treatment methods targeted on SPMs.

Inflammatory responses are activated both in the brain and in peripheral circulation after AIS and contribute to stroke prognosis.[21–24] DM is an independent risk factor for AIS, and often worsens the clinical symptoms and prognosis of AIS, where unresolved inflammation plays a key role therein [25–27]. SPM-mediated resolution of inflammation is a programmed process to counter-regulate pro-inflammatory responses [28]. The disturbed balance between SPMs and pro-inflammatory forces may contribute to inflammatory diseases. To evaluate the balance of these two forces, the ratios between SPMs and LTB_4 have been applied as an index in many studies[19, 29–31]. In our study, we showed that this index was reduced in macrophages from AIS patients compared to those from nonAIS patients. Such a reduction was further ananlyzed by dividing the patients into DM and nonDM groups. We then found that DM condition contributed to such a reduction prominently, as SPMs/LTB_4 ratios were significantly decreased in DM+AIS patients compared with DM patients without AIS, but there was no difference between control group and nonDM+AIS group. This result is consistent with our recent report that RvD2/LTB_4 is reduced in the plasma of AIS patients with DM compared to those without DM[32]. Such a finding revealed that disturbed resolution may be a critical cause for excessive inflammation in DM-related pathophysiological conditions, e.g. AIS.

We further explored possible causes for reduced SPMs secretion by analyzing the synthesis enzymes of SPMs. We found that macrophages from DM+AIS patients expressed less 15-LOX-1 comared to nonDM+AIS patients and DM patients. On the other hand, there was no difference with regard to 5-LOX among the patient groups. 15-LOX-1 is the key enzyeme involved in the biosynthesis of most SPMs[33]. It has been recently reported that α-hemolysin activates 15-LOX-1 in M2 macrophages to enhance the production of SPMs, and depletion of 15-LOX-1 expression abolished the effects of α-hemolysin on SPMs synthesis[34]. On the other hand, overexpression of 15-LOX-1 ameliorated diabetic peripheral neuropathy and improved nerve recovery via increasing the production of SPMs [35]. Thus, the reduced expression of synthesis enzyme 15-LOX-1 may be one of the causes for insufficient SPM production[36, 37].

The above results of disturbed resolution of inflammation is related to the excessive inflammation in AIS patients with DM. We found that macrophages from AIS patients with DM were more M1-polarized, and the pro-inflammatory pathway markers p-p38/p38 and p-p65/p65 were also increased in these patients. These data are consistent with previous studies reporting overactivated pro-inflammatory signals in DM. It has been demonstrated that macrophages are pushed towards a pro-inflammatory M1 spectrum in DM [38], which is in line with our data about lower CD206/iNOS ratio in DM patients with AIS. Furthermore, MAPK and NFκB pathways have been considered as the main activated inflammatory signals in DM [39, 40]. Thus, the decreased relative levels of SPMs may be a key etiology of unresolved inflammation in DM-related AIS. Our data explain the abnormal inflammation from a novel perspective, resolution of inflammation.

Our findings in peripheral macrophages of DM patients with AIS may represent a state of unresolved inflammation in the brain. During AIS, peripheral macrophages invade the brain and participate in the inflammatory response in the infarct area [41, 42]. Meanwhile, microglia, which serve as the resident tissue macrophages in central nervous system, are also activated after AIS [24, 43]. The invading

macrophages and resident microglia respond to the ischemia pathophysiology, including neuronal death, blood-brain barrier disrupt and brain edema. Similar to many other situations, the inflammatory response after AIS partially help to clean out the brain debris, and prepare the brain for further recovery of homeostasis in the resolution stage. However, unresolved inflammatory response can lead to secondary injury of the inflamed tissue, and thus exaggerate the primary injury, i.e. disruption of cerebral blood flow in AIS patients with DM [44, 45]. SPMs-based therapeutic strategy thus becomes promising in DM-related AIS.

Among the SPMs analyzed in our study, RvD2 has been previously implicated in DM-associated inflammatory disease. In a study of hind limb ischemia, RvD2 treatment rescued DM-impaired revascularization through activation of its receptor GPR18 [12]. In the aorta of $Apoe^{-/-}$ mice fed a high-fat diet, the level of RvD2 were reduced and correlated to plaque stability.[29] In mice from diet-induced obesity, treatment with RvD2 inhibited inflammation and adiposity, and improved glucose tolerance [11]. Thus, we chose RvD2 as a representative SPM to test the treatment effects on DM-related AIS. Firstly, we found macrophages in AIS patients with DM were skewed to M1 polarization, as shown by decreased CD206/iNOS ratio. And the pro-inflammatory MAPK and NFκB pathways were overactivated, as shown by increased ratios of p-p38/p38 and p-p65/p65. Similar result were found in microglia cells in the brain of DM+pMCAO mice. Then, we treated the human macrophages with RvD2 ex vivo, and injected RvD2 i.c.v in DM+pMCAO mice in vivo. The results showed that intervention with RvD2 switched macrophages/microglia to M2 polarization and downregulated the MAPK and NFκB inflammatory pathway markers ex vivo and in vivo. In turn, such an effect of RvD2 on macrophages/microglia contributed to the protection against brain injury caused by DM-related AIS. In DM+pMCAO mice, RvD2 reduced infarction volume, improved neurological function and increased neuron survival by inhibiting inflammation and promote resolution. These results were consistent with recent studies about the treatment effects of RvD2 on resolution of inflammation. Giannakis N and his colleague reported that pro-resolving $Ly6C^{lo}$ macrophages could release more SPMs and less pro-inflammatory mediators like LTB_4. RvD2 treatment increased the number of macrophages expressing $Ly6C^{lo}$, and thus benefit tissue repair in muscle injury [46]. In a cardiovascular study, treatment with RvD2 shifted macrophages to a pro-resolving phenotype, and prevented the progression of atherosclerosis [47].

