High Salt Diet Down Regulates TREM2 Expression and Blunts Efferocytosis of Macrophage After Acute Ischemic Stroke

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Research

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Abstract

Background: High salt diet (HSD) is one of the major risk factors for acute ischemic stroke (AIS). As a potential mechanism, surplus salt intake primes macrophage towards a proinflammatory phenotype. The study investigated whether HSD could blunt efferocytosis of macrophage after ischemic stroke, which was a vital process that alleviated post stroke neuroinflammation. Besides, the underlying mechanism was explored.

Methods: Wild type male C57/Bl6 mice were fed with fodder containing 8% sodium chloride for 4 weeks and subjected to transient middle cerebral occlusion (tMCAO). Disease severity, macrophage polarization as well as their efferocytic activities were evaluated. In *in vitro* study, bone marrow derived macrophages were cultured and the impact of high salinity environment on their efferocytic capacity, as well as their expression of phagocytic molecules were analyzed. The relationship of sodium concentration, macrophage phenotype, and disease severity in AIS patients with ischemic stroke was explored.

Results: HSD-fed-mice displayed increased infarct volume and aggravated neurological deficiency. Mice fed with HSD suffered exacerbated neural inflammation as higher level of inflammatory mediators and immune cells infiltration were documented. Polarization shift towards pro-inflammatory phenotype impaired efferocytosis of infiltrated macrophages within stroke lesion in HSD-fed-mice were detected. As was uncovered by PCR array, macrophage expression of triggering receptor expressed on myeloid cells 2 (TREM2), a receptor relevant with phagocytosis, was down regulated in high salt environment. Enhancing TREM2 signaling restored the efferocytosis capacity and cellular inflammatory resolution of macrophages in high salinity environment. In AIS patients, high concentration of urine sodium was correlated with lower expression of TREM2 and detrimental stroke outcomes.

Conclusions: HSD blunted efferocytic capacity of macrophages through down regulating the expression of TREM2, thus impeded inflammatory resolution after ischemic stroke. Enhancing TREM2 signaling in monocyte/macrophage could be a promising therapeutic strategy to enhance efferocytosis and promote post-stroke inflammatory resolution.

Background

High salt intake is highly associated with blood pressure, blood lipid concentration, the level of circulating alarmins and other factors affecting stroke prognosis, and considered as a risk factor of acute ischemic stroke (AIS) (1-3). Therefore, salt restriction is widely accepted as a vital step in efficient lifestyle intervention to prevent new vascular event, especially in AIS. Nevertheless, given that a large number of patients fail to convert diet habit before the arrival of acute vascular event, there is an unmet need to develop therapeutic strategy to tackle the already-exist high salinity and the associated pathophysiology.

As a pivotal part of innate immunity, macrophages characterize multiple roles in stroke lesion (4, 5). Whether they are involved in inflammatory resolution or serve as an inflammation amplifier depend on their specific phenotype and microenvironment (6, 7). Recent research has elucidated that surplus dietary

salt directed macrophages/microglia towards the classical activated pro-inflammatory phenotype, which is often referred to M1 subtype (8), indicating that excessive salt intake breaks the balance of macrophages and further aggravates the inflammatory response. *In vivo*, the pro-inflammatory property of macrophages in high salt diet (HSD) fed mice contributed to blood-brain barrier (BBB) disruption after stroke, and thereafter exacerbating stroke outcomes (9).

Orchestrating macrophage activities and enhancing the inflammatory resolution make it possible to develop a promising therapeutic strategy in AIS. The post stroke inflammatory resolution heavily depends on the efferocytosis function of macrophage, which eliminates dead cells or debris that enhance sterile immune reactions in stroke lesion (6). Nevertheless, the impact of high salt diet on the phagocytic activity and the subsequent anti-inflammatory functions of macrophages remains elusive.

The current study investigated the impact of excessive salt intake on the inflammatory resolution of macrophages in post stroke neural inflammation. Of particular interest, efferocytic capacity of macrophages was evaluated under this harmful condition. Meanwhile, corresponding countermeasure to fine-tune macrophage activities in high salinity environment was purposed.

Methods

Ethical statement

The clinical and the animal experimental studies were approved by the Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University and the Animal Care and Use Committee of Sun Yat-Sen University respectively. All participants had signed the informed consent according to the principles illustrated in Declaration of Helsinki.

Patients

In this study, a total of 38 stroke patients recruited in the Third Affiliated Hospital of Sun Yat-Sen University from July 2018 to October 2019 consecutively had an independently documented primary stroke event in combination with confirmed magnetic resonance imaging (MRI) evidence showing ischemic stroke. The inclusion and exclusion criteria have been published previously (19). Clinical data, including age, gender, and score of National Institute stroke scale (NIHSS), were recorded. We estimated dietary sodium intake by measurements of 24-hour urinary excretion of sodium. Patient demographics including co-morbidities were summarized in **Supplementary Table 1**.

MRI Scanning and Infarct Volume Analysis of Patients

Magnetic resonance imaging (MRI) was performed within 24h of admission using 1.5- or 3.0-T magnetic resonance imaging (Signa; GE Medical Systems, Milwaukee, WI, USA). In this study, the diffusion-weighted imaging (DWI) spin-echo planar sequence included 20 contiguous axial oblique slices (b = 0 and 1000s/mm2 iso-tropically weighted; repetition time/echo time, 6000/60.4ms; acquisition matrix, 128 × 128; slice thickness, 5mm; interslice gap, 1mm; field of view, 24cm). DWI lesions in 38 patients were

measured with Analyze 7.0 software (Analyze Direct, KS). Cerebral infarct sizes were assessed by largest infarct diameter determined on the image demonstrating the largest lesion (20-22). MRI scans of patients were assessed by experienced neurologist Zhengqi Lu, who was blinded to the patients' clinical features. All images were interpreted with the same window settings, same types of monitors and lighting conditions.

PBMC isolation

Anti-coagulated blood (3mL) was collected, and then diluted 2-fold with PBS, pipetted into centrifuge tube prefilled with Ficoll lymphocyte separation solution (TBDscience), followed by centrifuged at 2000rpm for 25 minutes at room temperature without deceleration. PBMCs from the buffy coat were washed twice with PBS, then stored at −80°C until further analysis.

Animals

C57/Bl6 wild-type mice (8 weeks old, weight 18–25 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China) and housed in a humidity- and temperature-controlled animal facility in Sun Yat-sen University with a 12-h light–dark cycle. Mice received normal chow (0.5% NaCl) and tap water ad libitum (normal diet) or sodium-rich chow (8% NaCl) and tap water containing 1% NaCl ad libitum (HSD) for 4 weeks according to the experiment.

Model of acute ischemic stroke

Mice were subjected to focal acute ischemic stroke induced with transient middle cerebral artery occlusion (tMCAO). Procedures of tMCAO were described previously (19). Briefly, mice were anesthetized with 1.5-2.0% isoflurane under conditions of spontaneous breathing. A filament was inserted into the external carotid artery (ECA) and was directed to the middle cerebral artery (MCA) through the internal carotid artery (ICA). Filament insertion into the ICA was maintained for 60min followed by reperfusion with maintenance of core body temperatures. Cerebral blood flow (CBF) during surgery was measured by laser Doppler flow cytometry. Mice with more than 70% reduction of blood flow in the ischemic core were included in the study and mice that died during surgery were excluded. Survival of mice were recorded.

Infarct volume analysis

For immunologic staining of NeuN, six equally spaced coronal brain sections encompassing the MCA territory were stained with NeuN antibodies. Infarct volume was analyzed with NIH Image J software on NeuN-stained sections. The infarct area was determined as the difference between the NeuN positive area of contralateral hemisphere and ipsilateral hemisphere. Brain infarct was determined by multiplying the mean area of tissue loss by the distances between the two adjacent stained brain slices.

Primary macrophage enriched culture and Stimulation

Primary macrophage-enriched cultures were prepared form the bone marrow of 6-8-week-old healthy C57/Bl6 wild-type mice using EasySep Mouse Monocyte Enrichment Kit (Stem cell) according to manufacturer's instructions. Macrophages were induced with MCSF (50ng/ml) for 6d in macrophage culture medium (RPMI1640 + 10%FBS). For polarization, macrophages were treated with lipopolysaccharide (LPS, 100ng/mL, Sigma), or IL-4 (20ng/mL, Peprotech) for 24 hours.

Primary microglia culture

Primary mouse microglia were obtained from BLUEFBIO company, and was cultured in culture medium (DMEM-HG + 10%FBS) until treatment.

Primary cortical neurons culture and OGD

Primary cortical neuronal cultures were prepared from E16–18 embryos of C57/Bl6 mice as previously described (23).

Neuronal ischemia was induced with OGD. Briefly, culture medium (Neural basal + B27 + 2% glutamate) was retreated

and was replaced by EBSS (Gibco). Neurons were then incubated in 95% N_2 + 5% CO_2 for 90 min.

Phagocytosis assay

For evaluation of efferocytic capacity, apoptotic neurons were labelled with the dead cell marker Propidium iodide (PI) in PBS (1ug/ml, 37℃, 15min) and treated to macrophages, with a ratio of dead neurons : macrophages = 5:1, for indicated time periods. For *in vitro* immunol staining experiments, macrophages were pre-grown on poly-l-lysine coated cover slips. The cover slips of macrophage were washed for 2 times to remove unengulfed neurons and fixed with 4% paraformaldehyde. The cover slips were then subjected to immunol staining and removed from wells using tweezers and mounted to the slides. F-actin of macrophage was then stained with Alexa Fluor488 phalloidin (A12379, 1:500 in PBS; Invitrogen) at room temperature in the dark for 30min. For flow cytometry experiment, macrophages were pre-seeded on 24-well plates and treated with the same ratio of dead neurons for indicated time periods. Macrophages were washed with PBS and detached from wells with trypsin and were subjected to flow cytometric analysis. Percentage of efferocytic macrophages (PI^+) was calculated with flow cytometric analysis.

Lentiviral infection of macrophage

Lenti virus was constructed and packaged by FenghBio (Changsha, China). The macrophage culture was infected for 3d with Lenti-TREM2 or the control vectors. The overexpression of TREM2 was confirmed by western blot and flow cytometry.

Flow cytometric analysis

Brain tissue was homogenized and prepared as single-cell suspensions for flow cytometric analysis (FACS). Briefly, brains were dissected, and ipsilateral hemispheres were collected. Each hemisphere was subjected to digestion with 0.25% trypsin-EDTA (Thermo Fisher, Carlsbad, CA, USA) at 37 °C for 25 min. Brain tissue was then pressed through a cell strainer (70 μm). Brain cells were separated from myelin debris by centrifugation in 30%/70% Percoll solution (GE Healthcare Biosciences AB, Uppsala, Sweden). Brain cells at the interface were collected, washed with HBSS, and subjected to further staining. The following antibodies were used: CD45-PE-Texas Red (1:400, Biolegend), CD11b-PE (1:400, Biolegend), CD3-PerCp/Cy5.5 (1:400, Biolegend), CD19-FITC (1:400, Biolegend), Ly6G-APC/Cy7 (1:400, Biolegend), TREM2-PE (1:200, R&D Systems), TNFα-PE (1:200, Biolegend), CD206-Alexa Fluor 647 (1:200, BD bioscience), Arg1-APC (1:200, R&D Systems). FACS was performed using a fluorescence-activated cell sorter flow cytometer (BD bioscience, San Diego, CA), and data were analyzed using FlowJo X 10.0.7r2 software. Appropriated isotype controls were stained following the manufacturer's instruction (Thermo Fisher, Carlsbad, CA, USA). Fluorchrome compensation was performed with single-stained OneComp eBeads (Thermo Fisher, Carlsbad, CA, USA). As for data presentation, when cells could be divided into negative or positive populations, percentage of cells was calculated. When expression of coordinated marker was consecutive and population separation was obscure, data were presented as mean fluorescence intensity (MFI).

