Resolution of Inflammation is Disturbed in Macrophages of Diabetic Patients with Acute Ischemic Stroke

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Original investigation

Keywords: resolution, inflammation, acute ischemic stroke, diabetes mellitus, macrophage

Posted Date: April 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-377994/v1

Abstract

Background: Inflammation plays an important role in diabetes mellitus (DM) and its related complications, including atherosclerosis and acute ischemic stroke (AIS). The mechanisms of excessive inflammation in DM are not fully understood. Specialized pro-resolving mediators (SPMs) are key regulators that promote resolution of inflammation. Unbalance of pro-resolving and pro-inflammatory signals may be the key pathogenesis in DM and DM-related diseases. We examined the resolution function in macrophages of DM patients with AIS.

Methods: Macrophages were derived from peripheral blood mononuclear cells of AIS and none-AIS patients with or without DM. The cultured macrophages were treated with oxidized low-density lipoprotein. Resolution and inflammatory pathway markers were analyzed in the culture medium and cells.

Results: In AIS patients, the ratios of SPMs to leukotriene B4 (LTB_4) released by macrophages were decreased in those with DM compared to those without DM. Oxidized low-density lipoprotein treatment further reduced the ratios of SPMs/LTB_4 in DM patients with AIS, but such a reduction was not observed in none-DM AIS patients. Analysis of M2/M1 polarization and MAPK/NFkB pathway markers also demonstrated un-resolved inflammation in macrophages of AIS patients with DM. Treatment with one of the SPMs, resolvin D2, rescued the resolution function and ameliorated inflammation in macrophages from these patients.

Conclusions: Our data demonstrated that resolution of inflammation is disturbed by DM in macrophages of AIS patients, implicating a novel mechanism of un-resolved inflammation in DM-related vascular disorder. Exogenous SPMs treatment can rescue resolution and downregulate inflammatory signals, and may thus be beneficial in DM-related AIS.

Background

Patients with type 2 diabetes mellitus (T2DM) 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) Although the mechanisms of T2DM have not been completely understood, inflammation has been shown to play crucial roles in T2DM pathophysiology and its complications, including atherosclerosis and AIS.(3) For example, in the pathogenesis of DM-associated atherosclerosis, macrophages are activated toward a pro-inflammatory phenotype and express higher levels of pro-inflammatory mediators.(4) The pro-inflammatory macrophages are more vulnerable to pro-atherosclerotic challenges, including oxidized low density lipoprotein (ox-LDL).(4, 5) Atherosclerosis is the most common risk for AIS. When AIS is combined with T2DM, the infarct volume in the brain is often increased, and the prognosis is usually worse.(2) Excessive inflammation plays a critical role therein. 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.(6, 7) 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.(6, 7) Monocytes/macrophages from the peripheral circulation also infiltrate the brain in AIS and contribute to the local inflammation.(8) Thus, to better understand the mechanisms of excessive inflammation in DM-associated atherosclerosis and AIS it is favourable to develop novel therapeutic methods against these diseases.

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).(9) 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). (9) Upon the challenge of acute inflammation, lipoxygenases (LOXs) are activated and promote the biosynthesis of SPMs from their precursors.(10) (11) (12) 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 T2DM and atherosclerosis. Studies on obesity mouse models showed that SPM levels were reduced in fat tissue, and treatment with the SPMs RvD1 and RvD2 counteracted local inflammation by regulating production of adipokines and monocyte function.(13) Moreover, it has been reported that ratios of SPMs to leukotriene B4 (LTB_4) are reduced in human vulnerable plaques compared to stable ones.(14) These studies implicate a critical role of resolution and SPMs in T2DM and atherosclerosis. In the present study, we set out to study resolution of inflammation in ox-LDL-challenged macrophages derived from peripheral blood mononuclear cells (PBMCs) of DM patients with AIS *ex 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.(15) 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.

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.

Human macrophage culture and stimulation

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll separation as previously described. (16) 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 divided into unstimulated group and ox-LDL group, and 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, $1 \subseteq 10^6$ cells/well monocytes 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 the ox-LDL group was stimulated with 10 μg/ml ox-LDL (Thermo Fisher Scientific) for 24 h in FBS/M-CSF-free 1640 culture medium, while the unstimulated group was incubated with PBS as vehicle. 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.

Western blot analysis

Macrophages were lysed and extracted with RIPA buffer supplemented with protease and phosphatase inhibitors. The protein concentration was quantified using a BCA kit. 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 G-protein coupled receptor (GPR) 18 1:1000 (Sigma-Aldrich); 5-LOX 1:200 (Cayman); 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 targeted bands 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, paired sample t 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 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 ± .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 between 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, total white blood cell count.