Conclusion

In conclusion, our data revealed that resolution of inflammation is disturbed in macrophages from AIS patients with DM, implicating a previously unreported perspective of uncontrolled inflammation in DM-related AIS. Treatment of one of the SPMs, RvD2, could rescue the disturbed resolution function both in human macrophages ex vivo and in mouse stroke model in vivo. Moreover, RvD2 treatment significantly reduced infarction volume, enhanced neuronal survival and improved neurological function in DM mice with ischemic stroke. Thus, targeting SPMs and resolution of inflammation could be a promising novel strategy in DM-related AIS.

Abbreviations

AIS, acute ischemic stroke; DM, diabetes mellitus; T2DM, type 2 diabetes mellitus; EPA, eicosapentaenoic acid; LOX, lipoxygenase; iNOS, inducible nitric-oxide synthase; LXA_4, lipoxin A4; LTB_4, leukotriene B4; MaR1, maresin 1; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; ox-LDL, oxidized low-density lipoprotein; pMCAO, permanent middle cerebral artery occlusion; RvD1, resolvin D1; RvD2, resolvin D2; SPMs, specialized pro-resolving mediators.

Declarations

Ethics approval and consent to participate

The study was approved by the ethical committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and written consent was obtained from all participants or their authorized relatives.

Consent for Publication

Consent for publication was contained from all authors.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XW and YZ conceived and designed the study. XT and LL performed the laboratory experiments, and analysed the data. ZM, LL and XC were involved in patient enrollment and data analysis. XT and XW drafted the article. BQZ, GC and MS gave critical suggestions on the study and manuscript writing. All authors approved the final version of the article.

