Original Article

Dual antiplatelet therapy improves functional recovery and inhibits inflammation after cerebral ischemia/reperfusion injury

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Background: Dual antiplatelet therapy with aspirin and clopidogrel (ASA + CPG) during the first 21 days has been shown to reduce the risk of major ischemic events in patients with transient ischemic attack (TIA) or minor stroke. However, the mechanisms underlying combination treatment with ASA + CPG in experimental ischemic stroke has not been fully elucidated.

Methods: Minor cerebral ischemia was induced in mice by transient distal middle cerebral artery occlusion (tdMCAO). Two doses of ASA + CPG (12 and 24 mg/kg/day) or vehicle were administered by gavage daily. Neurological behaviors were assessed using the modified Garcia scores, Rotarod test, Y maze, and open field test. Platelet function was assessed *in vitro* by flow cytometry and *in vivo* by bleeding and clotting time. The neutrophil ratio and the levels of inflammatory cytokines were measured by flow cytometry and the Meso Scale Discovery (MSD) electrochemilunimescence, respectively.

Results: Sensorimotor function was partially recovered with ASA + CPG treatment after ischemia. Anxiety levels and cognitive functions showed improvement in the ASA + CPG group at 12 mg/kg/day after 21 days. Both tail bleeding time and flow cytometry showed significantly decreased platelet function after ASA + CPG treatment. Notably, ASA + CPG at 12 mg/kg/day prolonged clotting time at 28 days after injury. Furthermore, the ratio of neutrophils, an indicator of inflammation, was reduced with 12 mg/kg/day ASA + CPG treatment in the bone marrow (BM) at 21 days and in the peripheral blood (PB) at 21 and 28 days after tdMCAO. Both doses of ASA + CPG decreased pro-inflammatory cytokine interleukin (IL)-6 expression 21 days after stroke. Taken together, these results demonstrated that combination treatment with ASA + CPG improved long-term neurological function after stroke and may inhibit platelet-neutrophil interaction by decreasing the concentration of pro-inflammatory cytokine, IL-6.

Conclusions: These findings indicate a neuroprotective effect of combination treatment with ASA + CPG for a duration of 21 days in an experimental acute minor stroke model. These findings provide further evidence that dual antiplatelet therapy may be a viable neuroprotective treatment to decrease the recurrence of stroke.

Keywords: Aspirin; clopidogrel; cerebral ischemia; platelet function; neutrophil

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Introduction

Minor stroke or transient ischemic attack (TIA) is the most common acute cerebrovascular disease and often leads to subsequent stroke events (1). Dual antiplatelet therapy with aspirin and clopidogrel (ASA + CPG) has been reported to decrease the risk of recurrent stroke among patients with TIA or minor ischemic stroke in the Clopidogrel in High-Risk Patients with Acute Non-Disabling Cerebrovascular Events (CHANCE) trial which enrolled 5,170 patients at 114 clinical centers in China and in the Platelet-Oriented Inhibition in New TIA and Minor Ischemic Stroke (POINT) trial which enrolled 4,881 patients at 269 international sites (2,3). Further pooled analysis from these two randomized, double-blind and placebo-controlled trials shows that the most optimal treatment duration for dual antiplatelet therapy is within the first 21 days after stroke (4). However, the mechanisms underlying the benefits of combination treatment with ASA + CPG are not fully understood.

Platelets are well-known for their roles in hemostasis and thrombosis. Antiplatelet therapy is the most commonly prescribed for secondary stroke prevention. However, bleeding is a major concern during anti-platelet treatment. In experimental intracerebral hemorrhage, antiplatelet pretreatment did not increase hematoma volume (5), but dual antiplatelet therapy for 3 days increased the risk for hemorrhagic transformation following intravenous thrombolytic treatment in experimental stroke (6). Notably, emerging studies indicate that platelets contribute to experimental stroke through signaling pathways which may be different from those involved in classic hemostasis function (7-9). Platelet P-selectin (also known as CD62P) binds to its high-affinity ligand P-selectin glycoprotein ligand-1 on neutrophils, which induces the activation of neutrophils and contributes to brain injury following ischemic stroke (10).

Neutrophils, a type of leukocyte, are the first immune cells that respond to acute cerebral ischemia insult. After ischemic stroke, neutrophil activation contributes to disruption of the blood brain barrier (BBB), cerebral edema, hemorrhagic transformation, and poor neurological outcomes by releasing free radicals and proteolytic enzymes (11,12). In humans, the degree of neutrophil accumulation is associated with cerebral micro-embolization and stroke severity (13,14). Colchicine inhibits neutrophil function and therefore reduces the risk of ischemic stroke (15). Moreover, elevated levels of neutrophils are an indicator of a higher risk for new strokes in patients with acute minor stroke and TIA (16). In addition, neutrophil cathepsin G promotes platelet aggregation, which suggests a relationship between neutrophil count and platelet activation (17). Furthermore, necrotic platelets mediated by cyclophilin D deletion interact with neutrophils to aggravate brain damage following ischemia reperfusion injury (7).

The current study investigated the role and the optimal duration of combination treatment with ASA + CPG in regulating platelet function and neutrophil-related inflammation in a minor ischemic stroke mouse model. We present the following article in accordance with the ARRIVE reporting checklist (18) (available at https://atm.amegroups.com/article/view/10.21037/atm-22-735/rc).

Methods

Animals and experimental groups

A total of 145 male C57BL/6 mice (8–10 weeks old) were supplied by Beijing HFK Bioscience Co., Ltd. All experimental procedures were approved by the Beijing Neurosurgical Institute Ethics Committee (No. 202002003) and were conducted in accordance with the guidelines of the National Institutes of Health on the care and use of animals. The mice were individually housed in polycarbonate cages at 22±2 ℃ on a regular 12-hour light/dark cycle. The body weight of all mice was measured daily until the end of the study. This study is a basic preclinical research, so a protocol was prepared before the study without registration.

Transient distal middle cerebral artery occlusion (tdMCAO) was performed on the mice. Reperfusion was performed at 3 different time points: 14 days (n=12 per group), 21 days (n=12 per group), and 28 days (n=11 per group) after the onset of tdMCAO. For each time point, mice with successful tdMCAO were randomly divided into four groups: sham-operated, tdMCAO, ASA + CPG at 12 mg/kg/day for each drug, and ASA + CPG at 24 mg/kg/day for each drug. In total, 5 mice died during chronic administration of drugs in this study, including 2 mice that died after vehicle administration, 2 mice that died after 24 mg/kg/day ASA + CPG treatment, and 1 mouse that died after 12 mg/kg/day ASA + CPG treatment. Surgery, drug administration, neurological assessment, and data analyses were performed by researchers who were blinded to the experimental conditions of the mice.

Transient distal middle cerebral artery occlusion (tdMCAO) model

To mimic acute minor ischemic stroke, tdMCAO surgery was performed in male mice weighing 24±0.5 g, as previously described (19). Briefly, the mice were anesthetized with 1.5% isoflurane. A skin incision was made between the right eye and ear. After opening a burr hole in the skull, the exposed distal middle cerebral artery (MCA) was compressed using a 30G blunted needle. After 1 hour of occlusion, the needle was removed, and the cerebral blood flow was partially restored. The body temperature of the mice was maintained at normothermia during surgery with a temperature-regulated heating pad (RWD Life Science, Shenzhen, China). To confirm ischemia and reperfusion in tdMCAO mice, regional cerebral blood flow (rCBF) was detected using laser Doppler flowmetry (RWD Life Science).