SPMs secretion from ox-LDL-stimulated macrophages was enhanced in patients with AIS, while the ratios to LTB $_4$ were decreased

First, we compared the secretion *ex vivo* of lipid mediators (LMs) by macrophages from patients with AIS (nonDM + AIS and DM + AIS groups) and without AIS (Control and DM groups). The analysis showed that the levels of RvD1, RvD2, LXA_4 and LTB_4 were all increased in the patients with AIS (nonDM + AIS and DM + AIS groups) compared to non-AIS patients (Control and DM groups) under unstimulated conditions, whereas there was no significant difference in MaR1 (Fig. 1A). We then explored changes in LMs produced by macrophages stimulated with ox-LDL. Ox-LDL stimulation increased the secretion of both pro-inflammatory LTB_4 and pro-resolving RvD1, RvD2, LXA_4 and MaR1 by macrophages cultured 24 h *ex vivo*. (Fig. 1C).

The ratio between SPMs and LTB_4 has been widely used as a marker reflecting the balance of resolution and inflammation.(17–19) We calculated the ratios between each SPM and LTB_4 to evaluate the function of resolution in macrophages from the different patient groups, and there was no difference between the AIS and nonAIS groups under unstimulated conditions (Fig. 1B). However, after challenge with ox-LDL, the ratios RvD1/LTB_4, RvD2/LTB_4 and MaR1/LTB_4 were significantly lower in AIS compared to nonAIS groups (Fig. 1D). The LXA_4/LTB4 ratio in AIS groups was not statistically different compared to nonAIS groups, although there was a trend of decrease (Fig. 1D).

DM disturbed the balance of SPMs to LTB_4 and impaired resolution of inflammation in unstimulated macrophages from AIS patients

We then explored the influence of DM on levels of LMs in the culture medium of unstimulated macrophages. Interestingly, the ratios of SPMs to LTB_4 were lower in the DM + AIS group than that in the AIS group. However, there was no significant difference between DM and the Control group (Fig. 2A).

To further explore the dysfunction of inflammation resolution, we assessed the levels of key SPM synthases in homogenates of macrophages by Western blot, including 5-LOX and 15-LOX-1, as well as the RvD2 receptor, GPR18. There was no significant difference in the levels of 5-LOX between the four patient groups. The levels of 15-LOX-1 and GPR18 were lower in the DM + AIS group than in the nonDM + AIS group, but there was no significant difference between control and DM group (Fig. 2B).

The inflammatory markers CD206 and iNOS were analysed by Western blotting in macrophage homogenates. The levels of the pro-inflammatory M1 marker iNOS was higher, while the levels of M2 marker CD206 was lower in DM + AIS compared to nonDM + AIS (Fig. 3C). To further evaluate the M2/M1 polarization, we calculated the ratio of CD206/iNOS, which was lower in DM + AIS than in nonDM + AIS (Fig. 2C). There was no difference between the DM and Control group (Fig. 2C).

The analysis of representative MAPK pathway markers p38 and NFκB pathway markers p65 exhibited similar results in that 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 than in nonDM + AIS (Fig. 2D). There was no difference between Control and DM with regard to these signal transduction markers nor in their ratios. There was no difference in the total levels of p38 and p65 between the four groups (Fig. 2D).

Ox-LDL stimulation exacerbated the disturbance in resolution function in macrophages from AIS patients with DM

We further explored the influence of DM on resolution function of macrophages stimulated with ox-LDL. The analysis revealed that the ratios RvD1/LTB_4, RvD2/LTB_4 and MaR1/LTB_4 were lower in DM + AIS than

in nonDM + AIS (Fig. 3A), consistent with the data for unstimulated macrophages. It is worth noting that ratios between SPMs and LTB_4 were lower in DM + AIS than in DM, while there was no difference between nonDM + AIS and Control (Fig. 3A). The difference between DM + AIS and DM was not seen in unstimulated macrophages, indicating that ox-LDL exacerbated the impairment of resolution in macrophages from AIS patients with DM.

The data from analysis of SPM synthases were consistent with the findings from unstimulated macrophages. The levels of 15-LOX-1 were lower in DM + AIS than in DM (Fig. 3B), whereas there was no significant difference in 5-LOX between the four groups (Fig. 3B). The RvD2 receptor GPR18 was upregulated in DM + AIS compared to DM (Fig. 3B), indicating a possible compensatory mechanism against decreased RvD2 production. Similar changes of 15-LOX-1 and GPR18 were also seen between nonDM + AIS and Control (Fig. 3B).