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Figures

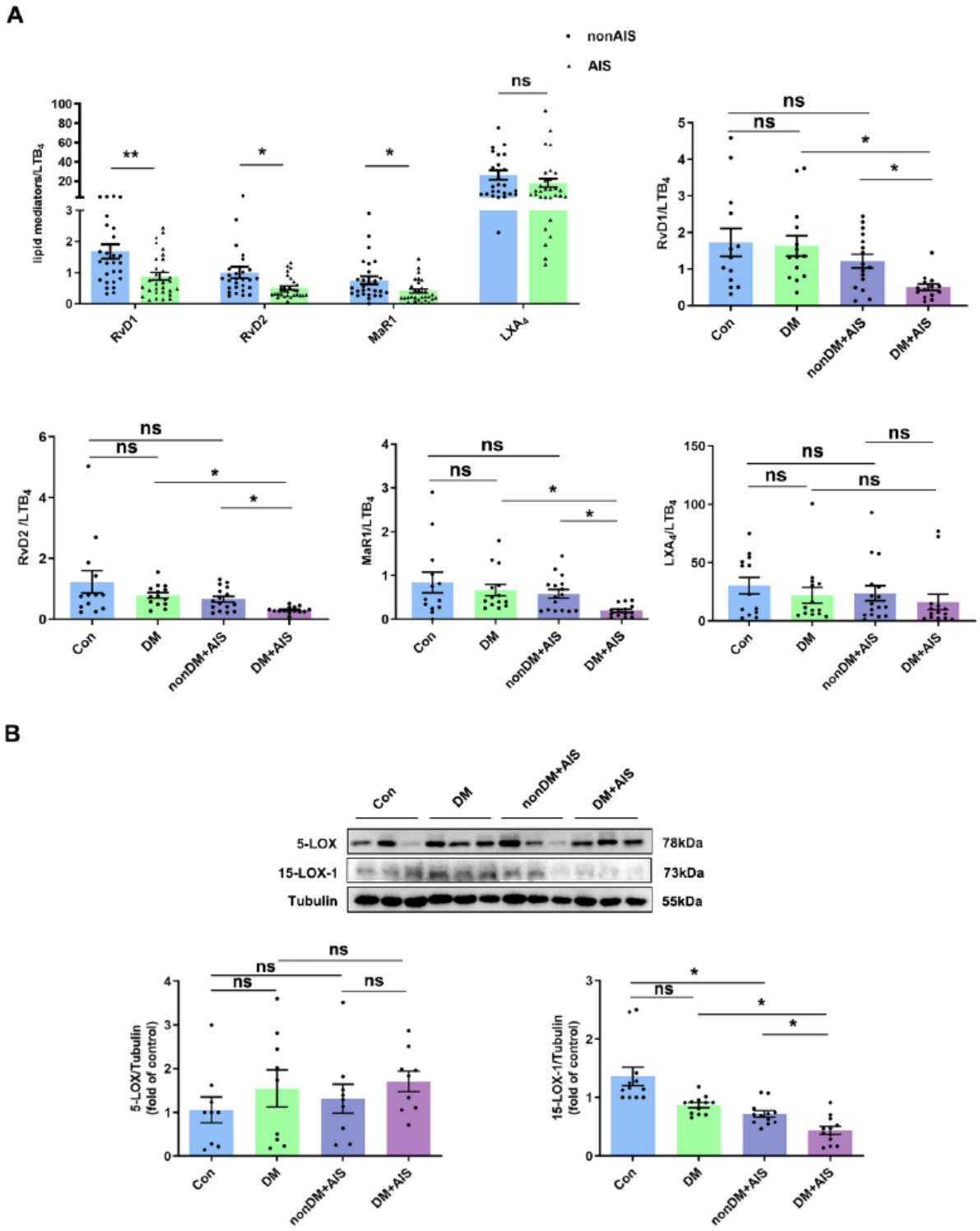

Figure 1

Levels of SPMs and their synthesis enzymes in ox-LDL-treated macrophages derived from PBMC of clinical patients. Macrophages were derived from PBMC of patients, and treated with ox-LDL for 24 hours. The cultured medium was collected for the analysis of lipid mediators by EIA, and the cells were collected for the analysis of synthesis enzymes of SPMs by WB. (A) Ratios of SPM/LTB4 were lower in AIS patients compared with that in nonAIS patients. Further analysis showed that ratios of RvD1, RvD2

and MaR1 to LTB4 were lower in DM+AIS group compared with DM and nonDM+AIS groups, while there was no difference between nonDM+AIS and Con groups. (B) The levels of 15-LOX-1 were lower in DM+AIS group compared with DM and nonDM+AIS groups, while the levels of 5-LOX were not changed among the four groups. Mann-Whitney U test was applied in the comparison between AIS and nonAIS groups, and ANOVA followed by Turkey post hoc test was applied for the comparison among the four groups. Error bars represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant. AIS, acute ischemic stroke; Con, control; DM, diabetes mellitus; EIA, enzyme immunoassay; LTB4, leukotriene B4; LXA4, lipoxin A4; MaR1, maresin 1; ox-LDL, oxidized low-density lipoprotein; PBMC, peripheral blood mononuclear cells; RvD1, resolvin D1; RvD2, resolvin D2; WB, western blotting.