Drug administration

A combination of aspirin (ASA; Bayer HealthCare, Milano, Italy) and clopidogrel (CPG; Sanofi, Paris, France) were concurrently administered in a fixed volume of 100 μL by gavage at the onset of reperfusion and continued until the day before euthanization. The dosage of ASA + CPG (12 mg/kg/day for each drug) was chosen based on the recommended human dosage (75 mg/day) for reducing the risk of subsequent stroke adjusted for body weight (2).

Modified Garcia score

A modified Garcia score test was used to evaluate sensorimotor function at 1, 3, 5, 7, 9, 11, 14, 21, and 28 days after tdMCAO, as described in our previous study (20). Five tests were conducted, including body proprioception, vibrissae touch, limb symmetry, lateral turning, and forelimb walking. The scores of each test ranged from 0 to 3, with a total maximum possible score of 15.

Rotarod test

The rotarod test was conducted to assess motor function at 3, 5, 7, 9, 11, 14, 21, and 28 days after ischemia as described previously (21). Briefly, the mice were trained on a rotarod apparatus (Panlab, Barcelona, Spain) for 3 successive days (speed from 4 to 40 rpm). The time for each mouse to fall onto the platform was recorded, with a maximum time of 300 seconds per trial.

Open field test

The open field test was performed to measure spontaneous locomotor and anxiety-like behavior in mice on days 14, 21, and 28 post-stroke as described previously (22). The animal was placed in the open field maze for free and uninterrupted exploration for 10 minutes. At the end of the test, the mouse was picked up and returned to its home cage. The movements of the mouse were tracked and analyzed by EthoVision XT system (Noldus, Wageningen, the Netherlands). The degree of thigmotaxis, which is the tendency of a mouse to stay close to the walls, is used as a measure of anxiogenic behavior in mice (22). In this study, thigmotaxis was determined as the time spent in the peripheral squares divided by the total time. After the animal was removed from the grid, the number of fecal boli was counted, as an increase in the number of fecal boli can be used as an indicator of higher anxiety (22). The maze was cleaned with 75% ethanol between tests.

Y maze

The Y maze was performed to test spatial working memory as previously described (23). The Y maze apparatus consisted of three arms, defined as the start arm, the other arm, and the novel arm. In the first trial, the mouse was placed into the start arm and allowed to explore the 2 open arms (start and other arm) for 5 minutes. After 1 hour, the mouse was allowed to explore all 3 open arms for 5 minutes (test trial). The discrimination ratio was calculated as the time spent exploring the novel arm divided by the total time spent in both the novel and other arm. Performance was monitored with the EthoVision XT system. The maze was wiped with 75% ethanol between tests.

Novel object recognition test

To investigate learning and memory/recognition in mice, the novel object recognition test was performed as previously described (24). Briefly, this two-trial test consisted of a training and a testing phase. During the training phase, the animal was allowed to explore 2 identical objects for 10 minutes. After a 6-hour interval, 1 of the training objects was replaced with a novel object, and the animal was allowed to explore the 2 different objects for another 10 minutes during the testing phase. Mice which did not reach a minimum exploration time (20 s) in the training phase were excluded from the analysis. The

recognition index was calculated as the time spent exploring the novel object divided by the total time spent exploring the novel object and the familiar object. The objects were cleaned between mice using 75% ethanol.

Bleeding time

Bleeding time was measured by a tail transection method as previously reported (25). Briefly, a distal 5-mm segment of the tail tip was amputated and the incised tail was blotted with filter paper every 15 seconds until the bleeding ceased. The period between the start and the arrest of bleeding was considered the bleeding time and was recorded for up to 900 seconds.

Clotting time

The blood clotting time was measured using the capillary tube method as previously reported (26). Briefly, a blood sample was collected from the venous plexus of the right eye with a glass capillary tube. Small portions of the capillary tube were broken off from one end at regular intervals of 30 seconds. The blood clotting time was recorded until blood streaks appeared. The maximum time allowed for clotting was 420 seconds.

Platelet function test using flow cytometry

The *in vitro* platelet function was tested based on the expression of CD41 (platelet indicator) and CD62p (platelet activation marker) (6). Blood samples were collected from the right angular vein and added to sodium citrate (final concentration 0.32%). A volume of 5 μL blood was diluted in 60 μL phosphate buffered saline (PBS). The diluted blood was then incubated with 30 μL of PBS or thrombin agonist buffer (Sigma-Aldrich) supplemented with $CaCl_2$ and GPRP at 37 ℃. After 5 minutes, the blood was incubated with CD41-FITC (BD Pharmingen™) and CD62P-APC (eBioscience) in the dark for 30 minutes at room temperature. After labeling, the samples were analyzed with CytoFLEX S (Beckman Coulter, CA, USA).

Flow cytometry

Cells were isolated from the bone marrow (BM), spleen, or peripheral blood (PB) of mice. The megakaryocyte (MK) precursor population was double-stained using FITC-anti-CD34 antibody (eBioscience) and BV650- anti-CD41 antibody (BD Biosciences). Mature MKs were double-stained using BV650-anti-CD41 (BD Biosciences) and PE-anti-CD42d antibodies (eBioscience). Positive neutrophils were double-stained using APC-anti-Mac1 (CD11b, eBioscience) and PE-anti-Gr1 (Ly-6G/Ly-6C, eBioscience) antibodies as previously described (27). T helper (Th) 17 cells were stained with FITC-anti-CD4 (Biolegend), PE-IL17a (Biolegend), and APC-anti-RORγt (eBioscience). Regulatory T cells (Treg cells) were stained with FITC-anti-CD4 (Biolegend), eFluor 450-anti-CD25 (eBioscience), and APC-anti-FoxP3 (eBioscience). The samples were analyzed with the CytoFLEX S flow cytometer (Beckman Coulter, CA, USA).

Meso Scale Discovery (MSD) electrochemiluminescence

The V-PLEX Pro-Inflammatory Panel 1 (Mouse) Kit (Meso Scale Discovery, Rockville, MD, USA) was used to detect serum cytokines levels including IL1-β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, interferon (IFN)-γ, KC/GRO [also named as chemokine (C-X-C motif) ligand 1, CXCL1] and tumor necrosis factor (TNF)α according to the manufacturer's instructions. Briefly, 25 μL of serum sample was added into the well of the assay plate and incubated for 2 hours. After washing the plate, the wells were incubated with detection antibodies for another 2 hours. The signals were read with the Meso QuickPlex SQ120 (Meso Scale Discovery). The concentration (pg/mL) of cytokines was calculated according to a standard curve.

Statistical analysis

Data are presented as means ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Significant differences were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's tests for multiple comparison. A P value <0.05 was considered statistically significant.

Results

The effects of ASA + CPG administration on body weight and cerebral blood flow in mice

Previous studies demonstrated that dual antiplatelet therapy with ASA and CPG for a duration of 21 days reduced the rate of major ischemic events and did not increase the

risk of major hemorrhagic outcomes in patients with TIA or minor ischemic stroke (2,4). In the present preclinical study, the effects of combination therapy with ASA + CPG were assessed in a tdMCAO mouse model, which provides a reproducible method using compression of the distal segment of the MCA to mimic acute minor ischemia (*Figure 1A*,*1B*). The administration of ASA + CPG once a day for 14, 21, or 28 consecutive days did not statistically impact the body weight of tdMCAO mice compared with mice in the vehicle group (*Figure 1C*). The rCBF was monitored before ischemia, at 10 minutes after ischemia, and at 10 minutes, 24 hours, and 28 days after reperfusion. There was no significant difference in the rCBF reduction between vehicle treatment and ASA + CPG treatment (12 or 24 mg/kg/day for each drug) (*Figure 1D*,*1E*). The rCBF after 28 days could not be quantified due to the hemorrhage of the skin incision during the detection procedure.