Further analysis of the macrophage M1/M2 phenotype revealed that under stimulation with ox-LDL the levels of CD206 were lower and the levels of iNOS higher in DM + AIS compared to DM (Fig. 3C). The CD206/iNOS ratio was significantly lower in DM + AIS than in DM (Fig. 3C).

The phosphorylated forms of the signal transduction factors (p-p38 and p-p65) and the p-p38/p38 and p-65/p65 ratios were higher in DM + AIS than in DM (Fig. 3D). The increase in p-p65/p65 in DM + AIS was not seen in unstimulated macrophages, indicating further activation of the NFκB pathway by ox-LDL stimulation of the macrophages from AIS patients with DM.

RvD2 alleviates ox-LDL-induced inflammatory response in macrophages from AIS patients with DM

The above results demonstrated impaired resolution and excessive inflammation in macrophages of AIS patients with DM. Next, we analysed the effect of one of the SPMs in these macrophages. Ox-LDL stimulation significantly reduced the ratio of CD206/iNOS in macrophages from DM 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. 4A).

Moreover, the MAPK pathway markers p-p38 and ratio of p-p38/p-38, as well as NFκB pathway markers p-65 and ratio of p-p65/p65, were all increased by ox-LDL stimulation (Fig. 4B), and treatment with RvD2 significantly downregulated these markers (Fig. 4B).

Discussion

Inflammatory responses are activated both in the brain and in peripheral circulation after AIS and contribute to stroke prognosis.(20–23) During the acute phase of AIS, resident microglia cells and circulating monocytes/macrophages are M1-polarized towards a pro-inflammatory phenotype, produing pro-inflammatory cytokines and other mediators of inflammation. We found that macrophages derived from AIS patients produced higher levels of both pro-resolving SPMs and pro-inflammatory LTB_4

compared to non-AIS individuals. SPM-mediated resolution of inflammation is a programmed process to counter-regulate pro-inflammatory responses, and the production of SPMs accompanies the initiation of pro-inflammatory mediator synthesis.(24) Thus, the increased release of both SPMs and LTB_4 in macrophages from AIS patients reflects such a co-activation of pro-resolving and pro-inflammatory programs. To evaluate the balance of these two forces, the ratios between SPMs and LTB4 have been applied as an index in many studies.(18, 25–27) The present study showed that this index was reduced in ox-LDL stimulated macrophages from AIS patients compared to those from nonAIS patients. This result may reflect defective resolution function and excessive inflammation in macrophages from patients with AIS.

We further explored whether DM plays a role in the disturbed resolution in these AIS patients. DM is an independent risk factor for atherosclerosis and AIS, and often worsens the clinical symptoms and prognosis of AIS.(28–30) Unresolved and increased inflammation contributes to advanced atherosclerosis and AIS prognosis.(18, 25) The analysis showed that the ratios of SPM/LTB_4 secreted from macrophages under both control and ox-LDL-stimulated conditions, were significantly lower in AIS patients with DM compared with those without DM, indicating that impaired resolution function in AIS was attributed to DM. This result is consistent with our recent report that RvD2/LTB_4 is reduced in the plasma of AIS patients with DM.(31) The decrease in 15-LOX-1 levels may be one of the upstream causes of lower SPM levels.(32, 33) Indeed, the aggravation of brain damage by DM has been demonstrated as a result of uncontrolled and perturbed inflammation.(34) Our data explain such an abnormality of inflammation from a novel perspective, resolution of inflammation. MAPK and NFκB pathways are over-activated as key inflammatory signals in DM.(35, 36) We also found that these two pathways were affected in macrophages from AIS patients with DM. It has been reported that macrophages are pushed towards a pro-inflammatory activation spectrum in DM,(37) which is consistent with our data on CD206/iNOS. Altogether, these findings indicate that resolution of inflammation is disturbed in AIS patients with DM, and that abnormal resolution may be an important etiology of excessive inflammation in DM-related AIS.