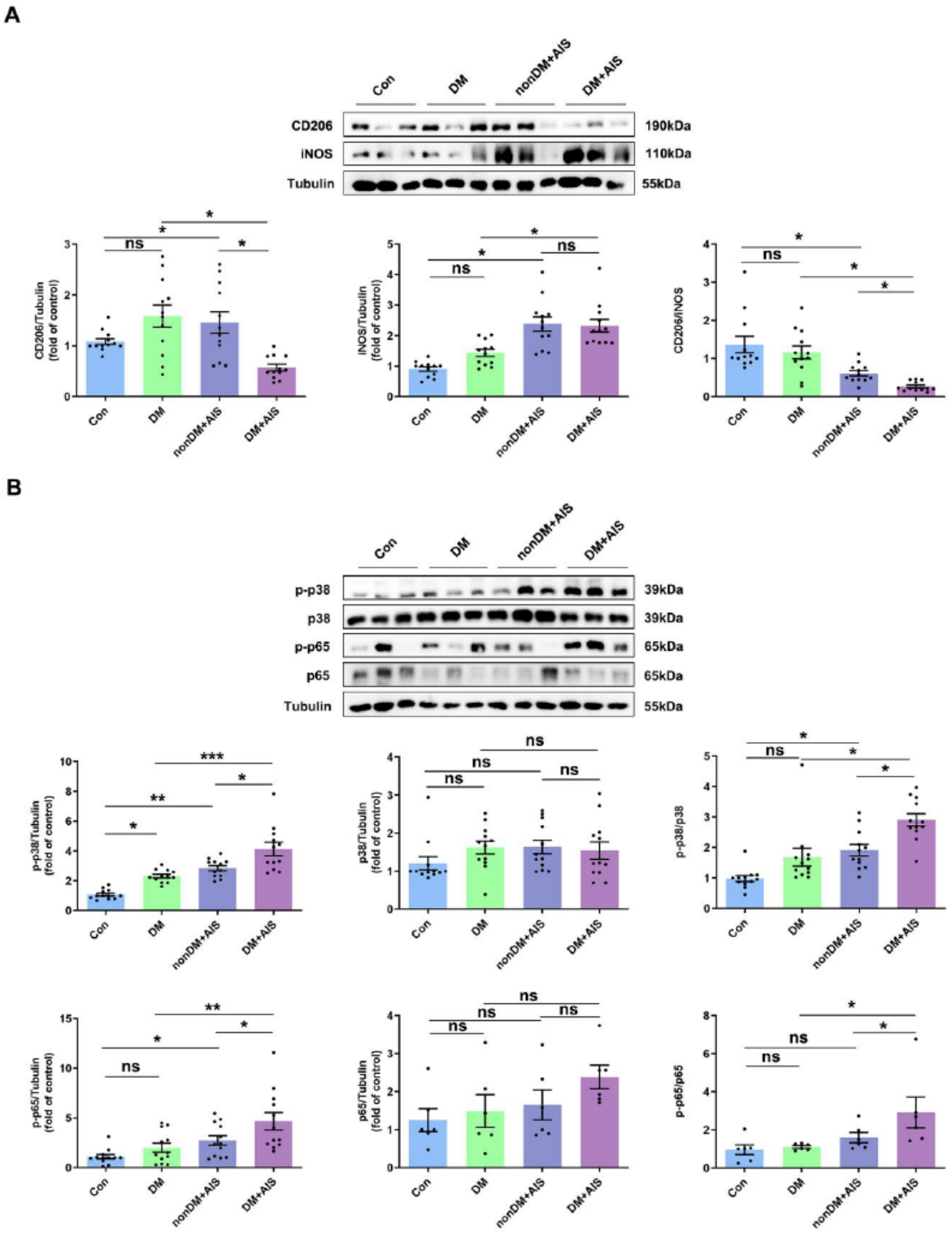

Figure 2

Changes of M1/M2 phenotype and other inflammatory pathway markers in ox-LDL-treated macrophages derived from PBMC of clinical patients. (A) Western blot analysis showed that the expression of CD206 was lower in macrophages from DM+AIS group compared with DM and nonDM+AIS groups. On the other hand, the expression of iNOS was increased when comparing DM+AIS with DM groups, as well as when comparing nonDM+AIS with Con groups. As an integrated marker of phenotype polarization, the ratio of

CD206 to iNOS was decreased in DM+AIS group compared with DM and nonDM+AIS groups. (B) Analysis of MAPK/NFκB pathway markers showed that the p-p38/p38 and p-p65/p65 ratios were higher in DM+AIS compared with DM and nonDM+AIS, while the total levels of p38 and p65 were not changes among the four groups. ANOVA followed by Turkey post hoc test was applied. Error bars represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** p□0.001. ns = not significant. AIS, acute ischemic stroke; Con, control; DM, diabetes mellitus; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; ox-LDL, oxidized low-density lipoprotein; PBMC, peripheral blood mononuclear cells.