Combination treatment with ASA + CPG improves motor impairment and anxiety-related behavior after tdMCAO

To evaluate the impact of ASA + CPG treatment on functional outcomes after tdMCAO, a range of neurobehavioral tests were conducted. As shown in *Figure 2A*, vibrissae touch was significantly improved at days 3 and 7 post-ischemia in mice treated with ASA + CPG at 12 mg/kg/day compared to mice treated with vehicle. Furthermore, mice treated with ASA + CPG at 24 mg/kg/day also showed significantly improved vibrissae touch at day 7 after stroke, compared with the vehicle group (*Figure 2A*). The differences in the other sensorimotor functions such as body proprioception, limb symmetry, lateral turning, and forelimb walking were not significantly different between vehicle and ASA + CPG treatment (*Figure 2B-2E*). The total neurological score, which includes the five assessments, was increased at days 3 and 7 post-ischemia in the 12 mg/kg ASA + CPG group, and at day 9 post-ischemia in the 24 mg/kg ASA + CPG group (*Figure 2F*). Rotarod tests were performed to assess behavioral deficits. The time to fall off the rotarod was significantly longer in the 12 mg/kg group compared with the vehicle group at 7 days post-stroke (*Figure 3A*). Notably, from days 11 to 28 after ischemia, treatment with ASA + CPG at 24 mg/kg revealed a trend of decreasing latency to fall which was not statistically different compared to the vehicle group.

The effects of ASA + CPG treatment on improvement of locomotor activity and anxiety were assessed using the open field test. ASA + CPG at 12 mg/kg significantly decreased the number of fecal boli at day 21 compared to day 14 post-ischemia (*Figure 3B*). However, peripheral locomotion (thigmotaxis) and distance traveled did not change after ASA + CPG treatment (12 or 24 mg/kg) at any reperfusion timepoint after stroke (*Figure 3C*,*3D*). Taken together, these behavioral results demonstrated that ASA + CPG treatment improved some aspects of neurobehavioral function at early timepoints and decreased anxiety levels at 21 days after acute minor ischemia.

Combination treatment with ASA and CPG protects against recognitive function impairment

Since cognitive neurological dysfunction can be induced by ischemic stroke (28), the effects of ASA + CPG on recovery of cognitive function was analyzed using the Y maze and novel object recognition tests. Discrimination ratio analysis showed that mice treated with ASA + CPG at 12 mg/kg/day spent significantly more time in the novel arm compared with mice treated with vehicle at day 21 after ischemia (*Figure 3E*,*3F*). This result demonstrated that short-term memory was restored in the 12 mg/kg ASA + CPG group on day 21, but not on days 14 or 28 after ischemia. Based on the novel object recognition test, there was no significant difference in the time spent exploring a new object at any of the three reperfusion times for both doses of the ASA + CPG groups compared with the vehicle group (*Figure 3G*,*3H*). These results suggested that treatment with 12 mg/kg/day ASA + CPG improved spatial memory 21 days after ischemia.

The effects of combination treatment with ASA + CPG on platelet function after tdMCAO

As ASA and CPG can irreversibly impair platelet function (29), bleeding times and clotting times were tested to evaluate the effects of ASA + CPG treatment on hemostasis after tdMCAO. As shown in *Figure 4A*, ASA + CPG at 12 mg/kg/day significantly increased the bleeding time compared with the vehicle group at all 3 reperfusion time points. The same prolongation was detected in mice treated with ASA + CPG at 24 mg/kg/day compared to mice treated with the vehicle at days 14 and 21, but not at day 28 after tdMCAO.

Combination therapy with ASA + CPG has been reported to synergistically increase the risk of major bleeding as detailed in a meta-analysis which combined data from 14 clinical trials (30). Clotting time is another parameter related to platelet function. Mice treated

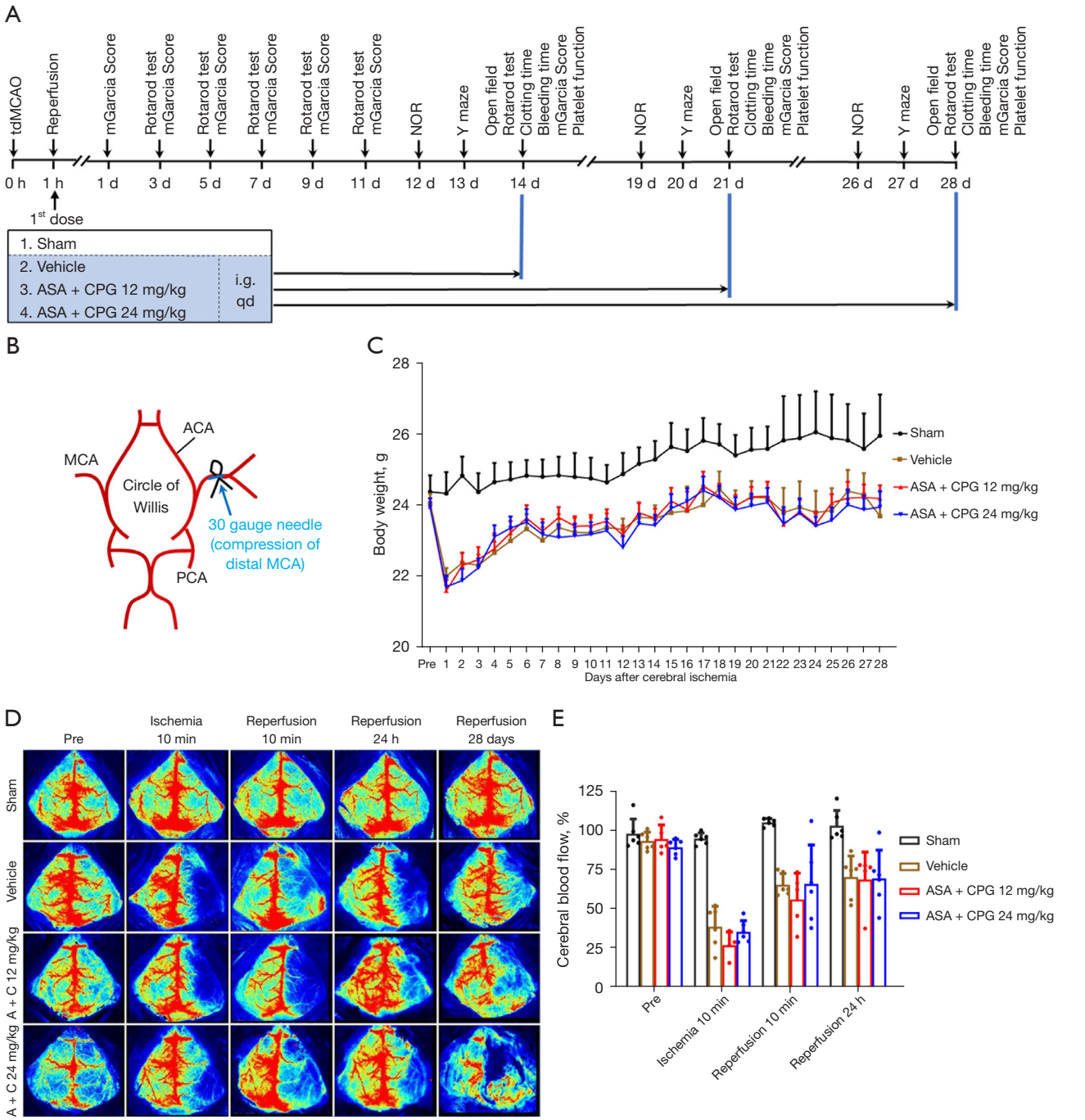

Figure 1 Combination treatment with ASA + CPG did not change body weight nor cerebral blood flow after tdMCAO in mice. (A) The timeline for evaluating the effects of combined ASA + CPG treatment in tdMCAO mice. (B) An illustration of the tdMCAO model. (C) Combination therapy with ASA + CPG did not have any impact on body weight. Data are represented as means ± SEM (n=6 for sham group, n=11 to 12 for the other three groups). (D) Representative images of the cerebral blood flow before cerebral ischemia, 10 minutes after ischemia, and 10 minutes, 24 hours, and 28 days after reperfusion in the four groups. (E) Quantification of cerebral blood flow. Data are presented as a percentage of baseline (pre-ischemia). Data are represented as means ± SD (n=4 to 6 mice per group). ASA, aspirin; CPG, clopidogrel; tdMCAO, transient distal middle cerebral artery occlusion; ACA, anterior cerebral artery; PCA, posterior cerebral artery; SEM, standard error of the mean; SD, standard deviation.