Ox-LDL is a key target in the primary and secondary prevention of AIS, and ox-LDL induced an increase in both SPMs and LTB_4 in the patient macrophages. Consistent with this, studies on endothelial cells showed that ox-LDL could trigger both pro-resolving and pro-inflammatory actions.(38) Therefore, the balance between pro-resolving and pro-inflammatory signals is crucial for the consequences of ox-LDL stimulation. Under ox-LDL conditions, the ratios between SPMs and LTB4 were lower in macrophages from AIS patients than in those from nonAIS patients. However, the SPMs/LTB_4 ratios in macrophages from AIS patients without DM were not different from ratios in control individuals, but were significantly lower in AIS patients with DM. These results indicate that resolution function in DM patients is more vulnerable to an AIS insult, while non-DM patients maintained a relative balance between resolution and inflammation after AIS. Thus, the results indicate that together with ox-LDL challenge, DM induces a disturbed resolution function in macrophages of AIS patients, reflecting the pathogenesis of exaggerated inflammation in AIS patients with DM.

Among the SPMs analyzed in this study, RvD2 has been implicated in DM-associated vascular damage and atherosclerosis. (25, 39) In a hind limb ischemia animal model, RvD2 treatment rescued DM-impaired revascularization through activation of its receptor GPR18.(39) LTB_4 levels were elevated in the aorta of $Apoe^{-/-}$ mice fed a high-fat diet, and correlated to instability of atherosclerotic plaques, while the RvD2 levels were reduced and correlated to plaque stability.(25) We demonstrate here that treatment with RvD2 reduced the pro-inflammatory phenotype of human macrophages and downregulated the MAPK inflammatory pathway marker p-p38/p38 and NFκB pathway marker p-p65/p65. These data demonstrated that RvD2 is a potent pro-resolving mediator that may be beneficial in the treatment of DM-related atherosclerosis and AIS.

The limitation of our study includes the observational design and lack of mechanistic experiments. The functional difference of resolution in diabetic macrophages in AIS may involve epigenetic factors not included in the present report. Further studies in vivo and in vitro to explore the upstream mechanisms of resolution dysfunction in DM patients with AIS is warranted.

Conclusions

our data showed dysfunction of resolution in macrophages from AIS patients with DM, implicating a previously unreported perspective of uncontrolled inflammation in DM-associated AIS. Treatment with one of the SPMs, RvD2, could rescue the disturbed resolution function, indicating a possible therapeutic role of SPMs in DM-related atherosclerosis and AIS.

Abbreviations

AIS: acute ischemic stroke; DM: diabetes mellitus; T2DM: type 2 diabetes mellitus; EPA: eicosapentaenoic acid; GPR18: G-protein coupled receptor 18; 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; RvD1: resolvin D1; RvD2: resolvin D2; SPMs: specialized pro-resolving mediators.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

XW and YZ conceived and designed the study. XT and JZ performed the laboratory experiments, and analysed the data. ZM, LL and XC were involved in patient enrollment. XT and XW drafted the article. BQZ

and MS gave critical suggestions on methodology and manuscript writing. All authors approved the final version of the artical.

Funding

The study is supported by the National Natural Science Foundation of China (Grant No. 81501089, 81974158, 31811530007), Shanghai Committee of Science and Technology (Grant No. 17411950103) and the Swedish Foundation for International Cooperation in Research and Higher Education (Grant No. CH2017-7308).

Availability of data and materials

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

Ethics approval and consent to participate

The study was approved by the ethical committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (No. 2019-040) and written consent was obtained from all participants or their authorized relatives.

Consent for publication

Not applicable.

Competing interests

None of the authors had competing interests in this trial.