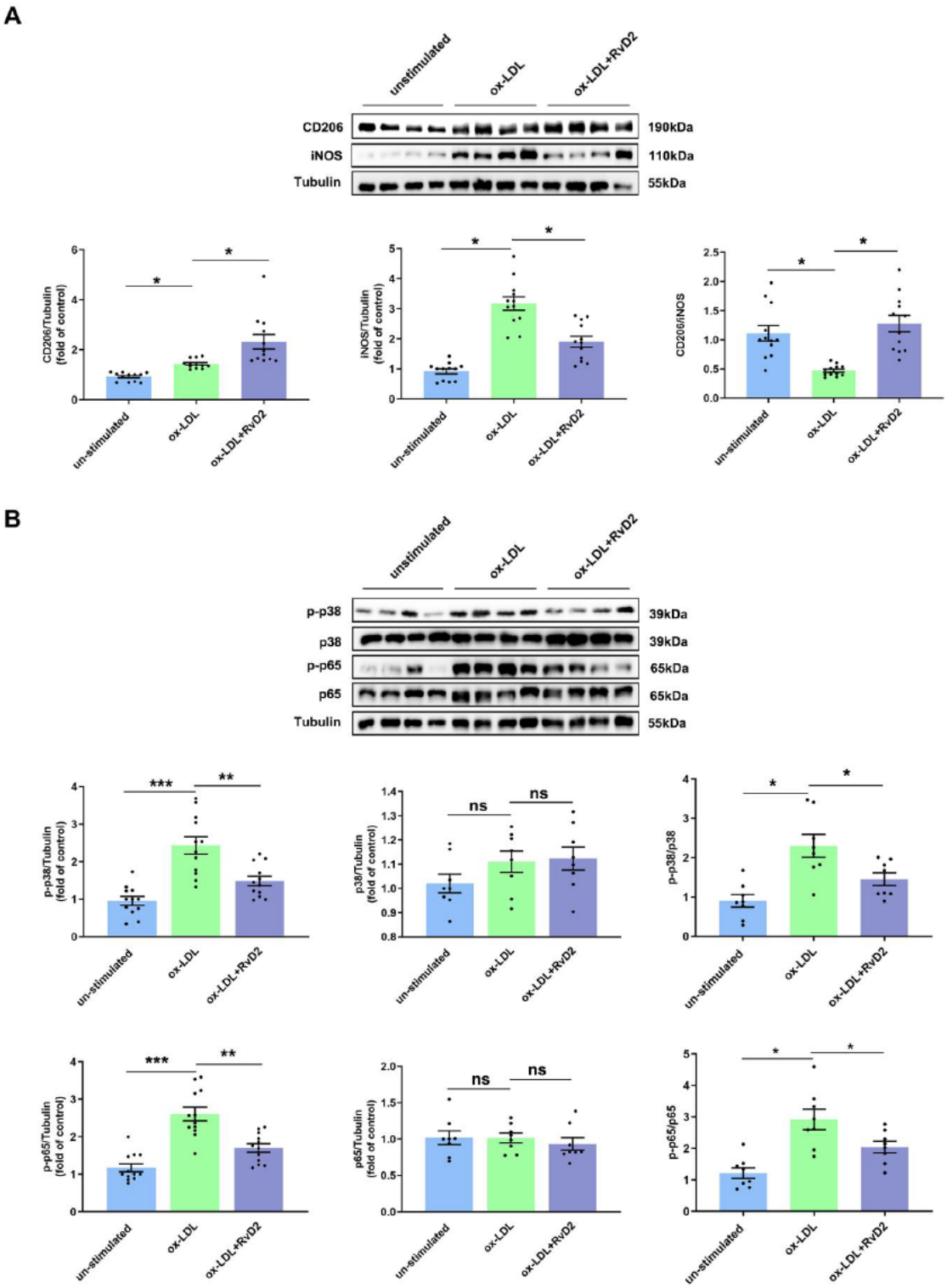

Figure 3

Treatment effects of RvD2 in ox-LDL-stimulated macrophages from AIS patients with DM. (A) Western blot analysis showed that RvD2 treatment increased CD206 levels and decreased iNOS levels in the macrophages from AIS patients with DM, and the CD206/iNOS ratio was also increased. (B) The expression of p-p38 and p-p65, as well as the p-p38/p38 and p-p65/p65 ratios, were all decreased by RvD2 treatment, while total p38 and p65 levels were unchanged. ANOVA followed by Turkey post hoc

analysis was applied. Error bars represent mean ± SEM. * p < 0.05, **, p < 0.01; ***, p < 0.001. ns = not significant. AIS, acute ischemic stroke; DM, diabetes mellitus; iNOS, inducible nitric oxide synthase; ox-LDL, oxidized low-density lipoprotein; RvD2, resolvin D2;

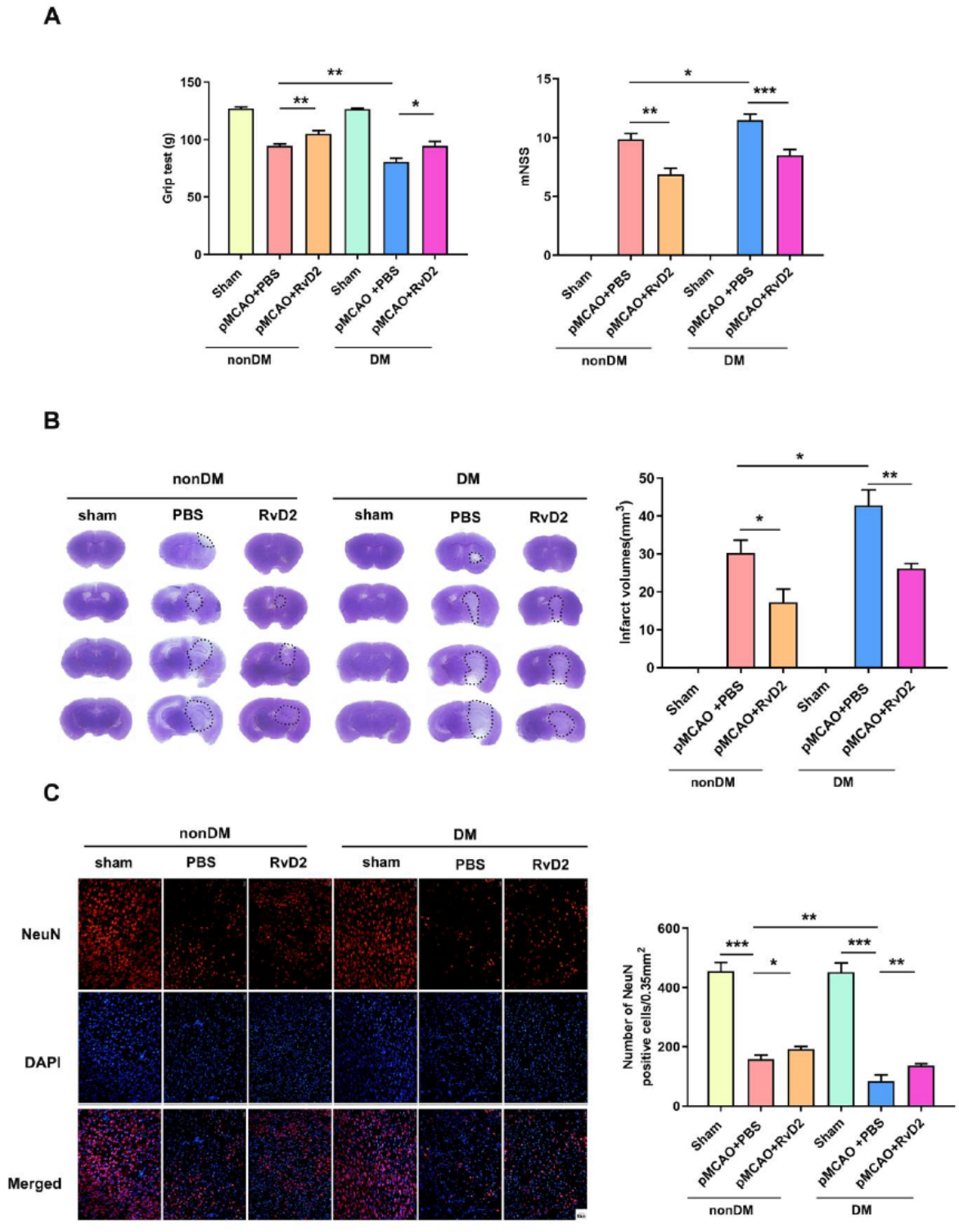

Figure 4

Treatment effects of RvD2 on neurological tests and pathological changes in DM mice challenged by pMCAO. (A) Neurological tests showed that DM decreased the score of grip test and increased the score