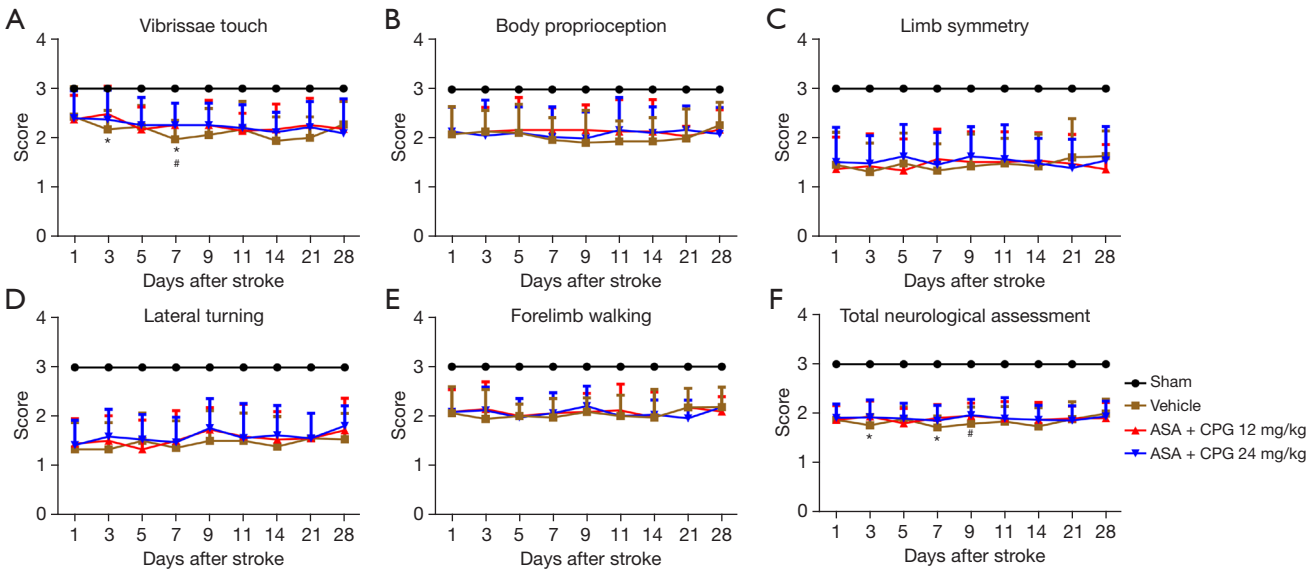

Figure 2 ASA + CPG treatment statistically improved sensorimotor functions after tdMCAO. (A) Vibrissae touch of the mice. (B) Body proprioception of the mice. (C) Limb symmetry of the mice. (D) Lateral turning of the mice. (E) Forelimb walking of the mice. (F) Total neurological assessment score of the mice. Data are represented as mean ± SD (n=11 to 12 mice per group). *P<0.05 between the vehicle group and the ASA + CPG group at 12 mg/kg/day; #P<0.05 between the vehicle group and the ASA + CPG group at 24 mg/kg/day. Statistical analysis was performed using two-way ANOVA followed by Tukey's tests. ASA, aspirin; CPG, clopidogrel; tdMCAO, transient distal middle cerebral artery occlusion; SD, standard deviation.

with ASA + CPG at 12 mg/kg/day showed significantly prolonged clotting time compared with the vehicle group at 28 days post-stroke, but not at 14 days nor 21 days post-stroke (*Figure 4B*). The difference in clotting time at the 3 reperfusion timepoints was further investigated. The results revealed that the clotting time was statistically increased at 28 days compared to 14 days after stroke in mice treated with either dose of ASA + CPG, and also compared to 21 days after stroke in the 12 mg/kg/day treatment group. These data from the clotting test times suggested that dual antiplatelet therapy with ASA + CPG for 21 consecutive days after ischemia onset did not impact hemostatic function, however, hemostatic function was disturbed at 28 days post-stroke.

The *in vitro* platelet function was assessed using flow cytometry. Platelets were distinguished by forward and side scatter gating, and CD41 signal parameter histograms. Activated platelet rates were identified by the percentage of positive staining with CD62p in the CD41-positive platelet population (*Figure 5A,5B*). In unstimulated and thrombin-stimulated blood *in vitro*, platelet activation was partially impaired at both doses of ASA + CPG treatment (12 and 24 mg/kg/day) at 7, 14, and 21 days post-ischemia, but not at 28 days after ischemia (*Figure 5C,5D*). Platelets are produced from the cytoplasm of megakaryocytes (MKs) in the BM (31). As shown in *Figure 6A,6B*, combination treatment with ASA + CPG decreased the ratio of MK precursor cells ($CD34^+CD41^+$) in the BM 14 days after ischemia. Moreover, the percent of mature MKs ($CD41^+CD42d^+$) declined at both doses of ASA + CPG treatment after 28 days (*Figure 6C,6D*). Taken together, this data demonstrated that combination treatment with the two doses of ASA + CPG during the 21-day period after tdMCAO inhibited platelet activation without interfering with the process of hemostasis.

Combination treatment with ASA + CPG reduces the ratio of neutrophils after tdMCAO

It has been well documented that elevated neutrophil counts are associated with an increased risk of new stroke in patients who have already had minor ischemia (16). Therefore, the effects of ASA + CPG treatment on the neutrophil ratio after tdMCAO were examined. Positive Mac1(+) Gr1(+) gates (neutrophils) in the BM and PB are shown in *Figure 7A,7B*. Compared with the vehicle group, ASA + CPG at