Immunofluorescence staining and cell quantification

Animals were euthanized and perfused with PBS followed by 4% paraformaldehyde. After sufficient perfusion, brains were removed and then cut into 25μm frozen cryo-sections using a microtome. Brain sections and were incubated with primary antibodies at 4°C overnight. After washing with PBS, sections were incubated with secondary antibodies for 1h at room temperature. Sections were then washed and mounted with DAPI Fluoromount-G (Thermo Fisher, Carlsbad, CA, USA). The following primary antibodies were used: rabbit anti-NeuN (1:500 Abcam), rabbit anti-Iba1 (1:1000, Wako Pure Chemical Industries), goat anti-CD206 (1:500, R&D Systems), and rat anti-CD16 (1:500, Santa Cruz Biotechnology). The following secondary antibodies were applied: anti-rabbit secondary antibody conjugated with Cy3 (1:1000, Jackson ImmunoResearch Laboratories), anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:1000, Jackson ImmunoResearch Laboratories), anti-goat secondary antibody conjugated with Alexa Fluor 488 (1:1000, Jackson ImmunoResearch Laboratories), and anti-rat secondary antibody conjugated with Alexa Fluor 488 (1:1000, Jackson ImmunoResearch Laboratories). For neuronal apoptosis analysis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was processed after NeuN labeling according to instructions from the manufacturer (Thermo Fisher). Confocal microscopy images were acquired using a Leica SP confocal microscope and Leica confocal software. Immunopositive cell quantification and area analysis were performed with the software of ImageJ (National Institutes of Health) by an investigator who was blinded to the experimental design. In quantification of cell in stroke penumbra, the stroke core was identified as the region in which the majority of DAPI-stained nuclei were shrunken, and the stroke penumbra was defined as the region of generally morphologically normal cells, approximately 450–500μm wide, surrounding the stroke core.

Quantitative determination of mRNA expression

Total RNA from cells was extracted with commercial kit (ESscience) according to the manufacturer's instructions. A total of 1ug RNA (OD260nm/280nm = 1.8-2.2) was applied to the first strand cDNA synthesis in a 40ul system using PrimeScript RT reagent kit (Takara). Real time polymerase chain reaction (RT-PCR) was performed on a QuantStudio 5 (ABI) quantitative PCR machine using TB green Premix Ex Taq kit (Takara) with 1ul of the synthesized cDNA in each reaction with addition of ROX. The following program was performed: 95℃ for 30s; 95℃ for 5s and 60℃ for 34s, repeated for 40 cycles; 95℃ for 15s, 60℃ for 1min and 95℃ for 15s (Melt curve). Primers used in the study are listed in **Supplementary Table 2**. Double delta CT were calculated, and the data presented as fold change normalized to PBS-treated contralateral brain, PBS-treated macrophage, or negative control lentivirus-treated macrophage. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizer housekeeping gene. In data analysis in **Figure 2**, **Figure 5**, and **Supplementary Figure 1B**, the mRNA expression level was visualized with heat map and clustered with the software of R using the "pheatmap" package.

Western blot

Protein isolation was performed as previously described (24). Western blots were performed using the standard SDS-polyacrylamide gel electrophoresis method and enhanced chemiluminescence detection reagents (GE Healthcare Biosciences AB, Uppsala, Sweden). Antibodies against TREM1 (1:1000, Abcam), TREM2 (1:1000, Abcam), TNFα (1:1000, Proteintech), IL-10 (1:1000, Proteintech), β-actin (1:3000, Abcam), and GAPDH (1:3000, Cell signaling technology) were used according to the manufacturer's directions. Immunoreactivity was semi-quantitatively measured by gel densitometric scanning and analyzed by the MCID image analysis system (Imaging Research, Inc.).

Statistical analysis

All results were presented as mean ± standard error of the mean (*SEM*). The differences in the means among multiple groups were analyzed using one- or two-way analysis of variance (*ANOVA*). When *ANOVA* showed significant differences, pair-wise comparisons between means were tested by *Dunnett's test*. The Student's *t* test was used for two-group comparisons. The software used for statistical analysis was R v3.6.3. In all analysis, $P < 0.05$ was considered statistically significant.

Results

Excess salt intake exacerbates disease outcomes of ischemic stroke

Healthy wild type (WT) C57/Bl6 male mice were fed with high salt diet (HSD) or normal diet (ND) for 28d. Mice were then subjected to 60min of transient middle cerebral artery occlusion (tMCAO) and sacrificed at 3d or 7d after cerebral ischemia (**Fig. 1A**). In consistent with previous study (9, 10), mice fed with high salt diet displayed increased lesion volume (**Fig. 1B-C**), detrimental neurological deficit (**Fig. 1G**) and poor

survival rate (**Fig. 1H**). As assessed with immunol staining, we recorded accumulated dead neurons ($NeuN^+TUNEL^+$) in stroke penumbra (**Fig. 1D-E**). Strikingly, at 7d after tMCAO, the number of dead neurons had a 67% reduction (vs. 3d) in mice fed with high salt diet, which was less than that of mice fed with normal diet (80%) (**Fig. 1F**). The results indicated that neurons in high salinity environment suffered processive injury and/or the injured neurons in mice fed with high salt diet were not eliminated in time after stroke.

Surplus salt intake amplifies post stroke neural inflammation

To examine the neural inflammatory status in mice fed with high salt diet after tMCAO, infiltration of immune cells in stroke lesion was analyzed with flow cytometry (**Fig. 2A**). We found that the percentages of T cells ($CD45^+CD3^+$), B cells ($CD45^+CD19^+$), neutrophils ($CD45^{hi}CD11b^+Ly6G^+$) and macrophages ($CD45^{hi}CD11b^+Ly6G^-$) among singlets increased in the ipsilateral hemisphere with ischemic stroke of HSD mice (**Fig. 2A**), while composition of neutrophils ($CD45^{hi}CD11b^+Ly6G^+$) and monocytes/macrophages ($CD45^{hi}CD11b^+Ly6G^-$) in peripheral blood and spleen remained to be comparable (**Supplementary Figure 1A**). With RT-PCR, we recorded that the level of multiple pro-inflammatory cytokines and chemokines elevated in the ipsilateral brain of HSD mice (*Ccl1*, *Cxcl1*, *Cxcl2*, *Cxcl9*, *Il1a* and *Il6*), while the anti-inflammatory markers, including *Il4* and *Arg1*, decreased at the meantime (**Fig. 2B** and **Supplementary Figure 1B**). The results illustrated that post stroke neural inflammation was amplified in mice fed with high salt diet.

Expression of inflammatory resolution associated molecules are down regulated in high salinity environment

To testify the macrophages' role in the drastic neural inflammation of HSD mice, we evaluated the inflammatory resolution of these cells. Immunol staining revealed that the inflammatory resolution associated marker CD206 was down regulated in $Iba1^+$ microglia/macrophages in the lesion of HSD mice at 3d after tMCAO (**Fig. 3A**). In contrast, the number of CD16 expressing $Iba1^+$ microglia/macrophages were up regulated (**Fig. 3A**). To explore the impact of high salinity environment on macrophage, bone marrow derived primary cultured macrophages were treated with 40mM of NaCl overnight in the presence of LPS (100ng/ml) or IL-4 (20ng/ml). We recorded that high concentration of NaCl alone reduced the expression of inflammatory resolution marker of CD206 as assessed with RT-PCR (**Fig. 3B**) and flow cytometry (**Fig. 3C**), and addition of LPS in the culture system further down regulated the expression (**Fig. 3B-C**). As was reported, IL-4 increased the expression of Arg1 and CD206 in primary cultured macrophages. Nevertheless, macrophages failed to response to the IL-4 signaling in high salinity environment (**Fig. 3B-C**). Macrophages pre-treated with NaCl, with or without the presence of IL-4, displayed high expression of TNFα (**Fig. 3B-C**). Our data indicated that high salinity environment undermined the anti-inflammatory or inflammatory resolution property of macrophages.

Efferocytosis of macrophages is impaired in high salinity environment

Efferocytosis represents an important biological process for inflammatory resolution mediated by macrophages. Therefore, we evaluated the impact of high salt environment on the phagocytic activities of macrophages. Clearance of dead/dying neurons was determined by detecting the neuronal marker NeuN within $Iba1^+$ microglia/macrophages in stroke penumbra with confocal microscopy (**Fig. 4A-B**). Under the premise of similar amount of $Iba1^+$ cells (**Fig. 4B**), the number of $Iba1^+NeuN^+$ cells, which indicated the microglia/macrophages that had engulfed neurons, was reduced in HSD mice at 3d after tMCAO compared to ND mice. Triple staining of Iba1/TUNEL/NeuN further revealed dampened phagocytosis of dead/dying neurons by microglia/macrophages as the engulfed dead neurons ($Iba1^+NeuN^+TUNEL^+$) decreased while the un-engulfed dead neurons ($Iba1^-NeuN^+TUNEL^+$) increased in HSD mice. Very few $Iba1^+NeuN^+TUNEL^-$ cells were observed in stroke penumbra in both HSD and ND mice (**Fig. 4A**). Consistently, the phagocytic index, which was calculated as the proportion of dead/dying neurons engulfed by microglia/macrophages, was lower in HSD mice (**Fig. 4B**). We further evaluated the impact of high NaCl concentration on the efferocytic activity of macrophages upon encountering dead/dying neurons *in vitro*. Primary cortical neurons were exposed to 90 min of oxygen-glucose deprivation (OGD), an *in vitro* model simulating ischemic injury. Propidium iodide (PI) was added into neurons 24-hour after OGD (before cell fixation) to label dead/dying cells. Macrophages pre-treated with 40mM of NaCl or equal volume of PBS were exposed to PI-labeled neurons at a ratio of 1:5. Efferocytic capacity of macrophages were evaluated over time with immunol staining and flow cytometry. Macrophages that pre-exposed to high salinity environment displayed reduced efferocytic capacity, as the engulfed dead/dying neurons per macrophage (**Fig. 4D**) or the proportion of phagocytic macrophages ($PI^+F4/80^+$) in high salinity environment (**Fig. 4E**) were lower than those in control group from 0.5-4h after the onset of co-cultured though no difference of cell viability between the two groups was observed (**Fig 4C**). To estimate the capacity of cellular inflammatory resolution, mRNA level of the pro-inflammatory cytokine *Tnfa* and inflammatory resolving molecule *Arg1* was assessed at 6h after the onset of efferocytosis. Macrophages pre-treated with high NaCl displayed increased expression of *Tnfa* and reduced expression of *Arg1* compared with those treated with PBS (**Fig 4F**). Our results revealed that efferocytosis and the subsequent cellular inflammatory resolution of macrophages were impaired in high salinity environment.

Excess salt down regulates TREM2 expression in macrophages and impairs inflammatory resolution

We went on to look into the mechanism of how excess salt suppressed efferocytosis of macrophages. Expression of phagocytosis-related receptors (PRRs) in macrophages treated with 40 mM of NaCl or equal volume of PBS was assessed with PCR array. We recorded that high salt environment down regulated the mRNA level *Trem2*, while expression of other PRRs, including *Trem1* and *Tim4*, remained stable (**Fig. 5A**). Moreover, molecules of downstream signaling of TREM2 were down regulated in high salt concentration including *Arp2*, *Vav3*, and *Rac* (11, 12) (**Supplementary Figure 2**). We confirmed the down regulation of TREM2 in macrophages exposed to excess salt *in vitro* on basis of western blot (**Fig. 5B**) and flow cytometric analysis (FACS) (**Fig. 5C**). We then examined TREM2 expression *in vivo* and recorded that the mRNA (**Fig. 5D**) and protein level (**Fig. 5E-F**) of TREM2 in the ipsilateral hemisphere of

HSD mice was lower than that in ND mice at 3d after tMCAO. Nevertheless, the level of TREM1 did not showed significant alteration in high salinity environment (**Supplementary Figure 3A-B**). When discovering the relationship of TREM2 expression and inflammatory phenotype of macrophages with FACS, we found that macrophages with high TREM2 expression ($CD45^+F4/80^+TREM2^{hi}$) displayed anti-inflammatory phenotype with higher CD206-MFI than those with low TREM2 expression ($CD45^+F4/80^+TREM^{lo}$), while CD16-MFI showed no difference between macrophages with high and low TREM2 expression in both HSD and ND mice (**Fig. 5G**).