of mNSS in mice after pMCAO, and treatment with RvD2 rescued these impairments in both DM and nonDM mice with pMCAO. (B) Crystal violet staining showed that DM mice had increased infarct volume after pMCAO, and RvD2 treatment reduced the infarct volume in both DM and nonDM mice with pMCAO. The infarct areas were circled by dotted line. (C) Immunofluorescent staining showed that the number of surviving neurons in the infarct area was decreased by DM, and RvD2 treatment increased the number of surviving neurons in both DM and nonDM mice with pMCAO. Sections were labeled by NeuN (red) to detect neurons and counterstained with DAPI (blue) to detect nuclei. ANOVA followed by Turkey post hoc analysis was applied. Error bars represent mean ±SEM. * $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$. ns = not significant. RvD2, resolvin D2; DM, diabetes mellitus; pMCAO, permanent middle cerebral artery occlusion.

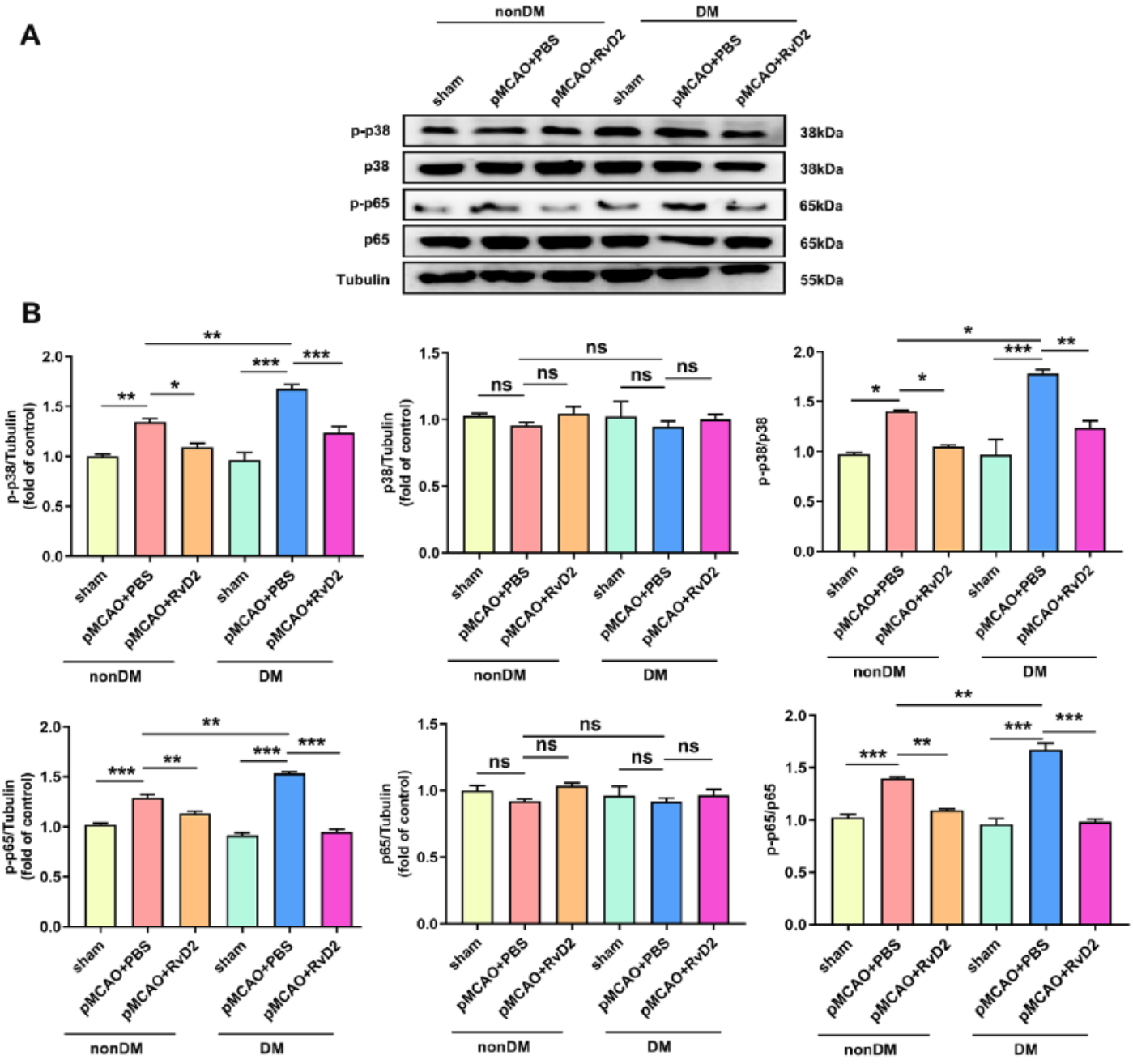

Figure 5

Treatment effects of RvD2 on MAPK/NFκB pathway markers in DM mice challenged with pMCAO. (A) Representative bands analysed by Western blotting. (B) Quantification analysis of Western Blotting showed that DM