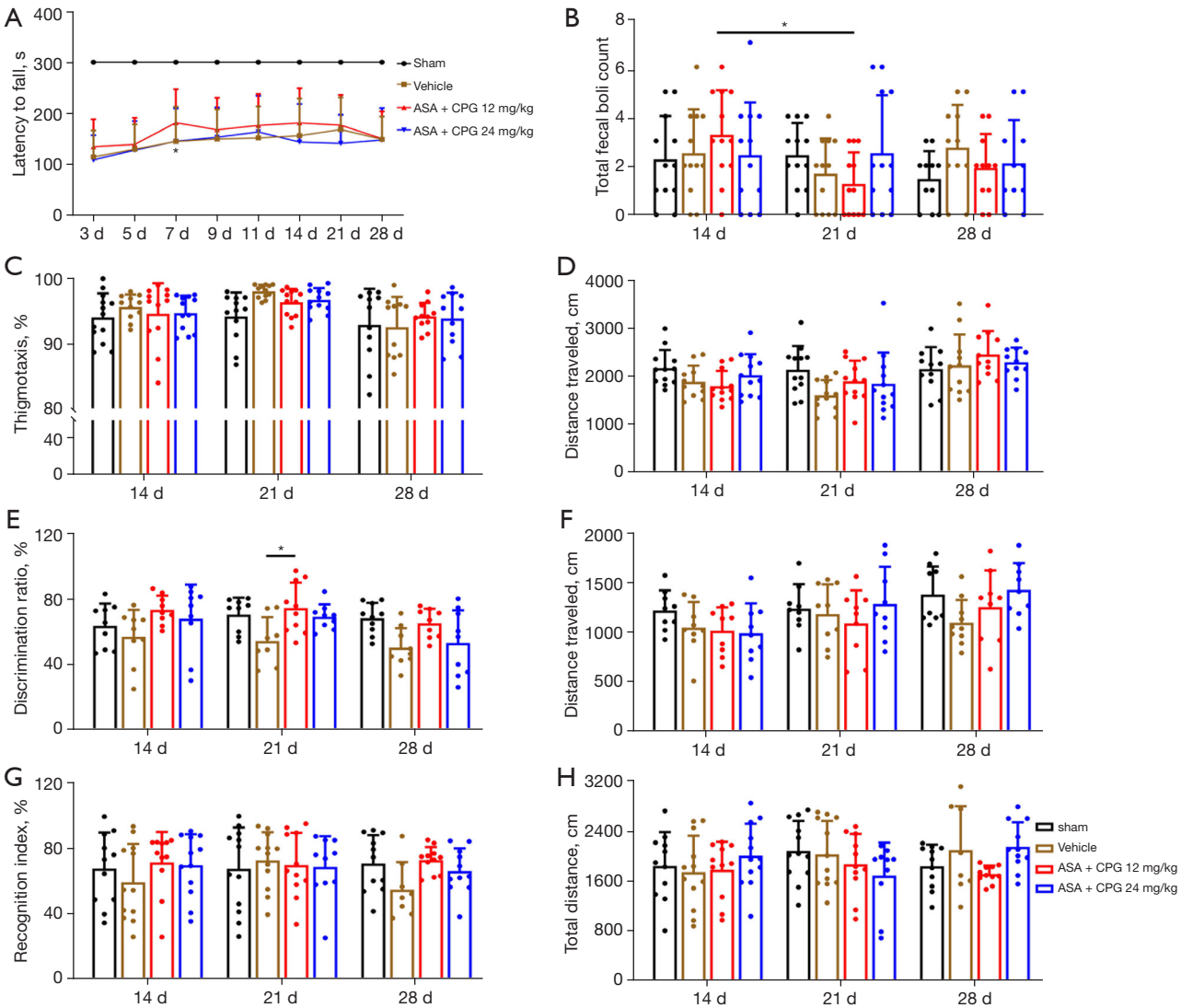

Figure 3 ASA + CPG at 12 mg/kg significantly improved motor and memory functions and decreased anxiety levels after tdMCAO. (A) Quantification of rotarod tests after cerebral ischemia. ASA + CPG at 12 mg/kg prolonged the time before mice fell off the rotarod. (B) The numbers of fecal boli deposits were statistically reduced by 12 mg/kg ASA + CPG at 21 days compared to 14 days after ischemia. (C) ASA + CPG treatment did not change the degree of thigmotaxis in the mice. (D) The total distance traveled in the open field. (E) Cognitive performance in the Y maze was evaluated using discrimination ratios in mice after ischemia. Increased ratio of discrimination can be an indicator of improved cognitive function in mice. Compared to the vehicle group, ASA + CPG at 12 mg/kg elevated the discrimination ratio 21 days after ischemia. (F) The total distance traveled in the Y maze. (G) There was no significant difference in the recognition index between groups of mice. (H) The total distance traveled during the novel object recognition test. Data are represented as means ± SD (n=8 to 12 mice per group). *P<0.05 between the vehicle group and the 12 mg/kg/day ASA + CPG group. ASA, aspirin; CPG, clopidogrel; tdMCAO, transient distal middle cerebral artery occlusion; SD, standard deviation.

12 mg/kg/day but not at 24 mg/kg/day, significantly decreased the fraction of neutrophils in the BM on day 21 only, but not on day 14 nor day 28 after ischemia (*Figure 7C*). ASA + CPG at 12 mg/kg/day significantly decreased the neutrophil ratio in the BM at 21 days after ischemia onset, followed by an increase at 28 days. Furthermore, the neutrophil ratio in the PB was dramatically reduced by 12 mg/kg/day ASA + CPG treatment on days 21 and 28 after tdMCAO

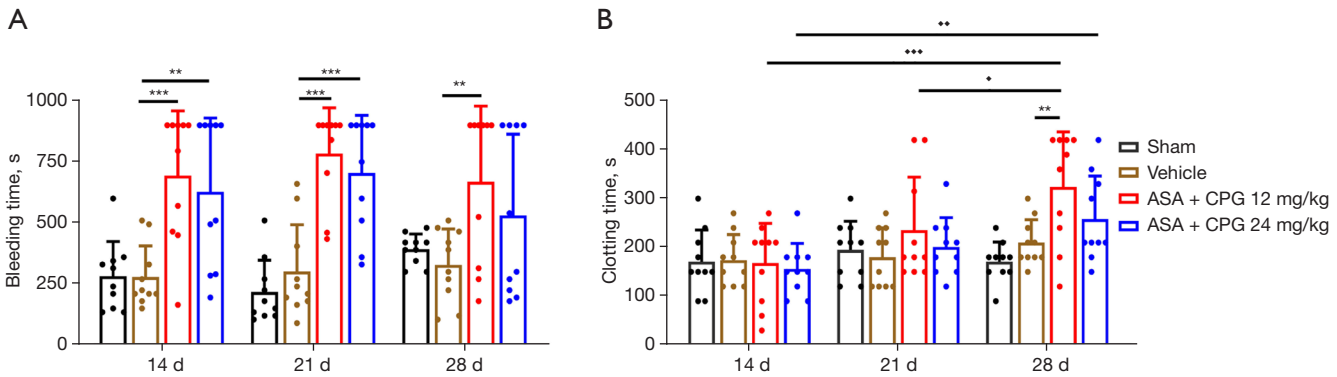

Figure 4 ASA + CPG treatment prolonged the bleeding time and the clotting time. (A) ASA + CPG at 12 mg/kg prolonged the bleeding time at all three reperfusion timepoints. ASA + CPG at 24 mg/kg increased the bleeding time at 14 days and 21 days after tdMCAO, but not at 28 days. (B) At day 28 after ischemia, 12 mg/kg ASA + CPG prolonged the clotting time compared to the vehicle group. For 12 mg/kg and 24 mg/kg ASA + CPG, the clotting time was prolonged at day 28 compared with the other reperfusion timepoints (n=10 mice per group). **P<0.01, ***P<0.001 versus vehicle; ˙P<0.05, ¨P<0.01, ˙˙˙P<0.001 at a particular dose between different reperfusion times. ASA, aspirin; CPG, clopidogrel; tdMCAO, transient distal middle cerebral artery occlusion.