Decreased TREM2 expression is correlated with pro-inflammatory property of circulating monocytes and detrimental stroke outcomes in AIS patients

We then tested the TREM2 level in monocytes of AIS patients and evaluated the relationship between TREM2 expression and stroke outcomes. Dietary salt intake of AIS patients was measured with 24-hour urine sodium with a normal limit of 170mmol (13). Thereafter, we found that patients with high urine sodium concentration had larger infarct scale (**Fig. 6A**) and higher NIHSS scores (**Fig. 6B**) than those with normal urine sodium concentration. To assess the impact of excessive salt on phenotypic shift of circulating monocyte in AIS patient during acute phase (0-3d after disease onset), expression of the pro-inflammatory marker CD80 and the anti-inflammatory marker CD206 (14-16) in monocyte ($CD11b^+CD14^+$) of their peripheral blood was analyzed with FACS. Detailed gating strategy is displayed in **Supplementary Figure 4**. As expected, monocyte from stroke patients with high urine sodium concentration expressed less CD206 compared with normal diet stroke patients, while no different expression of CD80 was recorded (**Fig. 6C, D** and **Supplementary Figure 5**). We documented that TREM2 expression in monocytes was down regulated in stroke patients with high urine sodium concentration compared with those with normal diet using FACS (**Fig. 6C, E** and **Supplementary Figure 5**). Moreover, we found that the *TREM2* mRNA level decreased in the peripheral blood mononuclear cells (PBMC) of patients with high urine sodium concentration (**Fig. 6F**), while expression of other PRRs remained to be stable (**Supplementary Figure 6**). Since PRRs are mainly expressed in monocytes in PBMC (17), our data indicated that high salinity environment specifically down regulated *TREM2* expression in monocytes of AIS patients. Through spearman correlation analysis, we recorded that CD206 MFI of peripheral blood monocyte showed significant positive correlation with TREM2 MFI in stroke patients, while CD80 MFI showed negative correlation with TREM2 MFI (**Supplementary Figure 7**), which was in consistent with our data in animal models. Interestingly, we found that TREM2 expression in the circulating monocyte of AIS patients was negatively correlated with the 24-hour urine excretion (**Fig. 6G** and **Supplementary Figure 7**), while decreased TREM2 level of macrophages was associated with increased NIHSS scores (**Fig 6G** and **Supplementary Figure 7**). The results indicated that TREM2 expression in monocytes/macrophages favored efferocytosis and the subsequent inflammatory resolution after ischemic stroke.

Enhancing TREM2 signaling restores the efferocytic capacity and cellular inflammatory resolution of macrophages in high salinity environment

TREM2 is a vital functional molecule implicated in the phagocytosis activity of macrophages. The efferocytosis capacity of macrophages plays a decisive role in inflammatory resolution after stroke and affects the disease outcomes. Therefore, we hypothesized that enhancing TREM2 signaling in macrophages could restore their efferocytic capacity and promote inflammatory resolution. Macrophages were infected with lent viral vectors carrying TREM2 cDNA or empty vector for 2d with or without addition of NaCl (40mM) and incubated with PI-labeled post-OGD neurons. Efficacy of transfection was confirmed with flow cytometry (**Fig. 7A**) and western blot (**Fig. 7B**). Gratifyingly, overexpressing TREM2 in macrophages exposed to excess salt restored the efferocytic capacity as the engulfed dead/dying neurons per macrophage (**Fig. 7C**) or the proportion of phagocytic macrophages ($PI^+F4/80^+$) (**Fig. 7D**) recovered to that of PBS treated macrophages at 1h after co-culture. Moreover, at 24h after co-culture, protein level of CD206 and Arg1 in TREM2 over-expressed macrophages treated with high concentration of NaCl resembled that treated with PBS (**Fig. 7E**). Our data revealed that enhancing TREM2 signaling could restore the efferocytic capacity and cellular inflammatory resolution of macrophages which were damaged by surplus salt concentration in the microenvironment.

Discussion

The current study documents that microenvironment with excess salt concentration could impair the inflammatory resolution property of macrophages after ischemic stroke. Mechanistically, surplus salt down regulates TREM2 expression in macrophages, which is associated with decreased efferocytic capacity, excessive neural inflammation and exacerbated stroke outcomes.

It has been reported that high salt diet could promote BBB injury after ischemic stroke (9). Consistently, we recorded that the increased infiltration of multiple leukocytes, including macrophage, neutrophil, T lymphocyte and B lymphocyte, in the stroke lesion of HSD mice at 3d after stroke, could be attributed to the exacerbated BBB damage. It was found that surplus dietary salt directed macrophages/microglia towards the classical activated “M1” phenotype, which further exacerbated stroke outcomes (10). In accordance, our data indicated that the inflammatory resolution property of macrophages were down regulated by excess salt, which led to postponed recovery of stroke lesion.

Efferocytosis represents a key process of inflammatory resolution. Elimination of the dead or injured components within stroke lesion arrests amplification of neural inflammation. We demonstrated that the efferocytic capacity, together with the subsequent cellular inflammatory resolution of macrophages, were impaired in high salinity environment, which could be the reason for accumulated dead cells in the stroke penumbra. It has been demonstrated that the function of TREM2 is indispensable for phagocytic activities of microglia and macrophages (18). Our data indicated that TREM2 was down regulated in macrophages by the high salinity environment. Decreased TREM2 expression was correlated with robust post-stroke neural inflammation and exacerbated stroke outcomes, which indicated that inhibition of TREM2 signaling in macrophages was the potential mechanism involved in the detrimental impact of high salt microenvironment.

It has been recognized that high salt diet is a key risk factor for ischemic stroke. Restriction of dietary salt intake serves as an efficient prevention of new vascular events. Nevertheless, no niched therapy that targets the already impaired inflammatory resolution property of macrophages in the high salt environment has been reported. Our study revealed that overexpression of TREM2 could restore the efferocytic capacity and cellular inflammatory resolution of macrophages in high salinity environment. The data appealed further research on the therapeutic potential of enhancing TREM2 signaling in patents of ischemic stroke, especially those with high salt intake.

Conclusions

Conclusively, HSD aggravated ischemic stroke outcomes by exacerbated neural inflammation, which was associated with the impaired inflammatory resolution property of macrophages. TREM2 expression in macrophages was down regulated by high salt environment, and enhancing TREM2 signaling could restore the efferocytic capacity and cellular inflammatory resolution of macrophages. Further study on the value of TREM2 signaling as a therapeutic target in AIS is warranted.

List Of Abbreviations

HSD	High salt diet
tMCAO	transient middle cerebral occlusion
TREM2	triggering receptor expressed on myeloid cells 2
BBB	blood-brain barrie r
ND	normal diet
WT	wild type
OGD	oxygen-glucose deprivation
PRRs	phagocytosis-related receptors
PBMC	peripheral blood mononuclear cell
MRI	Magnetic resonance imaging
ECA	external carotid artery
MCA	middle cerebral artery
ICA	internal carotid artery
CBF	cerebral blood flow
LPS	lipopolysaccharide
MCSF	macrophage colony stimulating factor
PI	propidium iodide
Msn	moesin
Rhoa	ras homolog family member A
Arp2	actin related protein 2
Myd88	MYD88 innate immune signal transduction adaptor
Itgam	integrin subunit alpha M
Syk	spleen associated tyrosine kinase
Tnfa	tumor necrosis factor alpha
Arp3	Actin-related protein 3
C3	complement C3
Alkbh5	alkB homolog 5, RNA demethylase
Itga4	integrin subunit alpha 4
Pten	phosphatase and tensin homolog

Mapk14	mitogen-activated protein kinase 14
Ager	advanced glycosylation end-product specific receptor
Tlr9	toll like receptor 9
Tlr4	toll like receptor 4
Trem1	triggering receptor expressed on myeloid cells 1
Fcgr1	Fc receptor, IgG, high affinity I
Pros1	protein S
Tlr7	toll like receptor 7
Vav3	vav guanine nucleotide exchange factor 3
Fasl	Fas ligand (TNF superfamily, member 6)
Il10	interleukin 10
Arg1	arginase 1
Rac	AKT serine/threonine kinase 1
Cd206	CD206 antigen
Pip5k1b	phosphatidylinositol-4-phosphate 5-kinase type 1 beta
Csk	C-terminal Src kinase
Csf1r	colony stimulating factor 1 receptor
Cd36	CD36 antigen
Cd44	CD44 antigen
Cd16	CD16 antigen
Pecam1	platelet and endothelial cell adhesion molecule 1
Ifng	interferon gamma
Mertk	MER proto-oncogene, tyrosine kinase
Stat1	signal transducer and activator of transcription 1
Apoe	apolipoprotein E
Csf1	colony stimulating factor 1
Tlr3	toll like receptor 3
Stat6	signal transducer and activator of transcription 6
Itgav	integrin subunit alpha V

Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Ccl1	chemokine (C-C motif) ligand 1
Ccl2	chemokine (C-C motif) ligand 2
Cx3cr1	C-X3-C motif chemokine receptor 1
Ccl6	chemokine (C-C motif) ligand 6
Cxcl1	C-X-C motif chemokine ligand 1
Cxcl2	C-X-C motif chemokine ligand 2
Cxcl5	C-X-C motif chemokine ligand 5
Cxcl7	C-X-C motif chemokine ligand 7
Cxcl9	C-X-C motif chemokine ligand 9
Cxcl10	C-X-C motif chemokine ligand 10
Cxcl11	C-X-C motif chemokine ligand 11
Il1a	interleukin 1 alpha
Il1b	interleukin 1 beta
Il1ra	interleukin 1 receptor antagonist
Il6	interleukin 6
Il12	interleukin 12
Il18	interleukin 18
Spp1	secreted phosphoprotein 1
Tgfb	transforming growth factor beta
Tgfbr1	transforming growth factor beta receptor 1
Il4	interleukin 4
Mfge8	milk fat globule EGF and factor V/VIII domain containing
Crp	C-reactive protein, pentraxin-related
Il1r1	interleukin 1 receptor type 1
Cd14	CD14 antigen
Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide
Fcgr2b	Fc receptor, IgG, low affinity IIb
Colec12	collectin sub-family member 12

Clec7a	C-type lectin domain containing 7a
Tim4	T cell immunoglobulin and mucin domain containing 4

Declarations

Ethics approval and consent to participate

The clinical and the animal experimental studies were approved by the Medical Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University and the Animal Care and Use Committee of Sun Yat-Sen University respectively. All participants had signed the informed consent according to the principles illustrated in Declaration of Helsinki. All animal experiments were approved by the Third Affiliated Hospital of Sun Yat-sen University and performed following the Guide for the Care and Use of Laboratory Animals and Stroke Treatment.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MH designed and performed the experiments, collected and analyzed data, and drafted the manuscript. YL and XM contributed to the experimental design and revised the manuscript. QZ and DL performed animal experiments and collected data. SL and BZ contributed to the experimental design and the manuscript. WC and ZL designed and supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