increased p-p38 and p-p38/p38 ratio, as well as p-p65 and p-p65/p65 ratio, in the mouse brain after pMCAO, and RvD2 treatment decreased the levels of these inflammatory pathway markers. ANOVA followed by Turkey post hoc analysis was applied. Error bars represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. ns = not significant. RvD2, resolvin D2; DM, diabetes mellitus; pMCAO, permanent middle cerebral artery occlusion.

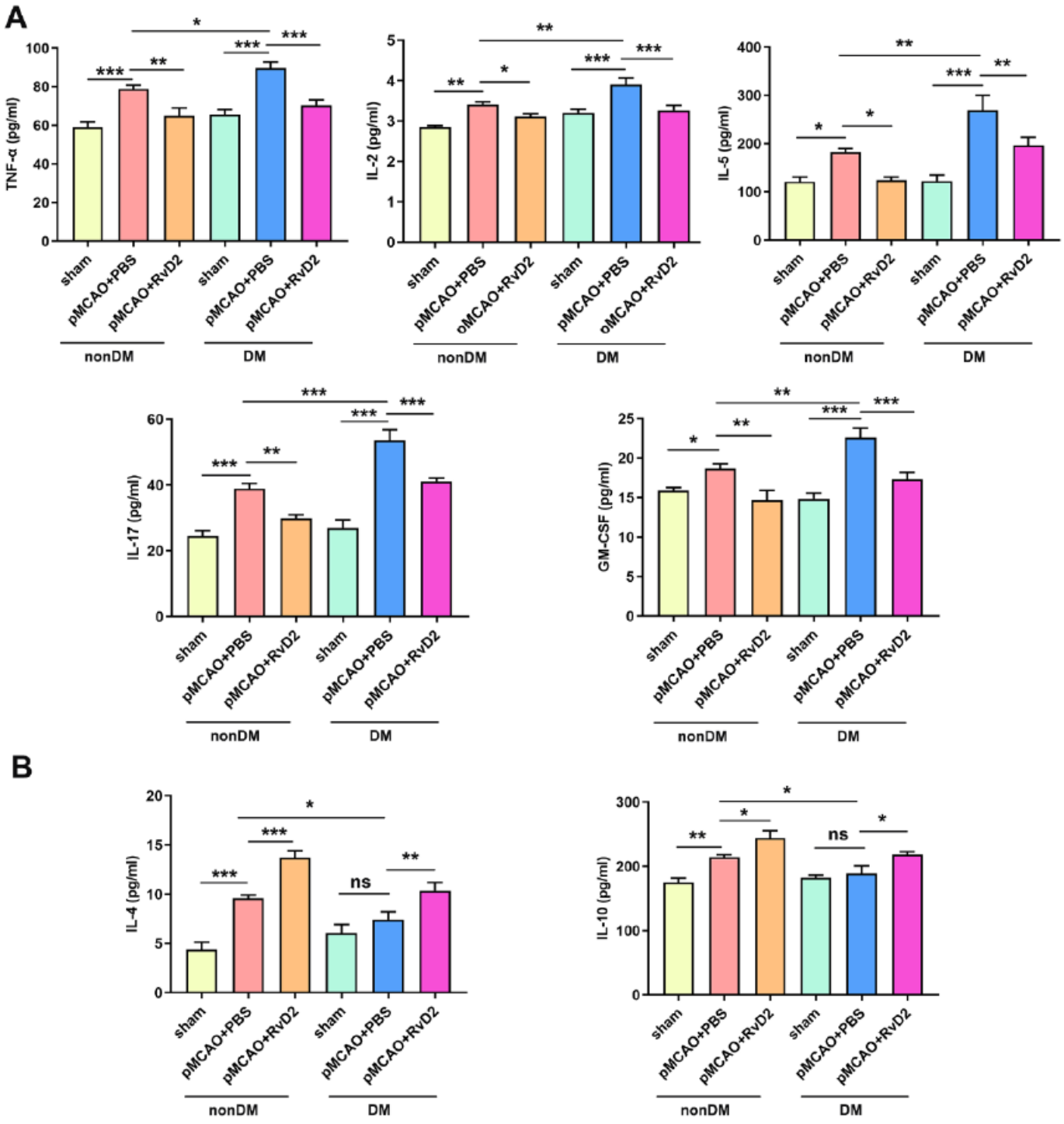

Figure 6

Treatment effects of RvD2 on inflammatory cytokines in DM mice challenged with pMCAO. (A) Quantification of cytokines by Raybiotech cytokine assay showed that DM increased the expression of GM-CSF, IL-2, IL-5, IL-17 and TNF-α, and RvD2 treatment decreased the expression of these pro-inflammatory cytokines. (B) DM decreased the expression of IL-4 and IL-10 in mice after pMCAO, and RvD2 treatment increased the expression of these anti-inflammatory cytokines. ANOVA followed by Turkey post hoc analysis was applied. Error bars represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. ns = not significant. RvD2, resolvin D2; DM, diabetes mellitus; pMCAO, permanent middle cerebral artery occlusion.