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Figures

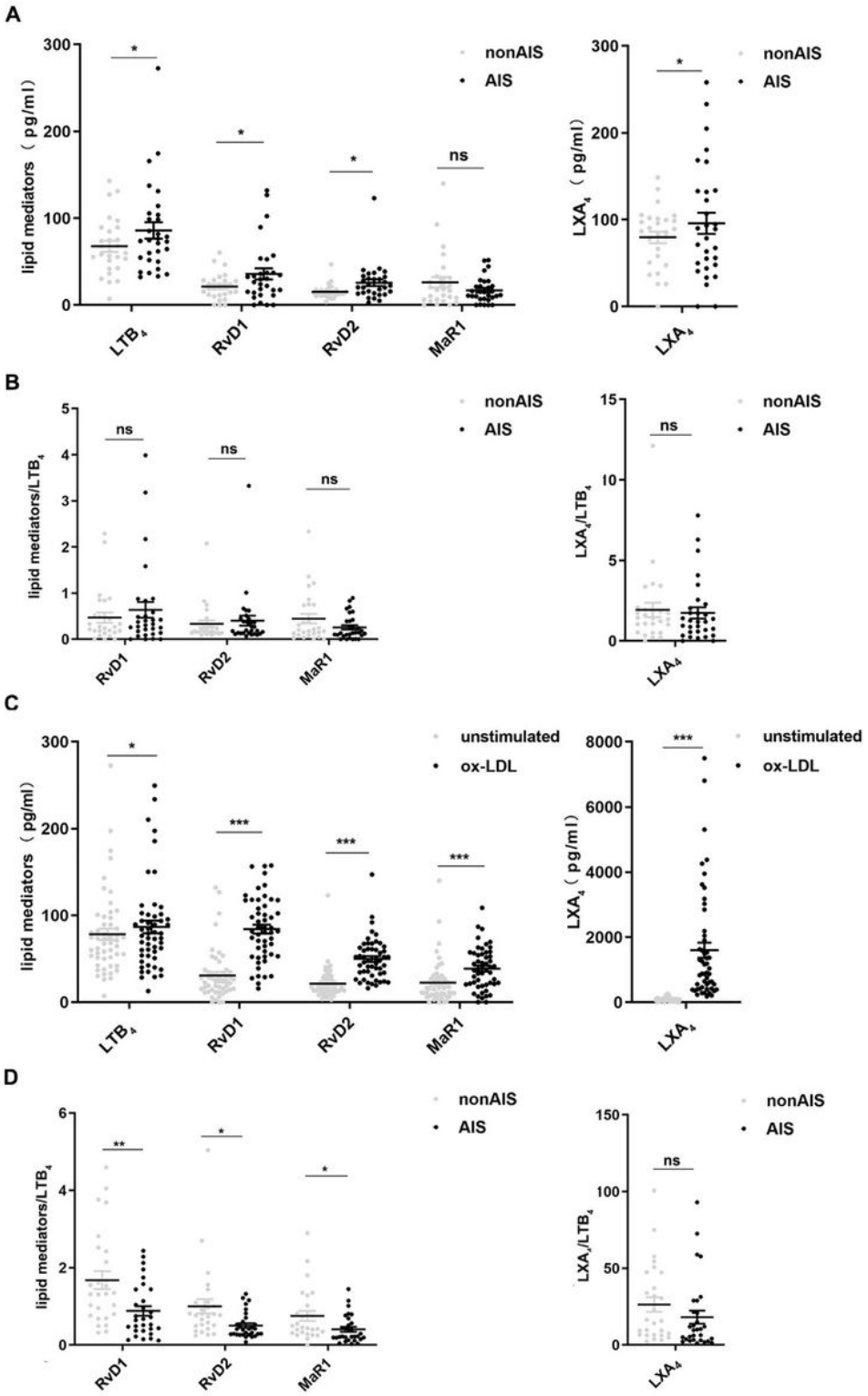

Figure 1

AIS and ox-LDL alter lipid mediators in macrophages from peripheral blood mononuclear cells. Levels of SPMs and LTB4 were higher in AIS patients compared to nonAIS patients (A), while the SPM/LTB4 ratios were unchanged (B). Stimulation by ox-LDL resulted in increased secretion of SPMs and LTB4 (C), while the SPM/LTB4 ratios were lower in AIS patients compared with nonAIS patients (D). Mann-Whitney U test or paired sample t test was applied. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ns = not

significant. AIS, acute ischemic stroke; Con, control; DM, diabetes mellitus; LTB4, leukotriene B4; LXA4, lipoxin A4; MaR1, maresin 1; RvD1, resolvin D1; RvD2, resolvin D2