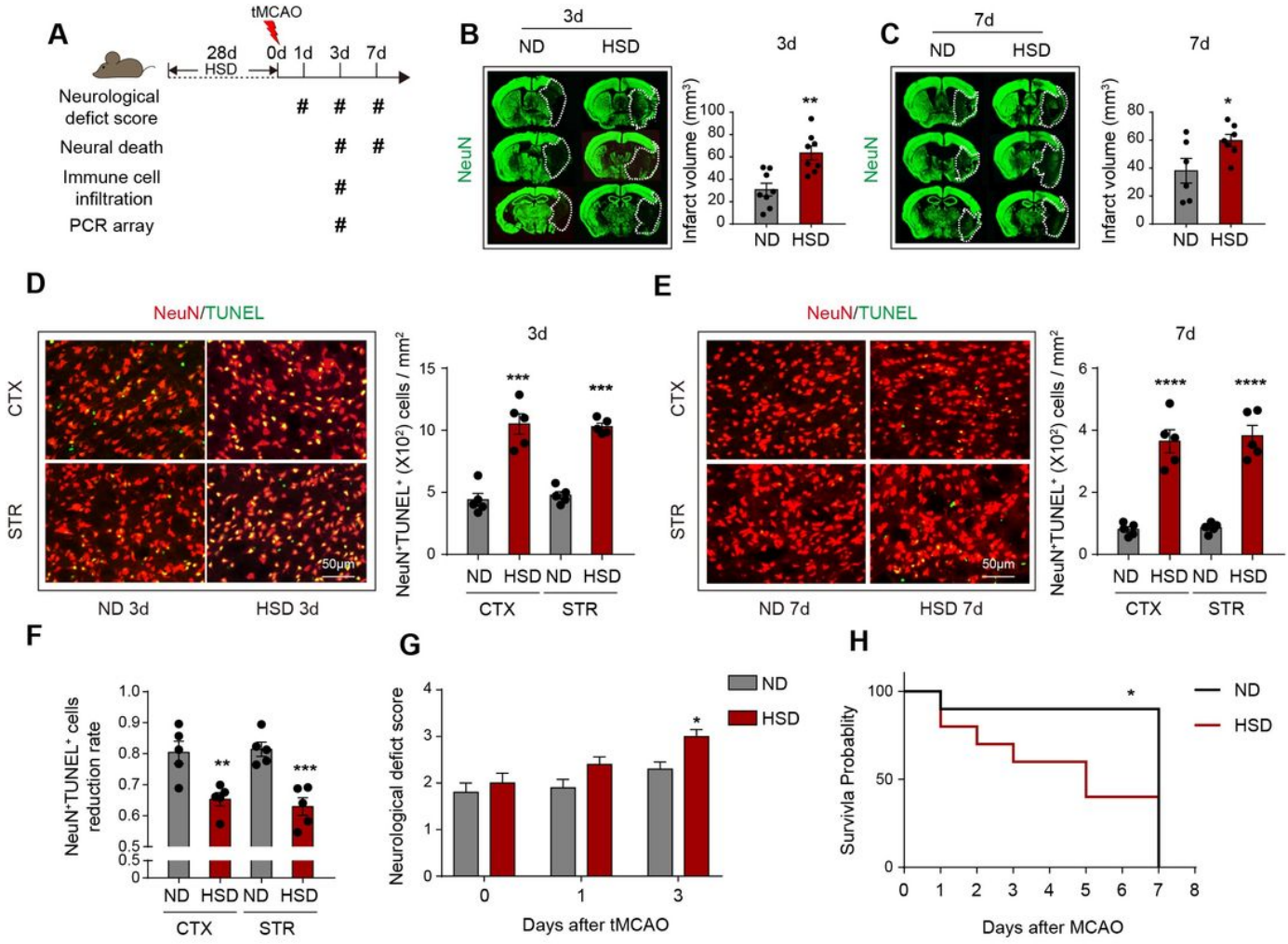
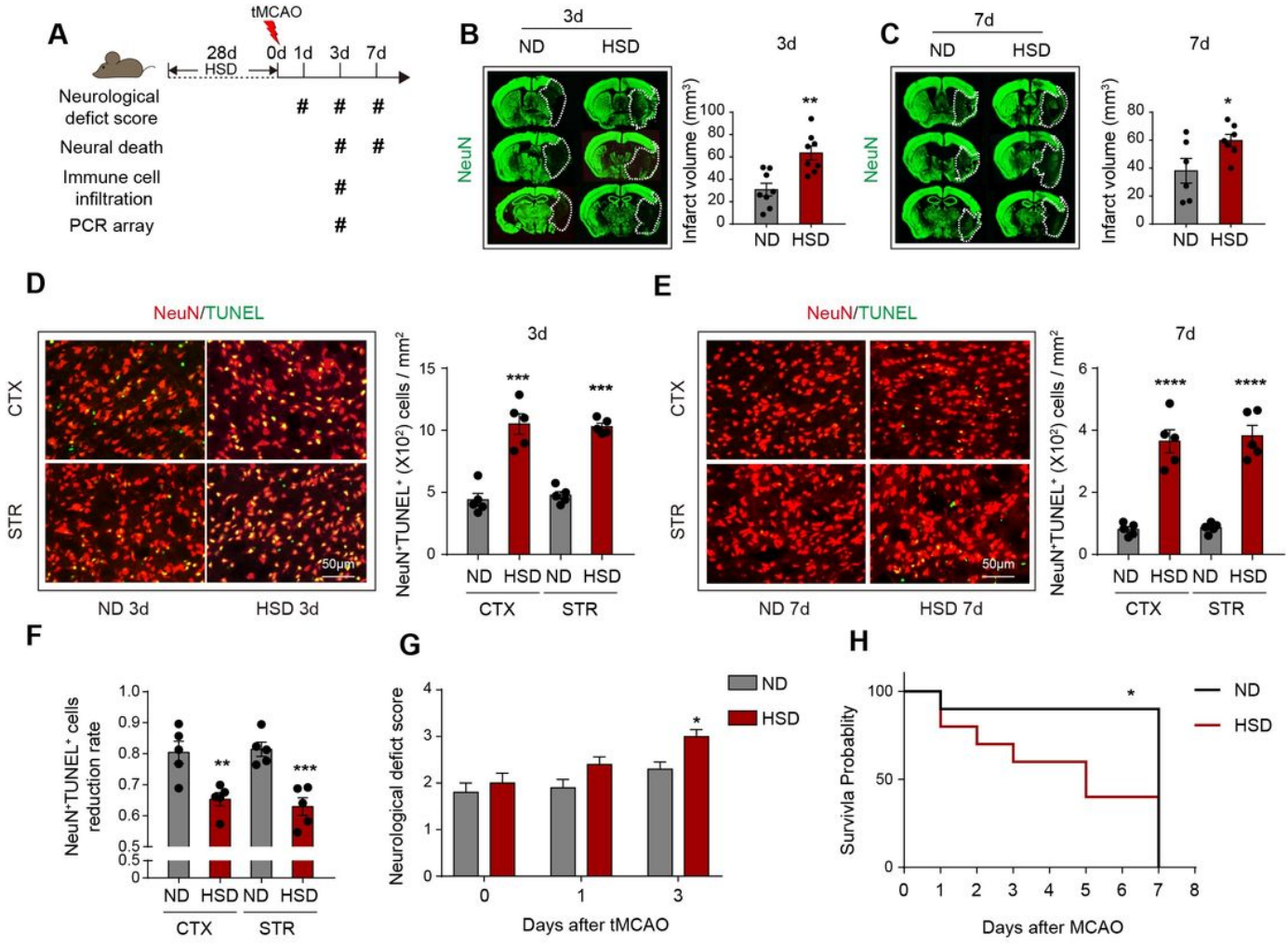


Figure 1

Excess salt intake exacerbates disease outcomes of ischemic stroke. C57/Bl6 mice were fed with ND or HSD for 28d and were subjected to 60min of tMCAO. Animals were sacrificed at 3d or 7d after tMCAO. (A) The timeline of experimental design. (B-C) Infarct volume of male mice was quantified on NeuN (green)-stained coronal sections at 3d and 7d. Dashed lines outline the infarct area. N = 6-8 mice per group, *P < 0.05, **P < 0.01 versus ND group in t-test. (D and E) Representative images demonstrating TUNEL (green) co-labeling with NeuN (red) in infarct penumbra at 3d and 7d after tMCAO. The number of NeuN+TUNEL+ neurons were quantified. N = 5 mice per group. ***P < 0.001, ****P < 0.0001 versus ND group in t-test. (F) The reduction rate of dead/dying neurons from 3d-7d after tMCAO was calculated. N = 5 mice per group, **P < 0.01, ***P < 0.001 versus ND group in t-test. (G) Neurological deficit score was assessed at 0-3d after tMCAO. N = 10 per group, *P < 0.05 versus ND group in t-test. (H) Survival of mice was recorded on 0-7d after tMCAO. N = 10 mice per group. *P < 0.05 versus ND group in t-test. ND, normal diet; HSD, high salt diet; STR, striatum; CTX, cortex.



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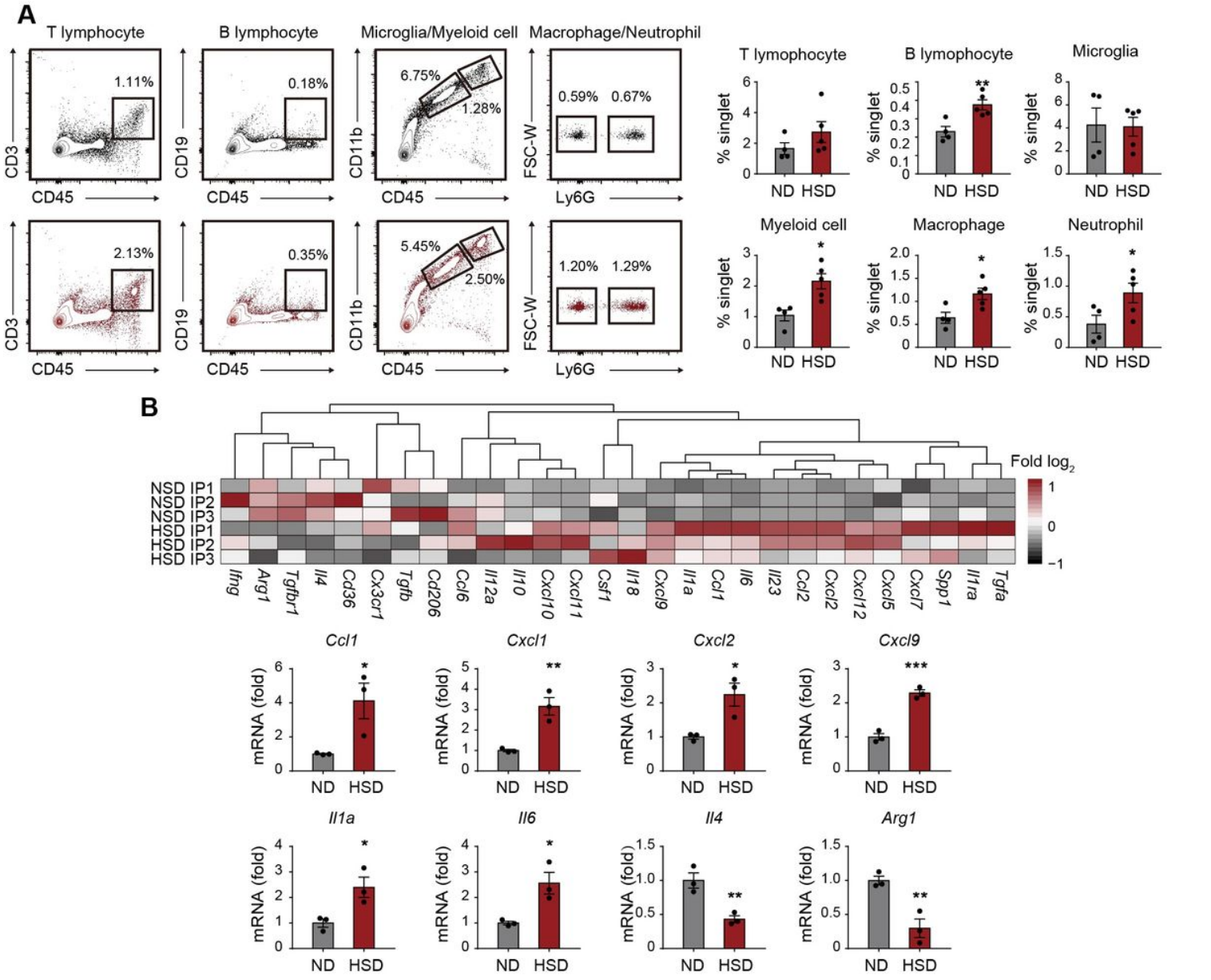
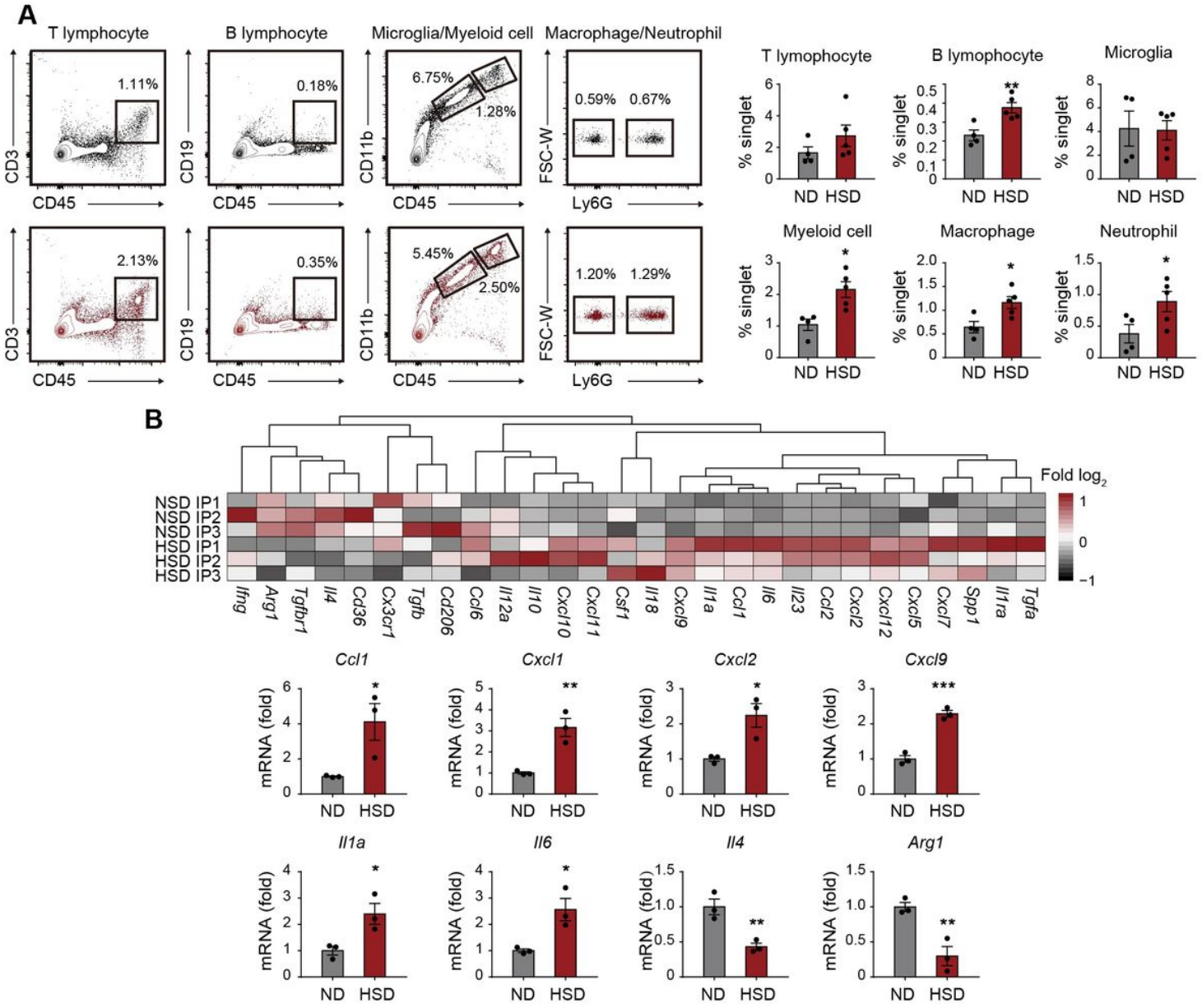