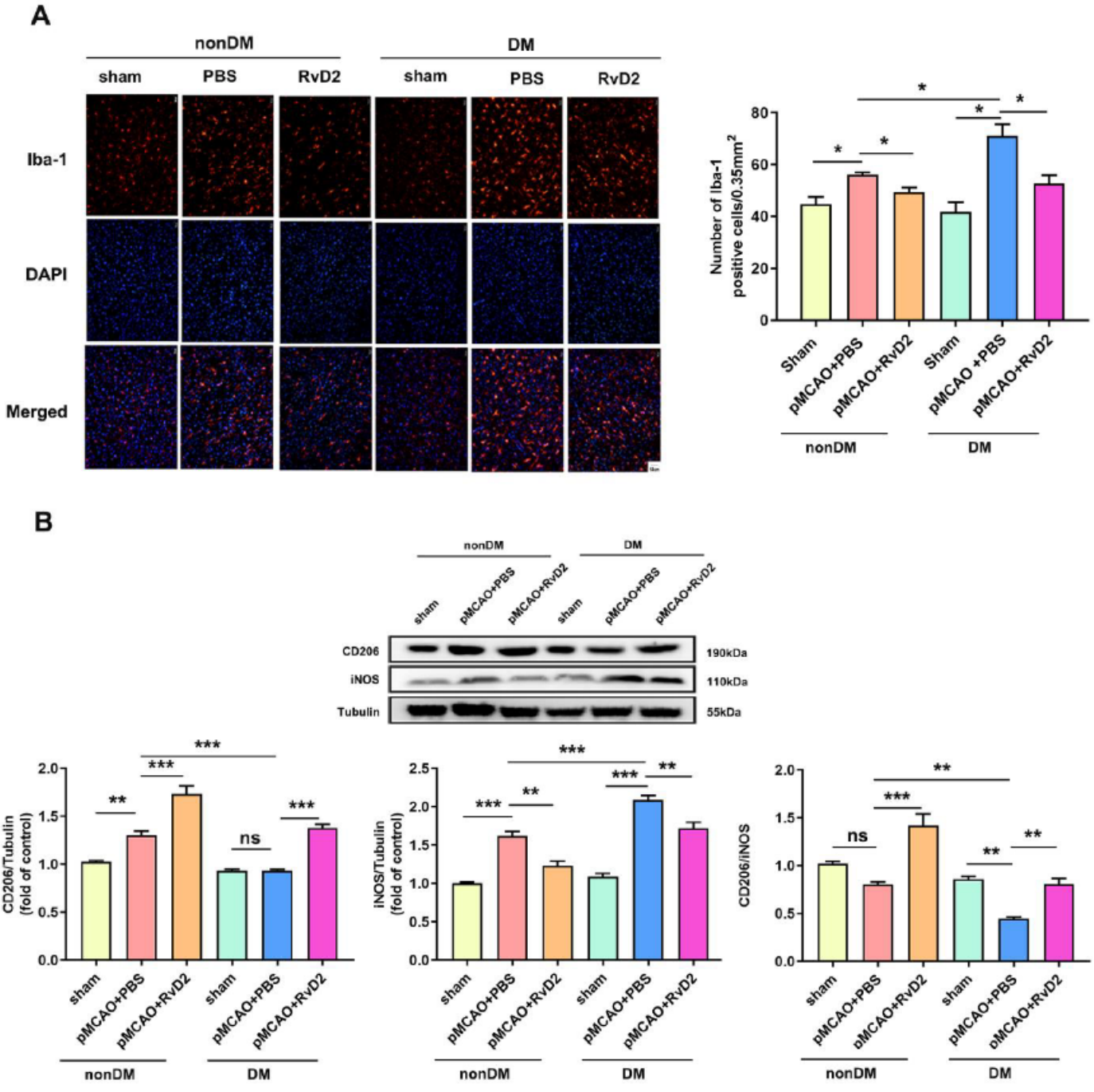

Figure 7

Treatment effects of RvD2 on microglia activation in DM mice challenged with pMCAO. (A) Immunofluorescent staining showed that DM increased the expression of Iba-1 in the mouse brain after pMCAO. Representative photomicrographs showed microglial activation labeled by Iba1 (red), and nuclei were stained with DAPI (blue). (B) Western blot analysis showed that DM decreased the expression of M2 marker CD206 and increased the expression of M1 marker iNOS in the mouse brain after pMCAO, and RvD2 treatment reversed these changes. ANOVA followed by Turkey post hoc analysis was applied. Error bars represent mean ± SEM. * $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$. ns = not significant. RvD2, resolvin D2; DM, diabetes mellitus; Iba1,ionized calcium-binding adaptor protein 1; iNOS, inducible nitric oxide synthase; pMCAO, permanent middle cerebral artery occlusion.