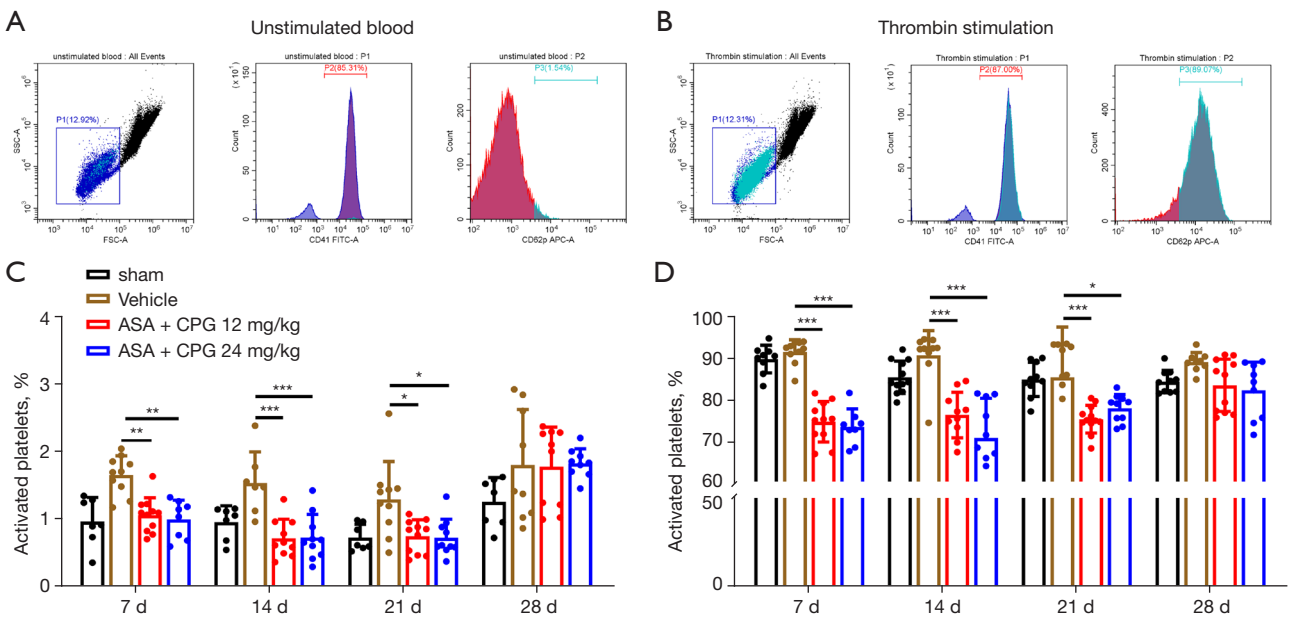

Figure 5 Platelet activation was decreased by ASA + CPG treatment at 21 days, but not at 28 days. (A,B) Gating strategy in unstimulated blood and thrombin-stimulated blood. P1: ungated whole blood; P2: cells positive for platelet identification maker FITC-CD41 in P1; P3: cells positive for platelet activation marker APC-CD62p in P2. Both doses of ASA + CPG inhibited platelet activation rates in unstimulated (C) and thrombin-stimulated blood (D) at 7, 14, and 21 days, but not at 28 days after ischemia (n=7 to 11 mice per group). *P<0.05, **P<0.01, ***P<0.001 versus vehicle. ASA, aspirin; CPG, clopidogrel.

compared to treatment with the vehicle (*Figure 7D*). Meanwhile, 12 mg/kg/day ASA + CPG increased the Th17/Treg ratio in the spleen but not in the PB (*Figure 7E-7J*). Taken together, these results indicated that 12 mg/kg/day ASA + CPG treatment reduced the neutrophil ratio in the BM and the PB 21 days after stroke.

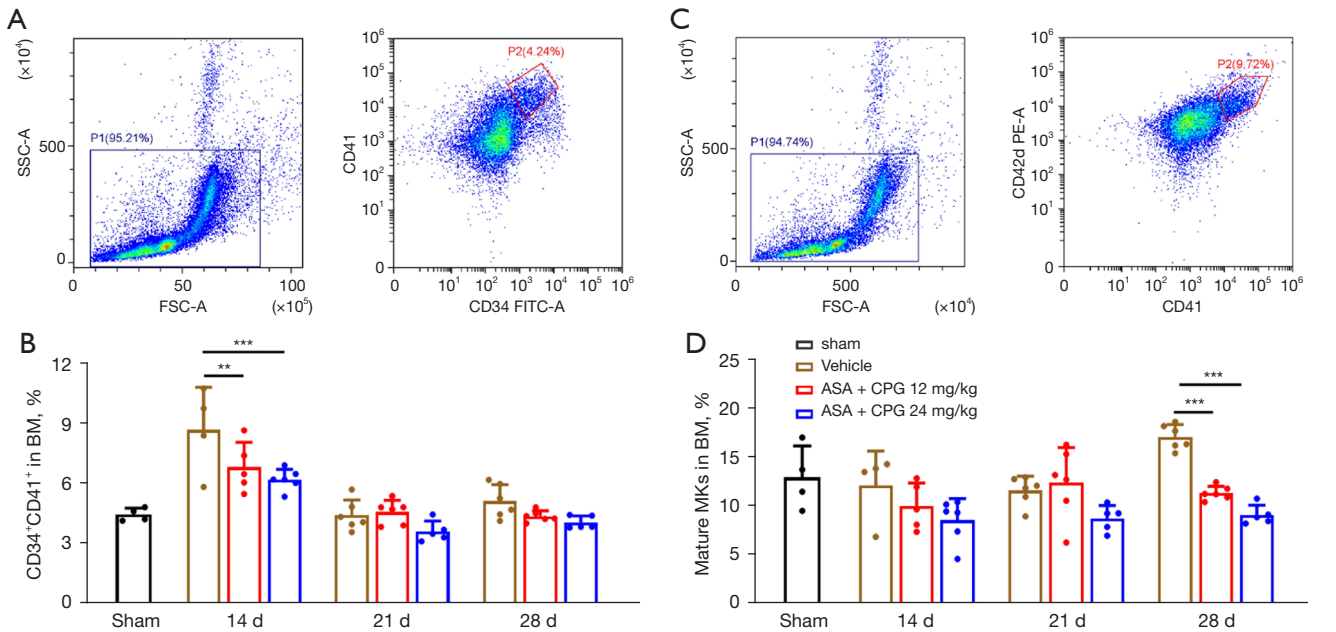

Figure 6 ASA + CPG treatment reduced the expression of megakaryocytes (MK) precursor cells and mature MKs. (A) Gating strategy of the MK precursor ($CD34^+CD41^+$) population in BM. (B) Compared with the vehicle group, both doses of ASA + CPG decreased the percentage of $CD34^+CD41^+$ cells in BM 14 days after ischemia. (C) Gating strategy of the mature MK ($CD41^+CD42d^+$) population in BM. (D) Both doses of ASA + CPG reduced the percentage of mature MK cells in BM 28 days after ischemia. n=4 to 6 mice per group. **P<0.01, ***P<0.001 versus vehicle. MK, megakaryocytes; BM, bone marrow; ASA, aspirin; CPG, clopidogrel.

Combination treatment with ASA + CPG decreases IL-6 levels 21 days after tdMCAO

Inflammation is a hallmark of ischemic stroke (32). MSD assays were performed to detect the release of pro-inflammatory cytokines in the drug-treated groups. Both doses of ASA + CPG treatment inhibited IL-6 secretion compared to the vehicle group 21 days after tdMCAO (*Figure 8A*). There were no statistically significant differences in the other 9 cytokines tested between groups at any reperfusion timepoint (*Figure 8B-8J*). Collectively, these data demonstrated that ASA + CPG at 12 mg/kg/day significantly reduced IL-6 levels at 21 days after tdMCAO without affecting the other 9 cytokines.