Figure 2

Surplus salt intake exacerbates post stroke neural inflammation. (A) Leukocyte infiltration in the ischemic brain at 3d after tMCAO was analyzed with flow cytometry. Representative flow plots showing infiltrated T lymphocytes (CD45hiCD3+), B lymphocytes (CD45hiCD19+), microglia (CD45inCD11b+), myeloid cells (CD45hiCD11b+), neutrophils (CD45hiCD11b+Ly6G+), and macrophages (CD45hiCD11b+Ly6G-) in ND and HSD brains, and the corresponding statistical analysis were displayed. N = 4 for ND and N = 5 mice for HSD group. *P < 0.05 **P < 0.01, versus ND group in t-test. (B) mRNA was extracted from the ipsilateral hemisphere of stroke mice at 3d after tMCAO and subjected to RT-PCR. N = 3 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, versus ND group in t test. IP, ipsilateral.



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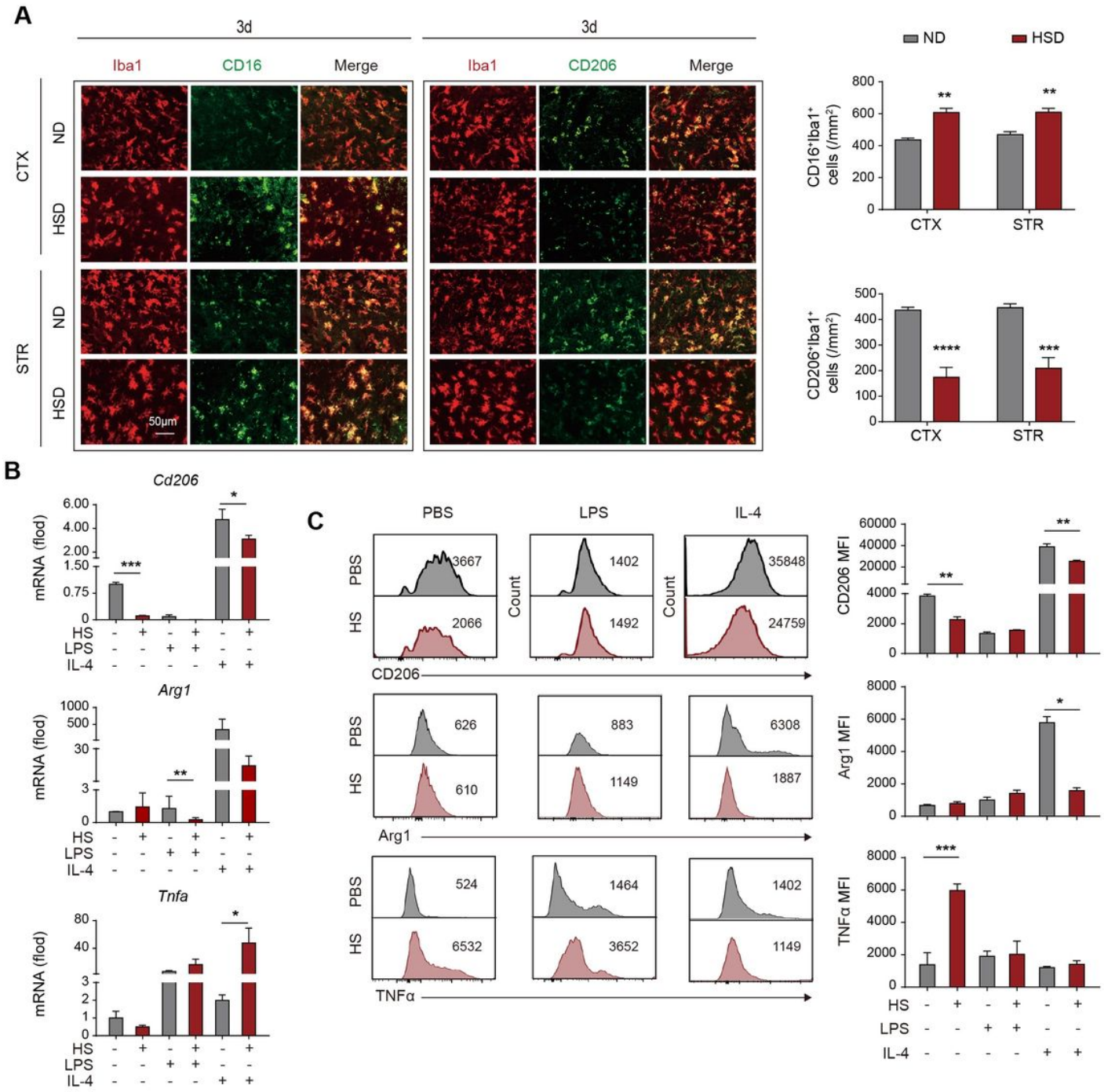

Figure 3

Expression of inflammatory resolution associated molecules in macrophage is down regulated in high salinity environment. (A) Brain sections were collected from ND and HSD mice at 3d after 60min tMCAO. Expression of CD16 (green) or CD206 (green) in Iba1+ cells (marker of microglia and macrophage) was analyzed with immunostaining. N = 3 mice per group. **P < 0.01, ***P < 0.001, ****P < 0.0001 versus ND group in t test. (B-C) Bone marrow derived primary cultured macrophages were treated with 40mM of NaCl overnight at the presence of LPS (100ng/ml) or IL-4 (20ng/ml). Expression of CD206, Arg1, and TNFα was assessed with RT-PCR (B) and flow cytometry (C). Comparable cell counts were analyzed

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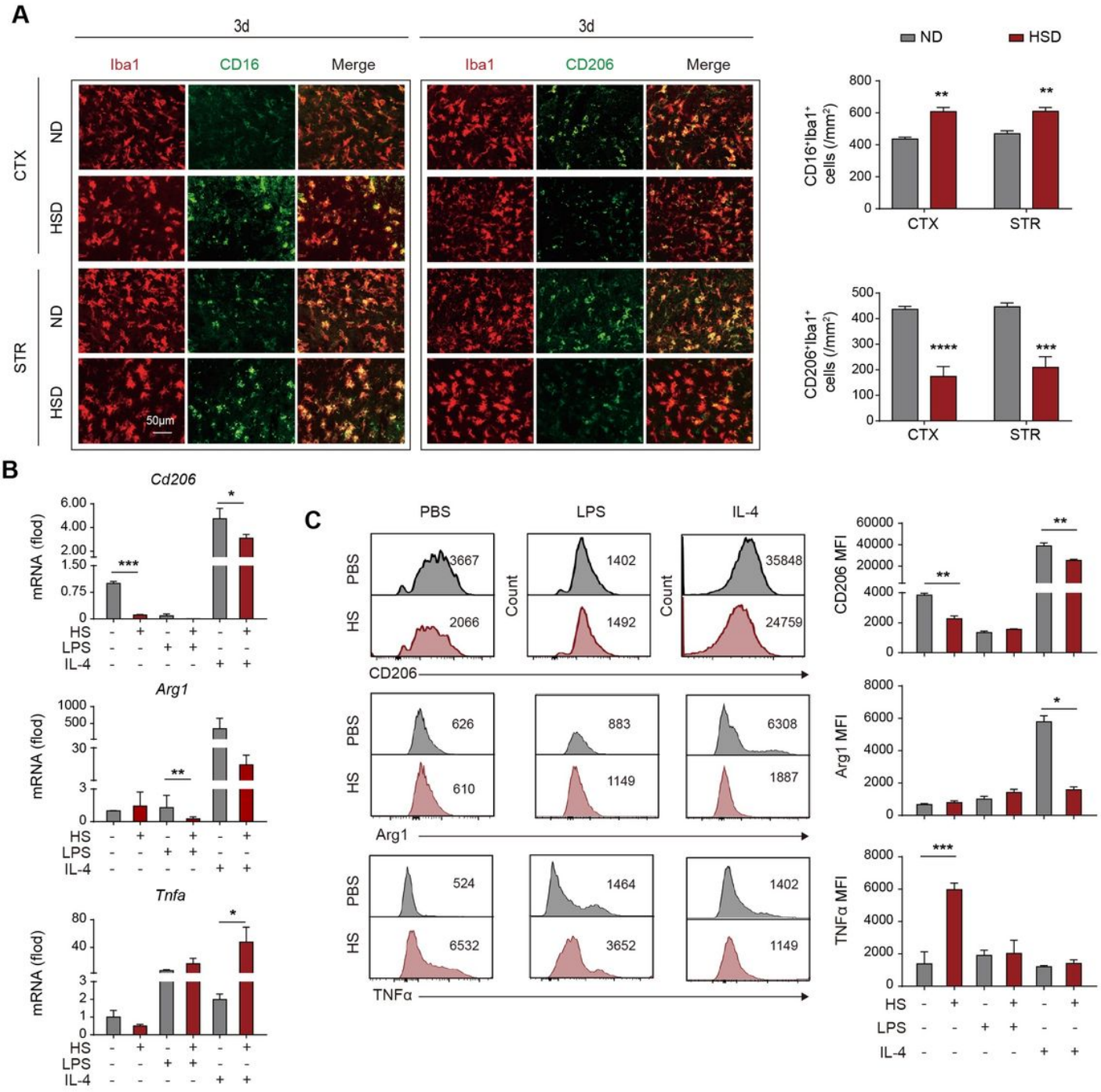