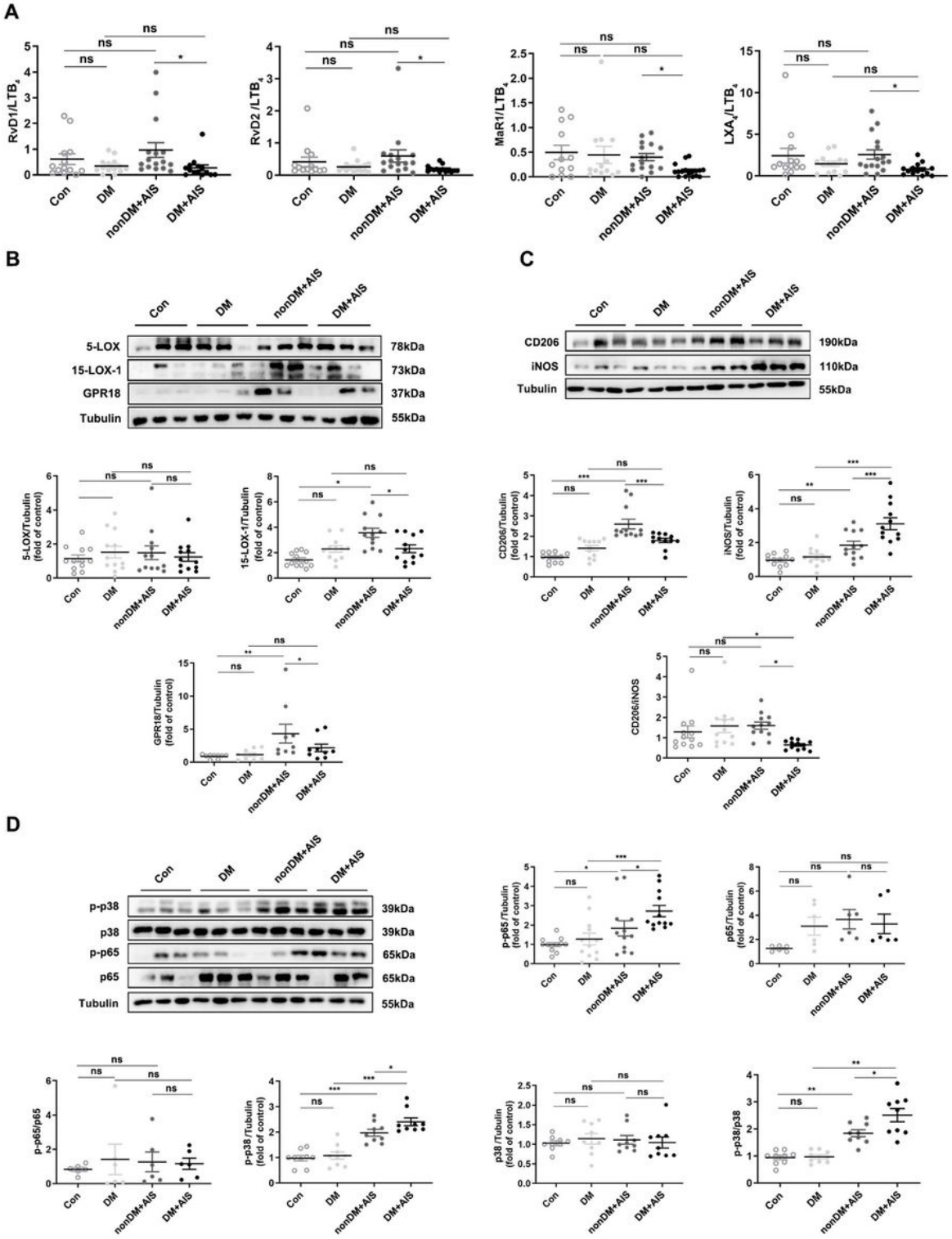

Figure 2

Profile of lipid mediators and inflammatory pathway markers in unstimulated macrophages. Ratios of SPM/LTB4 were lower in DM+AIS compared to the nonDM+AIS group (A). Similarly, levels of 15-LOX-1 and the SPM receptor GPR18 were lower in DM+AIS compared with nonDM+AIS (B). Analysis of

inflammatory markers revealed that the levels of CD206 were lower and iNOS higher in DM+AIS compared with nonDM+AIS (C). Analysis of MAPK/NFκB pathway markers showed that p-p38 and p-p38/p38 ratio, as well as p-p65 and p-p65/p65 ratio, were all higher in DM+AIS compared with nonDM+AIS (D). ANOVA followed by post hoc test was applied. Error bars represent 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; GPR18, G-protein coupled receptor 18; LOX, lipoxygenase; LTB4, leukotriene B4; LXA4, lipoxin A4; MAPK, mitogen-activated protein kinase; MaR1, maresin 1; NFκB, nuclear factor κB; RvD1, resolvin D1; RvD2, resolvin D2