Discussion

Data from the CHANCE and POINT trials suggested that dual antiplatelet therapy with ASA + CPG after the onset of symptoms had a lower risk of major ischemic stroke events as compared than aspirin alone in patients with acute minor stroke or TIA (2,3). Among patients with high risk TIA or mild to moderate ischemic stroke, combination treatment with aspirin and ticagrelor, a direct-acting antiplatelet agent, decreased the risk of stroke or death compared with those who received aspirin alone (33). The current study investigated the efficacy and potential mechanisms of combination treatment with ASA + CPG in a transient acute minor cerebral ischemia model. The dose of 12 mg/kg ASA + CPG was chosen based on the recommended human dose of 75 mg/day per body weight in the CHANCE trial. The results indicated that ASA + CPG at 12 mg/kg/day may exert a beneficial effect by regulating platelet function and inflammation if administered 21 days from the onset of symptoms after acute minor stroke.

To mimic acute minor stroke in mice, cerebral ischemia was induced by distal compression of the MCA (19). This model is easy to perform with low mortality rates and allows for well-controlled arterial reperfusion. Moreover, the infarct area in tdMCAO model is the barrel cortex, which is the whisker-related primary somatosensory cortex (34). In the current study, impairment of vibrissae touch was observed in the vehicle group, which was significantly improved in the ASA + CPG-treated group at 3 and 7 days after ischemia (*Figure 2A*).

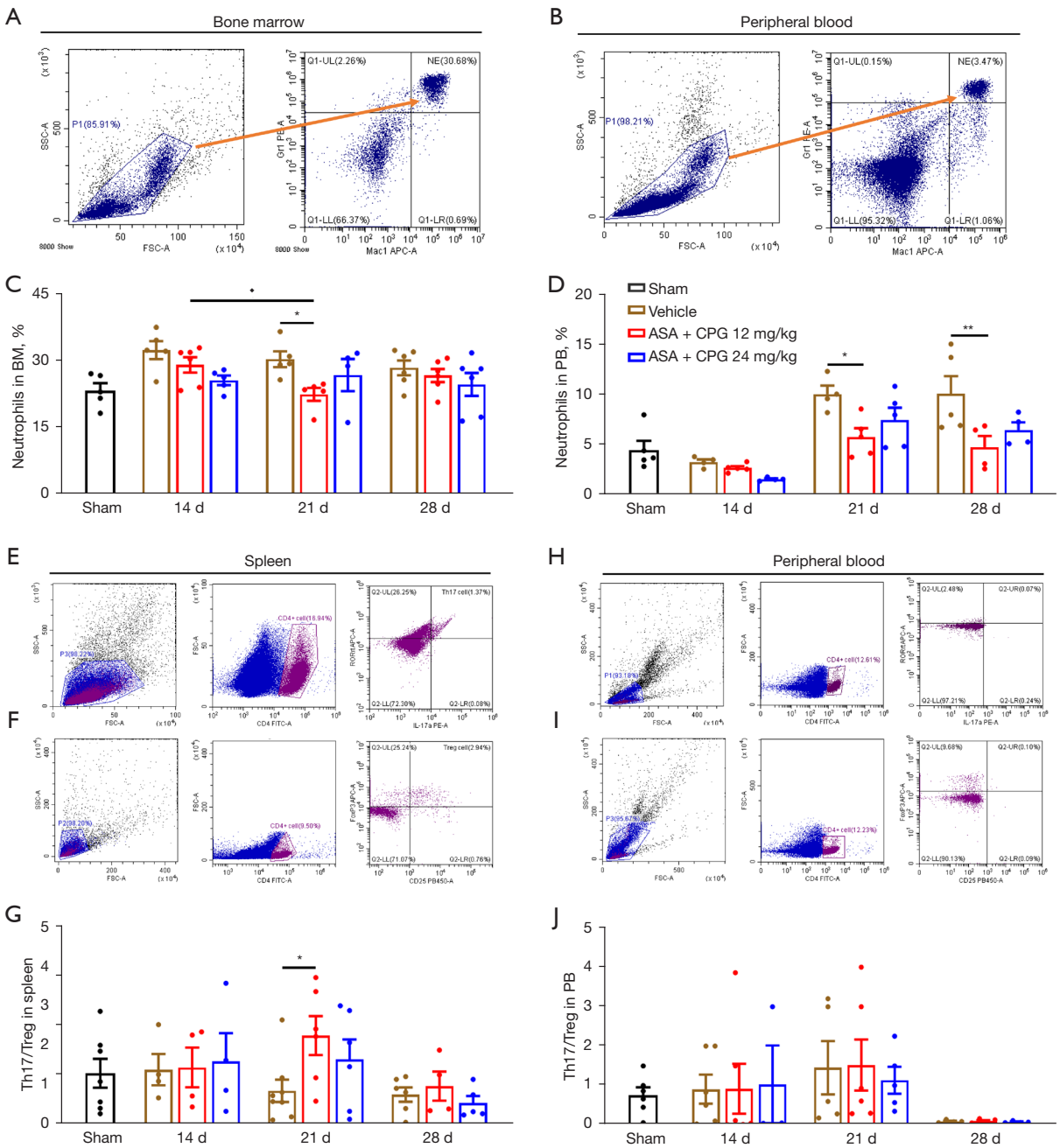

Figure 7 ASA + CPG treatment decreased the ratio of neutrophils in BM and PB and increased the ratio of Th17/Treg cells in the spleen. Gating strategy of neutrophils ($Mac1^+Gr1^+$) in BM (A) and PB (B). (C) Treatment with 12 mg/kg ASA + CPG decreased the ratio of neutrophils in the BM at day 21 after ischemia. The percentage of neutrophils was significantly lower in mice treated with 12 mg/kg ASA + CPG at 21 days compared to 14 days after ischemia. (D) 12 mg/kg ASA + CPG dramatically decreased the percentage of neutrophils in the PB at day 21 and 28 after ischemia. Gating strategy of Th17 ($CD4^+IL17a^+ROR\gamma t^+$) cells in the spleen (E) and the PB (H). Gating strategy of Treg ($CD4^+CD25^+FoxP3^+$) cells in the spleen (F) and the PB (I). (G) Treatment with 12 mg/kg ASA + CPG increased the ratio of Th17/Treg cells in the spleen 21 days after ischemia. (J) There was no difference in the percentage of Th17/Treg cells in the PB between ASA + CPG-treated mice and vehicle-treated mice (n=4 to 6 mice per group). *P<0.05, **P<0.01 versus vehicle; •P<0.05 at a particular dose between different reperfusion times. ASA, aspirin; CPG, clopidogrel; BM, bone marrow; PB, peripheral blood; Th17, T helper cell 17; Treg, regulatory T cell.