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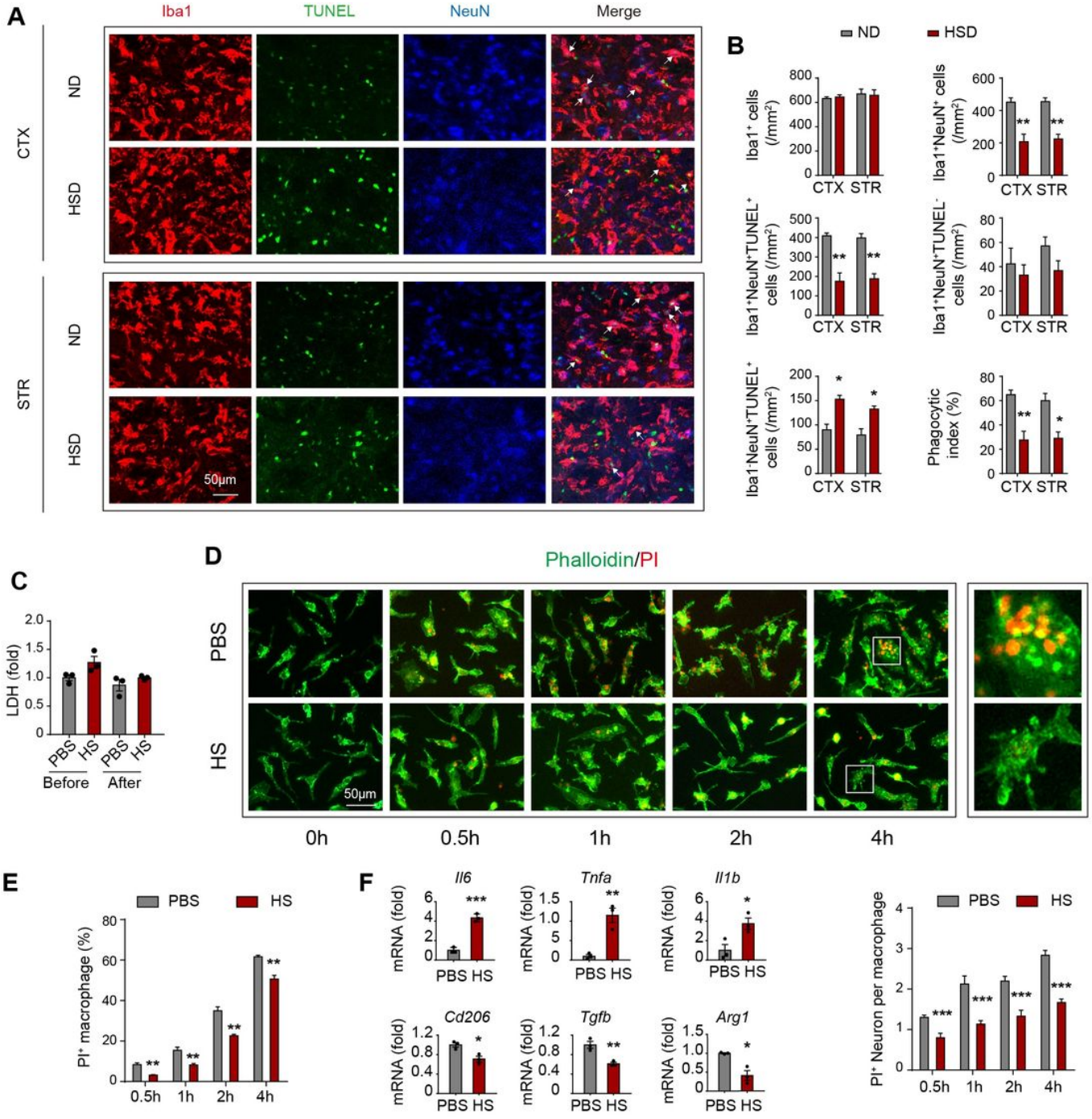
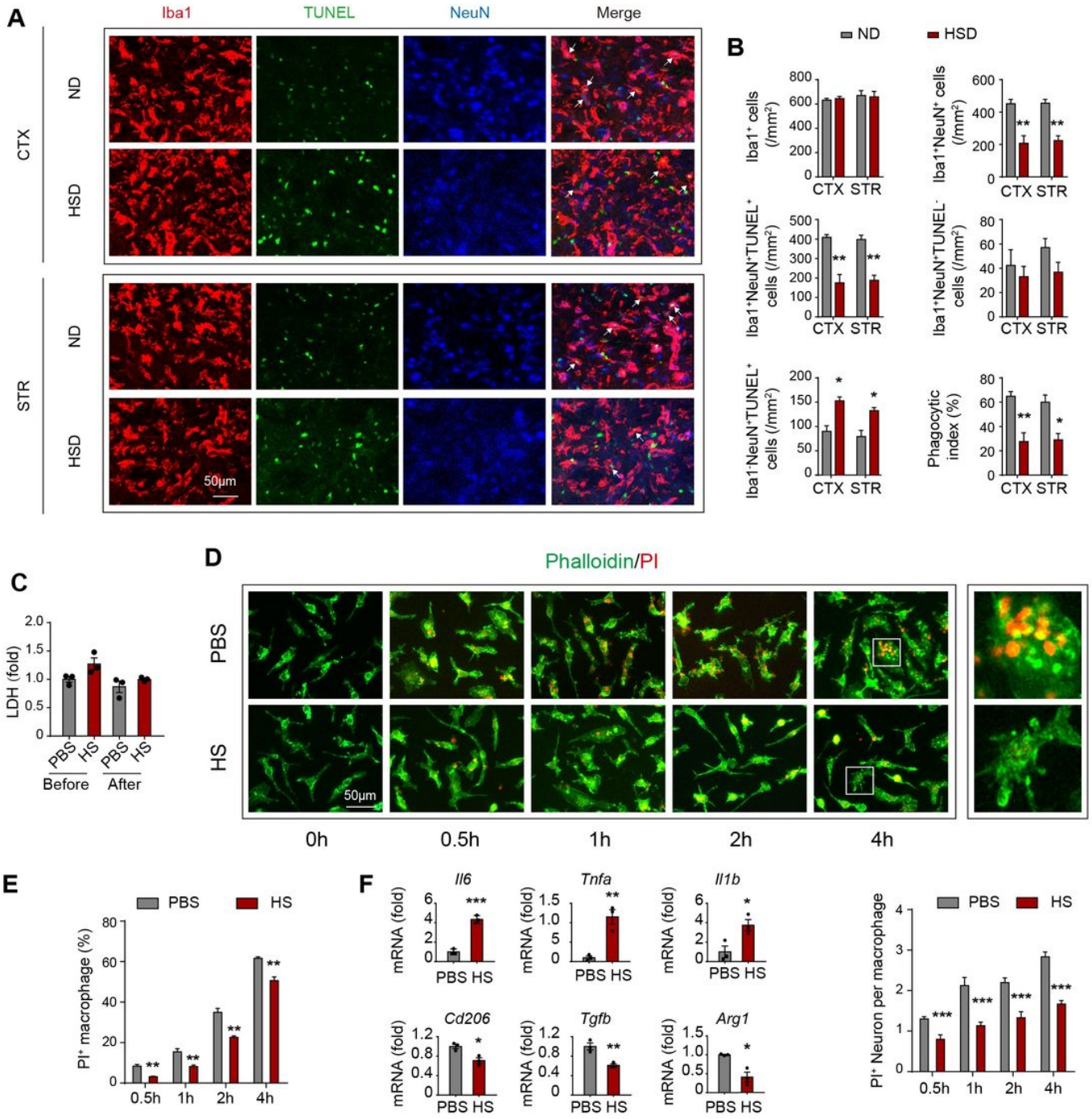


Figure 4

Efferocytosis of macrophages is impaired in high salinity environment. (A-B) ND and HSD mice were subjected to 60min of tMCAO. Brain sections were collected at 3d after cerebral ischemia. (A) Confocal microscopy analysis of NeuN (blue), TUNEL (green), and Iba1 (red) triple-staining. White arrows indicate

microglia/macrophages that engulfed dead/dying neurons (Iba1+NeuN+TUNEL+). (B) Quantification of the total number of Iba1+ microglia/macrophages, Iba1+NeuN+ cells (microglia/macrophages that have engulfed neurons), Iba1+NeuN+TUNEL+ cells (microglia/macrophages that engulfed dead/dying neurons), Iba1+NeuN+TUNEL- cells (microglia/macrophages that colocalize with TUNEL- neurons), Iba1-NeuN+TUNEL+ cells (not engulfed dead neurons), and phagocytic index (the proportion of dead/dying neurons engulfed by microglia/macrophages) in ischemic areas. N = 3 mice per group. *P < 0.05, **P < 0.01, versus ND group in t test. (C) Macrophage viability before and after 4h of neuron-efferocytosis was quantified by LDH-assay. Data were collected from 3 independent experiments. (D-E) Phagocytosis of PI+ dead/dying neurons by macrophages was evaluated with immunostaining (D) and flow cytometry (E). Engulfed dead/dying neurons (PI+) per macrophage (Phalloidin labelled) or the proportion of phagocytic macrophages (PI+) was quantified at indicated time points. The right images enlarged the boxed area. Data were collected from 3 independent experiments. **P < 0.01, ***P < 0.001 versus PBS group in t test. (F) Dead neurons were treated to macrophages at a ratio of neuron : macrophage = 5:1. After 6h, expression of pro-inflammatory (Tnfα, Il6 and Il1β) and inflammatory resolving molecules (Arg1, CD206, TGFβ) in the efferocytic macrophages were analyzed with RT-PCR. Data represent 3 independent experiments in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 versus PBS group in t test.



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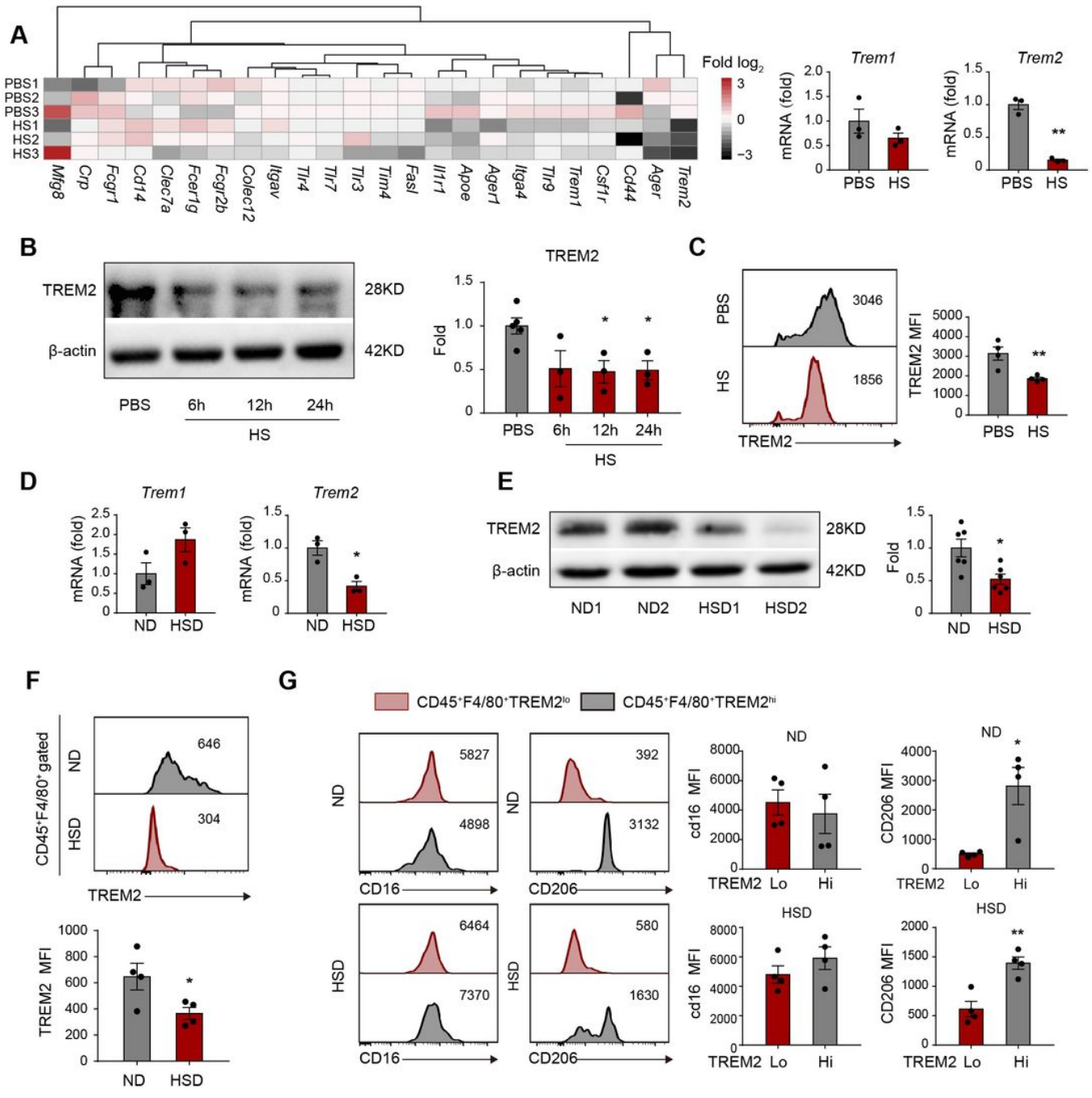
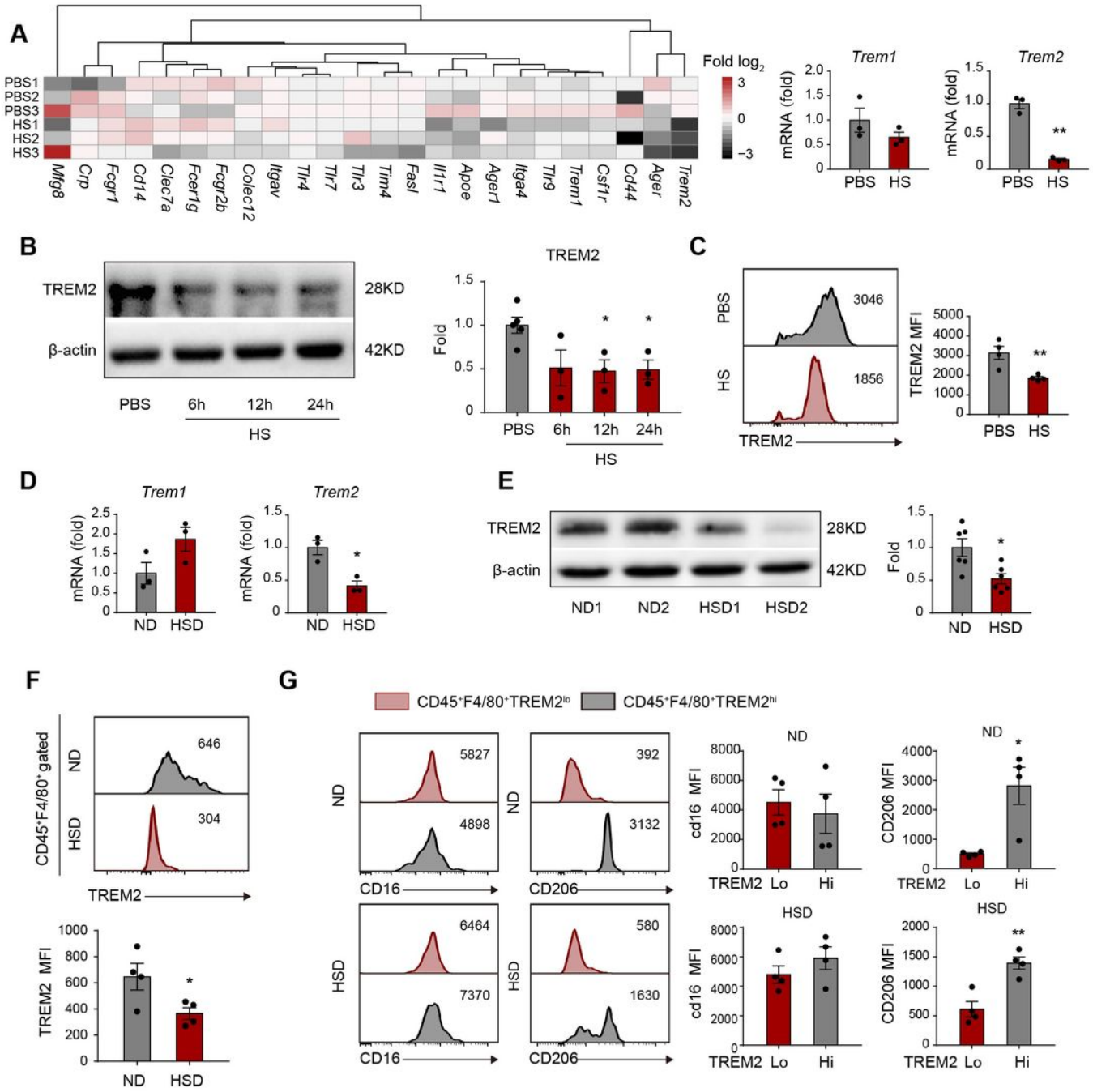