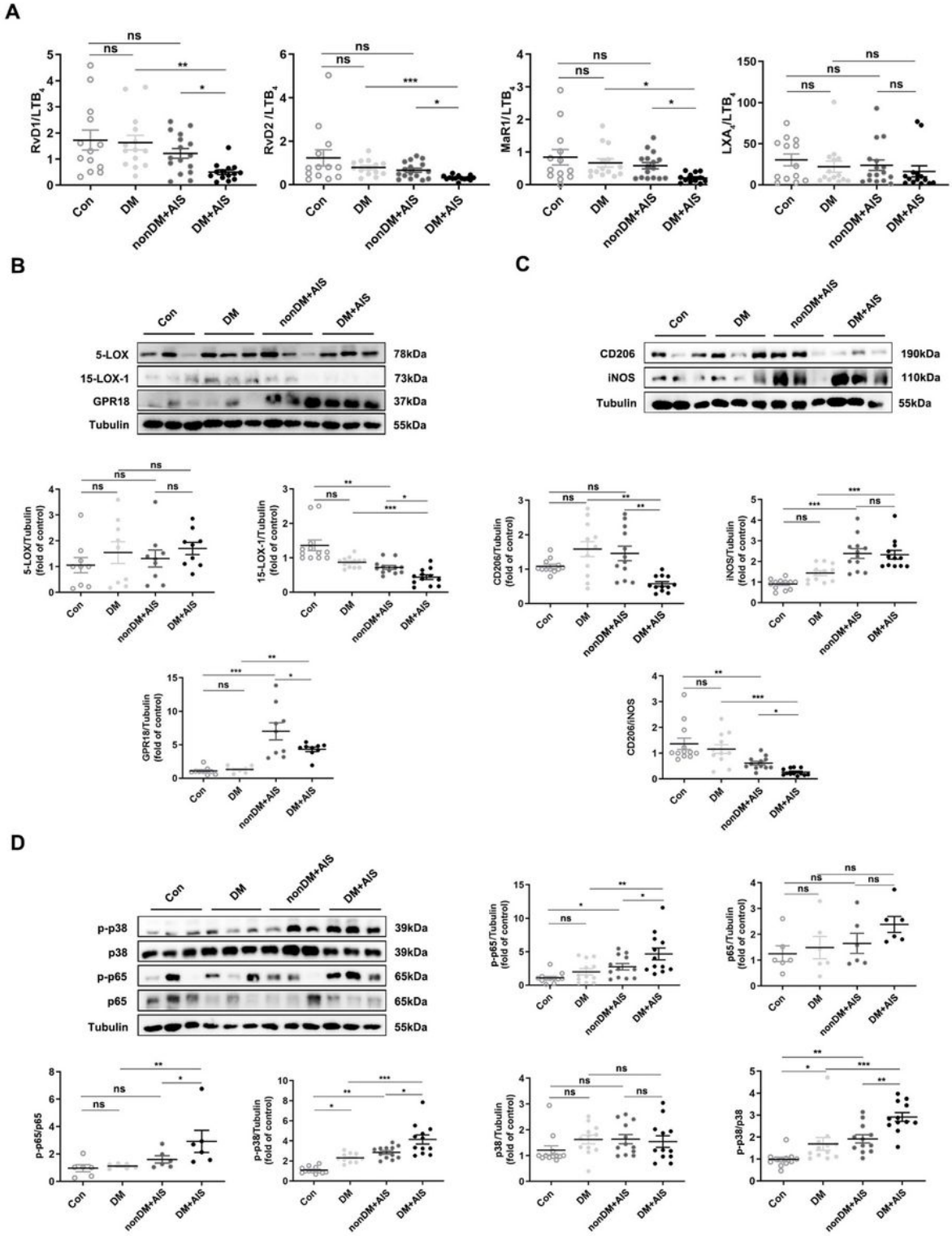

Figure 3

Profile of lipid mediators and inflammatory pathway markers in macrophages stimulated with ox-LDL. Ratios between RvD1, RvD2 and MaR1 to LTB4 were lower in DM+AIS compared with DM, while they were not altered in nonDM+AIS compared with Con (A). The levels of 15-LOX-1 were lower in DM+AIS compared with nonDM+AIS, as well as in nonDM+AIS compared with Con (B). The levels of the SPM receptor GPR18 were higher in DM+AIS compared with nonDM+AIS, as well as in nonDM+AIS compared

with Con (B). Analysis of inflammatory markers revealed that CD206 was lower and iNOS was higher in DM+AIS compared with DM (C). Analysis of MAPK/NFκB pathway markers showed that the p-p65/p65 and p-p38/p38 ratios were higher in DM+AIS compared with DM (D). ANOVA followed by post hoc test was applied. Error bars represent 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; GPR18, G-protein coupled receptor 18; LOX, lipoxygenase; LTB4, leukotriene B4; LXA4, lipoxin A4; MAPK, mitogen-activated protein kinase; MaR1, maresin 1; NFκB, nuclear factor κB; RvD1, resolvin D1; RvD2, resolvin D2