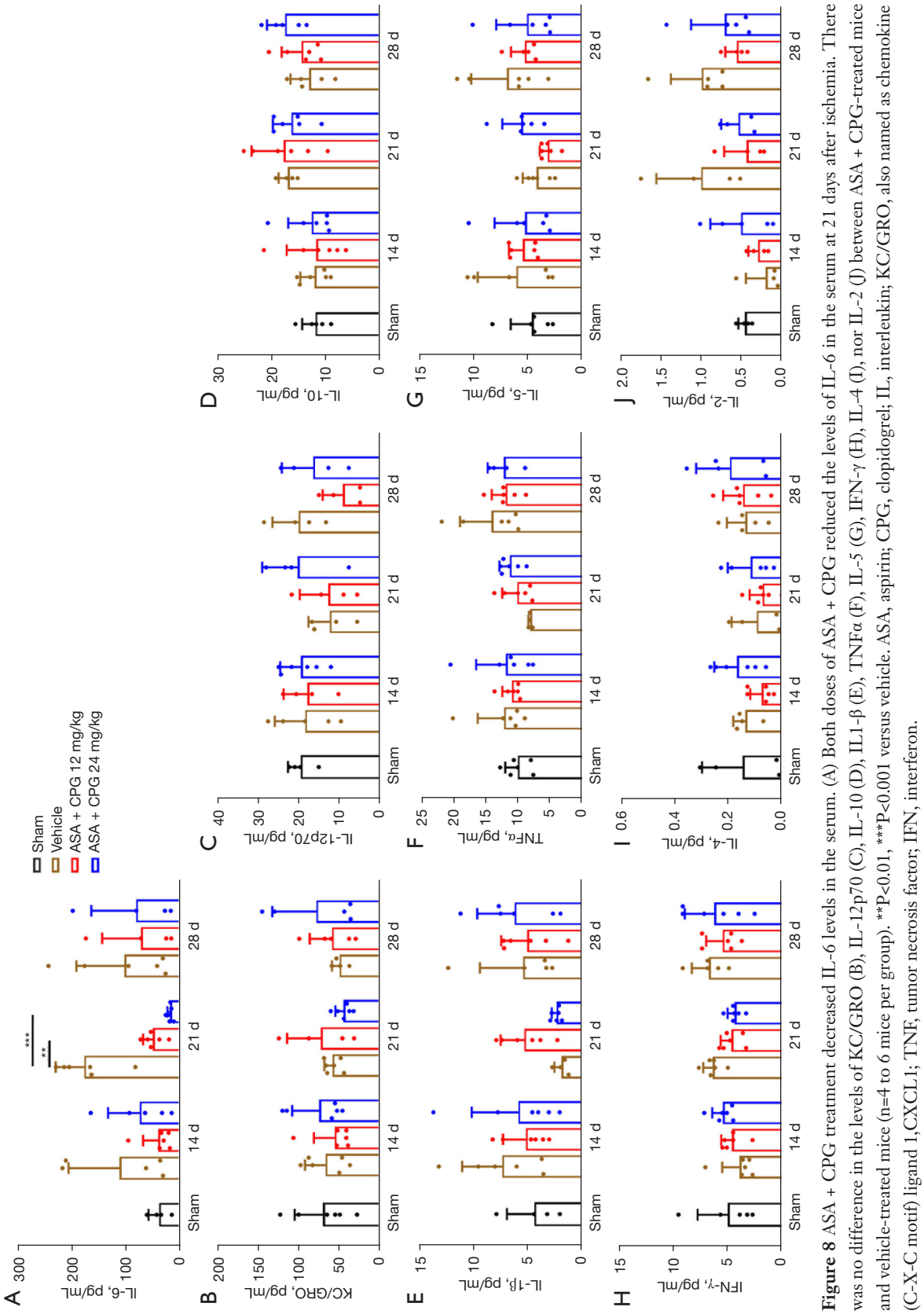

Figure 8 ASA + CPG treatment decreased IL-6 levels in the serum. (A) Both doses of ASA + CPG reduced the levels of IL-6 in the serum at 21 days after ischemia. There was no difference in the levels of KC/GRO (B), IL-12p70 (C), IL-10 (D), IL1-β (E), TNFα (F), IL-5 (G), IFN-γ (H), IL-4 (I), nor IL-2 (J) between ASA + CPG-treated mice and vehicle-treated mice (n=4 to 6 mice per group). **P<0.01, ***P<0.001 versus vehicle. ASA, aspirin; CPG, clopidogrel; IL, interleukin; KC/GRO, also named as chemokine (C-X-C motif) ligand 1,CXCL1; TNF, tumor necrosis factor; IFN, interferon.

Platelets are central players in maintaining hemostasis of the blood. Bleeding is a major concern after combination treatment with dual antiplatelet therapy. Clotting time, a parameter of coagulation (25), was prolonged with ASA + CPG treatment at 28 days, but not at 14 days nor 21 days (*Figure 4*). The results revealed that long-term (28 days) treatment with ASA + CPG increased the risk of bleeding. Aside from classic hemostasis, platelets have also been reported to mediate the development and progression of ischemic stroke (7). Neutrophils are released from the BM after acute cerebral ischemia and play an important role in post-stroke inflammation (35). Accumulating data show that neutrophils are related to ischemic injury (11,12). Disruption of the BBB plays a key role in post-stroke cerebral hemorrhagic transformation (HT) and edema. Stimulation of neutrophil activation has been shown to disrupt the integrity of the BBB in experimental stroke models. In contrast, depleting or inhibiting neutrophils decreases BBB disruption and the rate of HT. Thus, targeting neutrophils may inhibit HT after stroke. Moreover, a higher neutrophil count is associated with an elevated risk of future strokes (36,37). The neutrophil membrane protein FLAP (five lipoxygenase activating protein) contributes to increased risk of stroke (38). Our current study demonstrated that the rate of neutrophil activation was inhibited by 12 mg/kg ASA + CPG treatment at 21 days after stroke, but not at 14 days nor 28 days (*Figure 7*). These results suggest that the optimal dose and duration for treatment with ASA + CPG is 12 mg/kg/day for 21 days.

The role of pro-inflammatory cytokine IL-6 after ischemia is still controversial. It has been reported that IL-6 can dose-dependently protect neurons against N-methyl-D-aspartate (NMDA) toxicity (39) and IL-6 deficient mice has larger infarct areas and reduces survival (40). However, other studies have indicated that the rapid increase in IL-6 is associated with a larger infarct area. In human stroke patients, increased serum levels of IL-6 accelerates neutrophil release (41). Activated neutrophils secrete significant amounts of IL-6 for a pro-inflammatory response (42). The interaction between platelets and neutrophils is mediated by platelet P-selectin (CD62p). It has been reported that P-selectin deletion exerts a neuroprotective effect after cerebral ischemia (43). Increased P-selectin expression is associated with an increase in platelet necrosis and is thus a good marker for platelet necrosis (44). Moreover, necrotic platelets prefer to interact with neutrophils (7). The current study showed that 12 mg/kg ASA + CPG inhibited CD62p expression, decreased the neutrophil ratio and IL-6 levels (*Figures 5*,*7*,*8*). Based on our findings and other reports, we hypothesized that ASA + CPG reduced IL-6 secretion from neutrophils by inhibiting platelet-neutrophil interaction through decreasing CD62p expression, thus alleviating the inflammatory response, and promoting the recovery of neurological function. This hypothesis warrants thorough investigation in future studies. A previous study showed that the levels of TNFα and IL-8 significantly increased in acute stroke patients compared to controls, which were statistically inhibited by aspirin and clopidogrel treatment. This data suggest that antiplatelets treatment may have a neuroprotective role against the acute ischemic stroke through inhibiting the proinflammatory cytokine secreted by stroke patients (45). How dose ASA + CPG impair platelet function and decrease IL-6 expression? CPG, an antiplatelet agent, blocks the P2Y12 receptor on platelets and inhibits platelet function (33). Suppressor of cytokine signaling 3 (SOCS3) specifically binds to gp130, the co-receptor of IL6 family cytokines, and inhibits IL-6 signaling (46). A previous study has shown that aspirin increases the expression of SOCS3, which is a potent anti-inflammatory molecule. We supposed that aspirin decreased IL-6 through regulating SOCS3 expression (47). Further study is warranted.

In summary, this data demonstrated a neuroprotective effect of combination treatment with ASA + CPG for a duration of 21 days in an acute minor stroke mouse model, likely through inhibition of platelets and neutrophil inflammation. These findings provide further evidence that dual antiplatelet therapy may be a viable neuroprotective treatment to decrease the incidence of recurrent strokes.

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Footnote

Reporting Checklist: The authors have completed the

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-735/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were approved by the Beijing Neurosurgical Institute Ethics Committee (No. 202002003) and were conducted in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

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