Figure 5

Excess salt down regulates efferocytic molecule TREM2 in macrophages and impairs their inflammatory resoluting functions. (A) Macrophages cultured in high salt environment were subjected to PCR array to analyze expression of efferocytosis associated receptors. Data are displayed as fold change to macrophage from PBS-treated group. Experiments were repeated for 3 times. **P < 0.01 versus PBS group in t test. (B-C) Protein expression of TREM2 in macrophage after HS treatment was evaluated with western blot (B) and flow cytometry (C). Experiments were repeated for 3 times. *P < 0.05, versus PBS-treated group in t test. (D-G) ND and HSD mice were subjected to 60min of tMCAO. Brains were collected

at 3d after tMCAO. (D) mRNA expression of Trem1 and Trem2 in ipsilateral hemisphere of stroke mice was analyzed with RT-PCR. CT value was normalized to that of ND mice. N = 3 mice per group. *P < 0.05, versus ND group in t test. (E) Protein expression of TREM2 in ipsilateral hemisphere of stroke mice with western blot. N = 6 mice per group. *P < 0.05, versus ND group in t test. (F) Protein expression of TREM2 in infiltrated macrophages (CD45hiCD11b+Ly6G-) of stroke mice was analyzed with flow cytometry. N = 4 mice per group. *P < 0.05, versus ND group in t test. (G) The expression of CD16 and CD206 in TREM2lo and TREM2hi macrophages (CD45hiCD11b+Ly6G-) in ipsilateral hemisphere from ND and HSD mice was measured by flow cytometry. Mean fluorescent intensity (MFI) of CD16 or CD206 was quantified. N = 4 mice per group. *P < 0.05, **P < 0.01 versus TREM2lo macrophages in t test.



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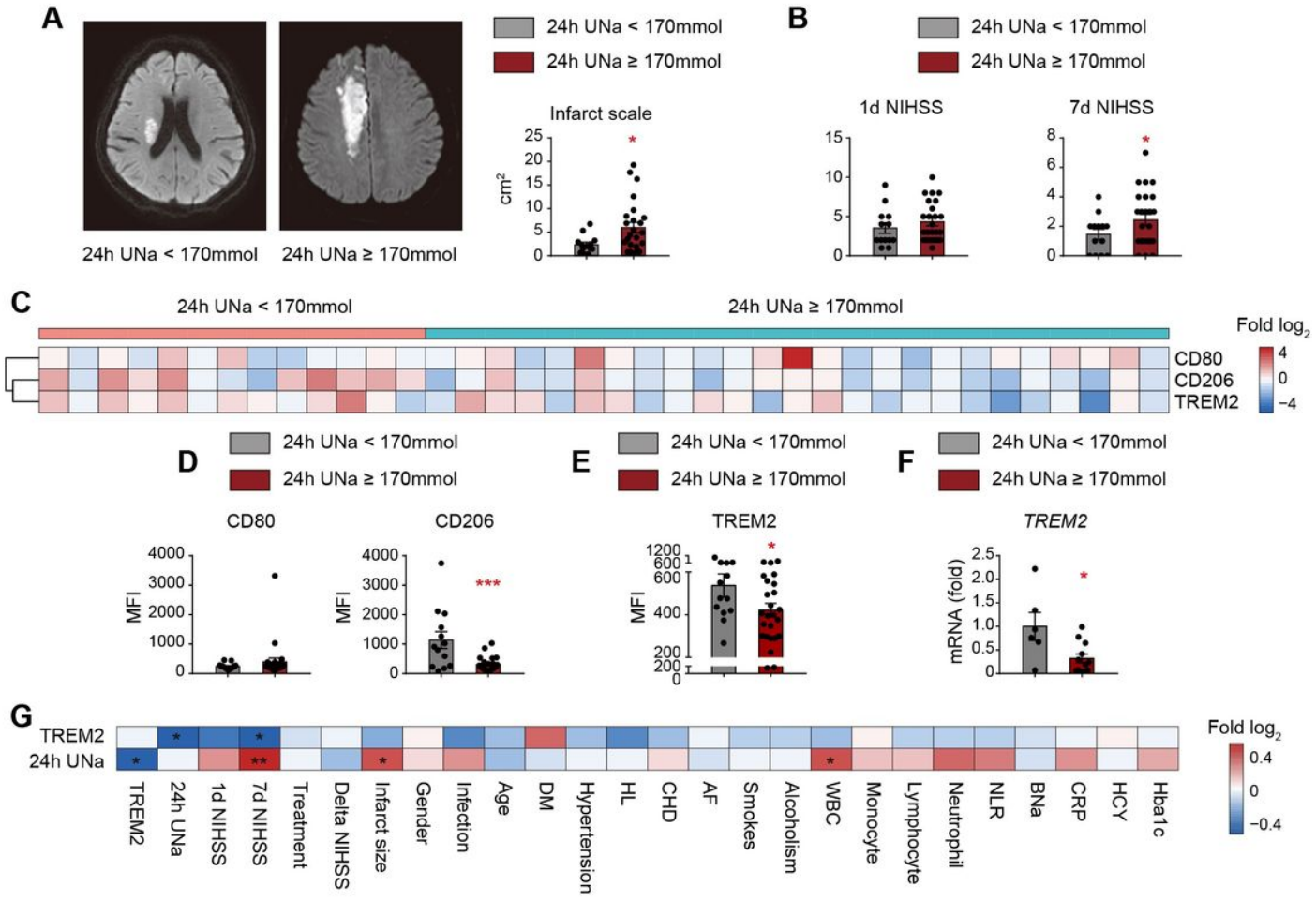


Figure 6

TREM2 expression is decreased in AIS patients with high salt intake and is correlated with the pro-inflammatory property of circulating monocytes and detrimental stroke outcomes. AIS patient dietary salt intake was measured with 24-hour urine sodium (24h UNa) with a normal limit of 170mmol. (A) Representative images of the MRI-DWI of AIS patients with normal or high urine sodium concentration. Comparison of infarct scale of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration. *P < 0.05 by Student's t test. (B) Comparison of 1d NIHSS, 7d NIHSS of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration. *P < 0.05 by Student's t test. (C) Heat map showing the TREM2, CD206, CD80 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration using flow cytometry. The function of "scale" was applied for value normalization. (D) Comparison of CD206 and CD80 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high urine sodium concentration (N = 25) using flow cytometry . *P < 0.05 by Student's t test. (E) Comparison of TREM2 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high urine sodium concentration (N = 25) using flow cytometry. *P < 0.05 by Student's t test. (F) Comparison of TREM2 mRNA expression in PBMC of AIS patients with normal (N = 6) or high urine sodium concentration (N = 12). *P < 0.05 by Student's t test. (G) Correlation of clinic parameters, the protein expression of TREM2 and 24h UNa was assessed with Spearman correlation analysis. *P < 0.05, **P < 0.01. DWI, diffusion-weighted imaging; DM, diabetes mellitus; HL, hyperlipidemia; CHD, coronary heart disease; AF, atrial fibrillation; WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; BNa, blood sodium; CRP, C-Reactive Protein; HCY, homocysteine; Hba1c, glycated hemoglobin.