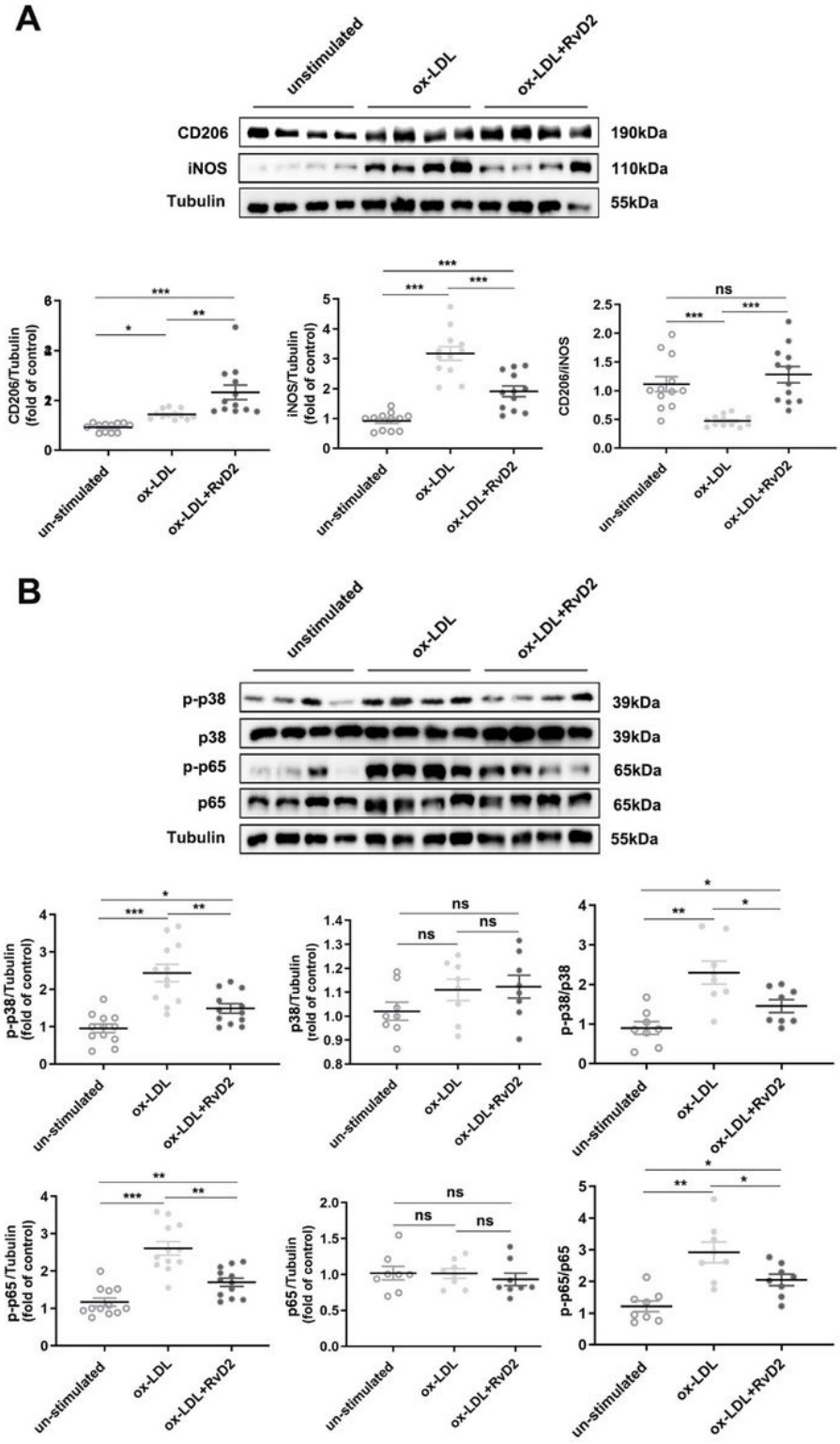

Figure 4

Effects of RvD2 treatment in ox-LDL-stimulated macrophages from AIS patients with DM. Western blot analysis of inflammatory markers showed that CD206 levels were higher and iNOS was lower, while the CD206/iNOS ratio was higher after treatment of the microphages with RvD2 (A). p-p38 and p-p65 levels and the p-p38/p38 and p-p65/p65 ratios were all decreased by RvD2 treatment, while total p65 and p38 levels were unchanged (B). ANOVA followed by post hoc analysis was applied. Error bars represent SEM. * $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$. ns = not significant. AIS, acute ischemic stroke; iNOS, inducible nitric oxide synthase; RvD2, resolvin D2