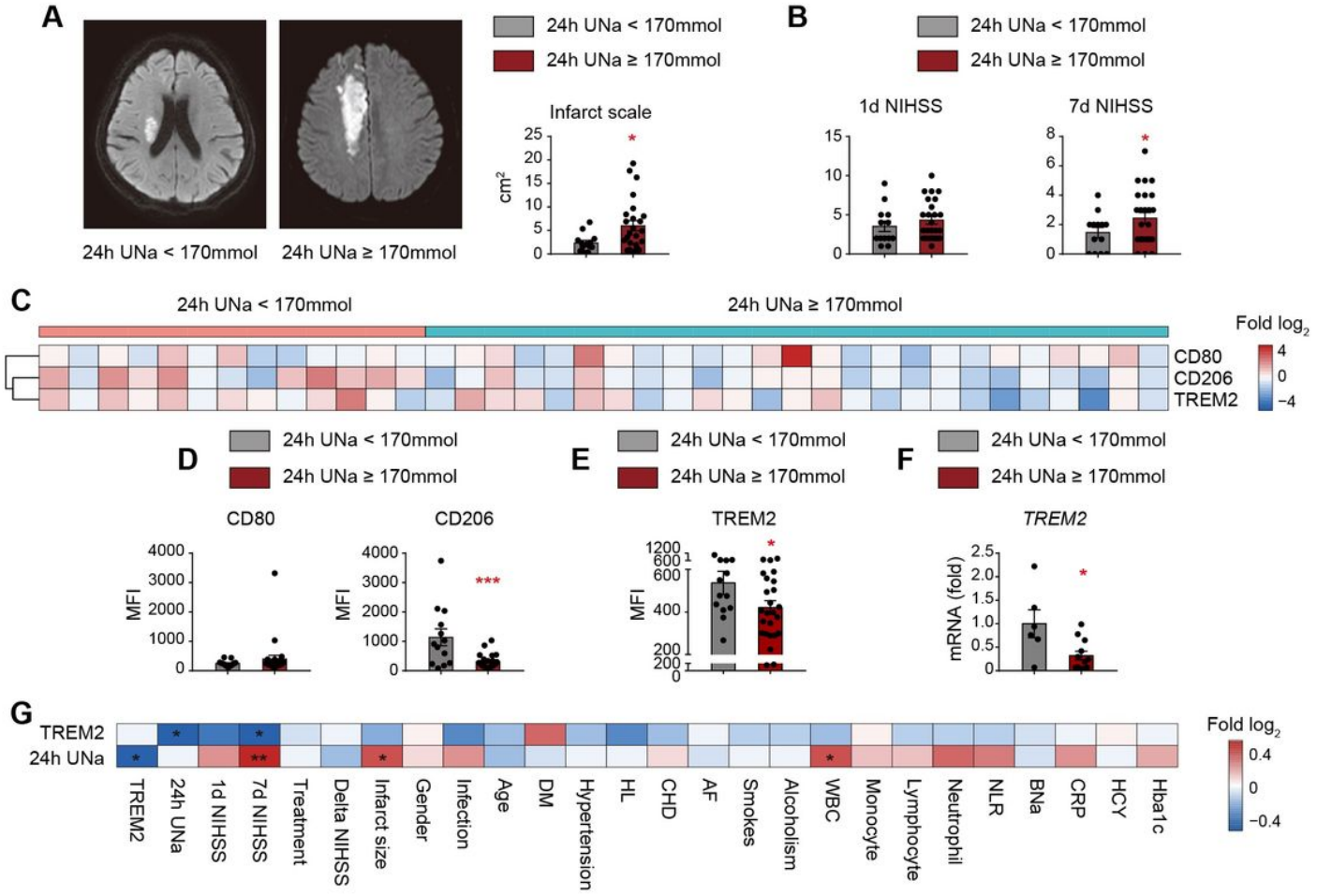


Figure 6

TREM2 expression is decreased in AIS patients with high salt intake and is correlated with the pro-inflammatory property of circulating monocytes and detrimental stroke outcomes. AIS patient dietary salt intake was measured with 24-hour urine sodium (24h UNa) with a normal limit of 170mmol. (A) Representative images of the MRI-DWI of AIS patients with normal or high urine sodium concentration. Comparison of infarct scale of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration. *P < 0.05 by Student's t test. (B) Comparison of 1d NIHSS, 7d NIHSS of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration. *P < 0.05 by Student's t test. (C) Heat map showing the TREM2, CD206, CD80 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high (N = 25) urine sodium concentration using flow cytometry. The function of "scale" was applied for value normalization. (D) Comparison of CD206 and CD80 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high urine sodium concentration (N = 25) using flow cytometry . *P < 0.05 by Student's t test. (E) Comparison of TREM2 protein expression in peripheral monocyte of AIS patients with normal (N = 13) or high urine sodium concentration (N = 25) using flow cytometry. *P < 0.05 by Student's t test. (F) Comparison of TREM2 mRNA expression in PBMC of AIS patients with normal (N = 6) or high urine sodium concentration (N = 12). *P < 0.05 by Student's t test. (G) Correlation of clinic parameters, the protein expression of TREM2 and 24h UNa was assessed with Spearman correlation analysis. *P < 0.05, **P < 0.01. DWI, diffusion-weighted imaging; DM, diabetes

mellitus; HL, hyperlipidemia; CHD, coronary heart disease; AF, atrial fibrillation; WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; BNa, blood sodium; CRP, C-Reactive Protein; HCY, homocysteine; Hba1c, glycated hemoglobin.

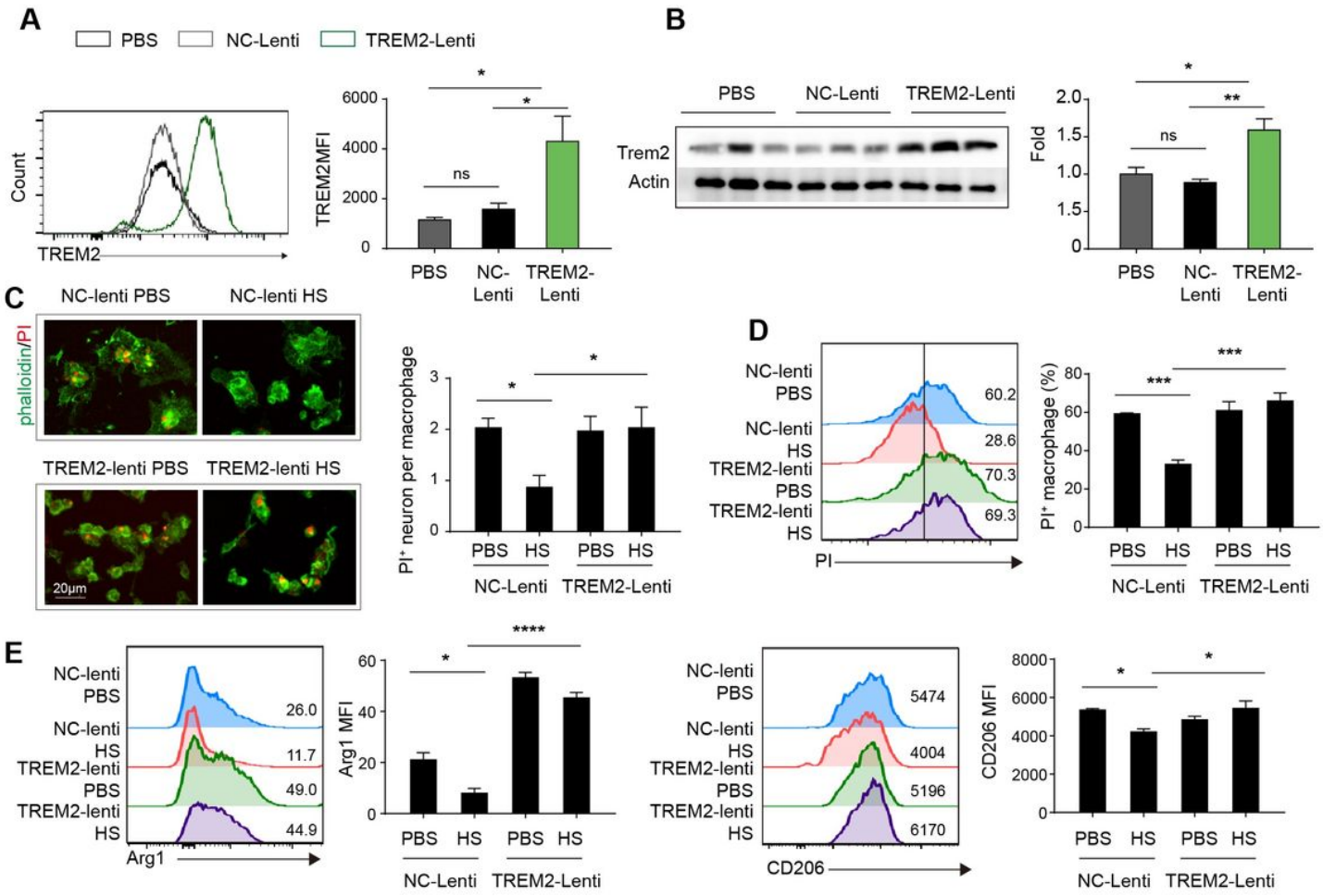
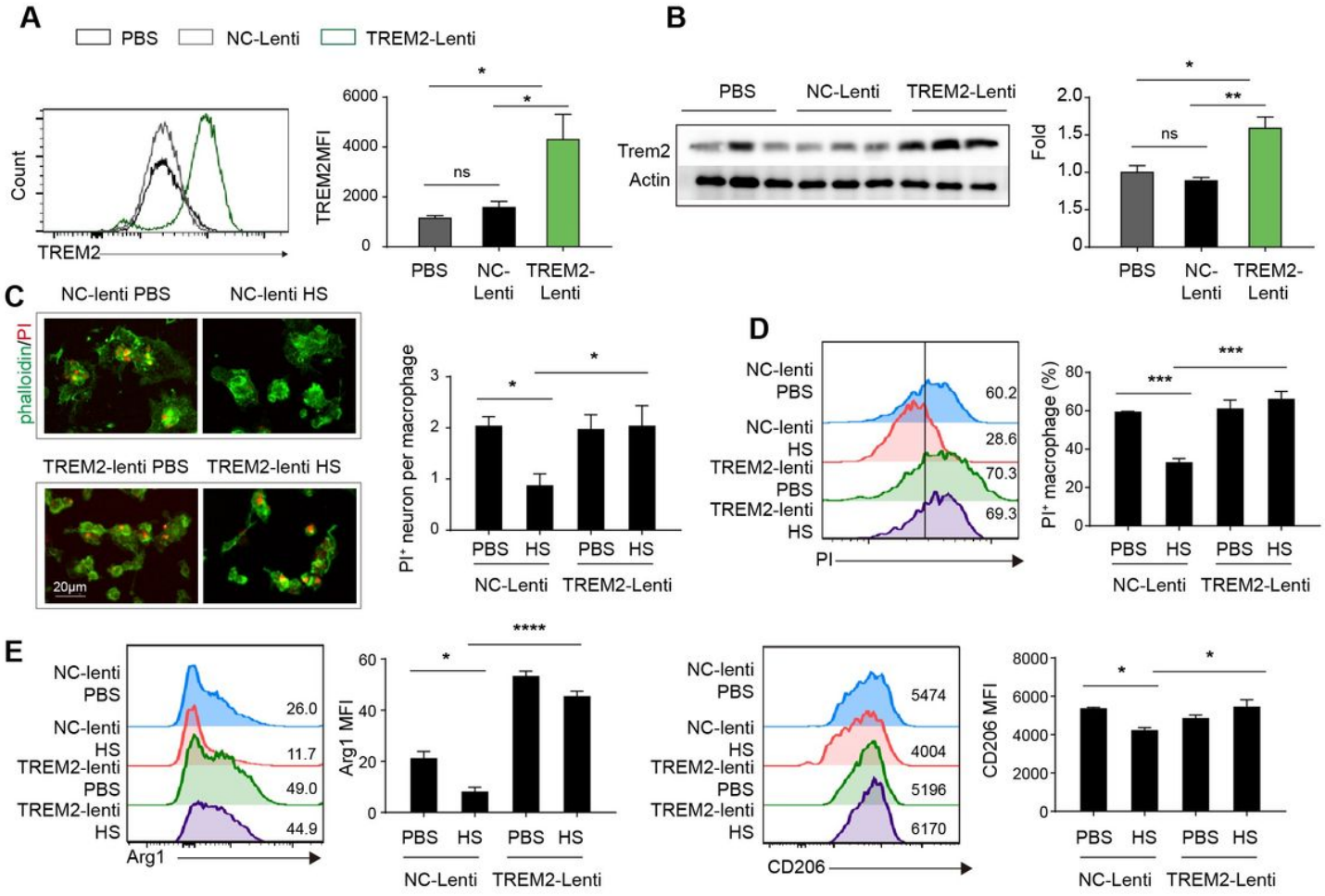


Figure 7

Enhancing TREM2 signaling restores the efferocytic capacity and cellular inflammatory resolution of macrophages in high salinity environment. Bone marrow derived primary cultured macrophages were infected with lentiviral vectors carrying TREM2-GFP cDNA (TREM2-Lenti) or control lentivirus carrying GFP only (NC-Lenti). Macrophages were subjected to analysis at 2d after infection. (A-B) Protein expression of TREM2 was analyzed with flow cytometry (A) and western blot (B). Experiments were repeated for 3 times. *P < 0.05, **P < 0.01 versus PBS-treated group in t test. (C-E) Transfected macrophages were treated with HS or PBS overnight then subjected to efferocytic analysis with PI+ dead/dying neurons. Efferocytic efficiency of macrophages were evaluated with immunol staining (C) and flow cytometry (D). Engulfed dead/dying neurons (PI+Phalloidin+) per macrophage or the proportion of phagocytic macrophages (PI+F4/80+) was calculated at corresponding time points. Data were collected from 3 independent experiments. *P < 0.05, ***P < 0.001 versus NC-Lenti PBS group in one-way

ANOVA. (E) Protein expression of Arg1 and CD206 in transfected macrophage with HS or PBS treatment overnight was analyzed with flow cytometry. *P < 0.05 versus NC-Lenti PBS group in one-way ANOVA.



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Supplementary Files

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