The HDAC3 inhibitor RGFP966 ameliorated ischemic brain damage by downregulating the AIM2 inflammasome

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Abstract
Histone deacetylases 3 (HDAC3) modulates the acetylation state of histone and non-histone proteins and could be a powerful regulator of the inflammatory process in stroke. Inflammasome activation is a ubiquitous but poorly understood consequence of acute ischemic stroke. Here, we investigated the potential contributions of HDAC3 to inflammasome activation in primary cultured microglia and experimental stroke models. In this study, we documented that HDAC3 expression was increased in microglia of mouse experimental stroke model. Intraperitoneal injection of RGFP966 (a selective inhibitor of HDAC3) decreased infarct size and alleviated neurological deficits after the onset of middle cerebral artery occlusion (MCAO). In vitro data indicated that LPS stimulation evoked a time-dependent increase of HDAC3 and absent in melanoma 2 (AIM2) inflammasome in primary cultured microglia. Interestingly, AIM2 was subjected to spatiotemporal regulation by RGFP966. The ability of RGFP966 to inhibit the AIM2 inflammasome was confirmed in an experimental mouse model of stroke. As expected, AIM2 knockout mice also demonstrated significant resistance to ischemia injury compared with their wild-type littersmates. RGFP966 failed to exhibit extra protective effects in AIM2−/− stroke mice. Furthermore, we found that RGFP966 enhanced STAT1 acetylation and subsequently attenuated STAT1 phosphorylation, which may at least partially contributed to the negative regulation of AIM2 by RGFP966. Together, we initially found that RGFP966 alleviated the inflammatory response and protected against ischemic stroke by regulating the AIM2 inflammasome.

KEYWORDS
AIM2 inflammasome, HDAC3, microglia, STAT1, stroke
Histone-modifying enzymes are epigenetic modulators of inflammatory responses in various central nervous diseases, including ischemic stroke. Emerging evidence has demonstrated that histone deacetylase 3 (HDAC3) deletion dampens inflammatory processes in cultured macrophages and microglia. Screening for different HDAC isoforms in a stroke model revealed a substantial increase in HDAC3 during the early phases of experimental stroke, indicating its potential contributions to stroke pathogenesis. However, the functional properties of HDAC3 in microglia after stroke are currently unknown. These findings spurred our interest in exploring the protective efficacy of HDAC3 inhibitor in ischemic stroke and profile its underlying mechanisms in microglia.

Recently, emerging evidence has pointed to critical roles of the inflammasome in regulating immunological and inflammatory cascades after ischemic stroke and neurodegenerative diseases. Absent in melanoma 2 (AIM2) belongs to the interferon-inducible gene HIN-200 family and structurally consists of an N-terminal pyrin domain (PYD) and a C-terminal oligonucleotide-binding HIN domain. When the HIN domain senses damaged-associated molecular patterns (DAMPs), AIM2 recruits apoptosis speck-like protein (ASC) and caspase-1 to form a molecular platform for the maturation and secretion of IL-1β and IL-18. This multiprotein complex was also associated molecular patterns (DAMPs), AIM2 recruits apoptosis speck-like protein (ASC) and caspase-1 to form a molecular platform for the maturation and secretion of IL-1β and IL-18. This multiprotein complex was also named the AIM2 inflammasome. However, activation of the AIM2 inflammasome and its physiological relevance to ischemic stroke are poorly understood. A previous study demonstrated that activation of the AIM2 inflammasome in cultured rat embryonic cortical neurons mediated pyroptotic neuronal cell death. Microglial activation is a prominent feature of inflammation after ischemic stroke. Targeting the functional repertoire of microglia may hold novel approaches for further stroke management. The properties and regulation of the AIM2 inflammasome in microglia are unknown.

Therefore, in the present study, we explored the cellular distribution and expression characteristics of HDAC3 with or without ischemic challenge. RGFP966 is a selective HDAC3 inhibitor with an IC50 of 0.08 µM and could cross the blood-brain barrier when administered peripherally. We confirmed that HDAC3 inhibition by RGFP966 protected against ischemic brain injury. We also investigated whether RGFP966 could regulate the AIM2 inflammasome and explored its potential mechanisms in vivo and in vitro. Thus, we concluded that the negative regulation of the AIM2 inflammasome in microglia by RGFP966 alleviated the inflammatory response and protected against ischemic stroke.

## Materials and Methods

### Animals and drug treatment

AIM2 knockout mice generated by the CRISPR/Cas9 system were obtained from the Model Animal Research Center of Nanjing University. Age-matched C57BL/6J littermates were used as controls. Experiments were carried out in 12- to 16-week-old male mice. In reference to previous studies, RGFP966 (Selleck Chemicals, Houston, TX, USA) at 10 mg/kg was administered intraperitoneally immediately after ischemic stroke injury and then twice per day until the mice were sacrificed. WT mice or AIM2 KO mice were randomly assigned to the sham, middle cerebral artery occlusion (MCAO)+Vehicle, MCAO+RGFP966 groups. All animal experiments were approved by the Animal Care Committee of Nanjing University. Drugs and animal strains were arranged and labeled by an independent researcher according to the randomization plan.

### Middle cerebral artery occlusion model in mice

Transient MCAO was induced by the intraluminal filament technique as previously described. Briefly, after mice were anesthetized with 1% sodium pentobarbital (45 mg/kg, i.p.), a midline cervical incision was made under a dissecting microscope. Then, the right common carotid artery and external carotid artery (ECA) were isolated. A 6-0 monofilament nylon suture with a heat-rounded tip was introduced into a wedge-shaped incision on the ECA and arranged to obstruct the origin of the middle cerebral artery in advance. After 60 minutes of occlusion, reperfusion was initiated by withdrawing the filament. Mice were included if the Doppler laser reading was below 20% of baseline and they exhibited neurological deficits after anesthesia recovery. Sham-treated mice were subjected to the same procedure without MCAO. Rectal temperature was maintained at 37.0 ± 0.5°C during the procedure.

### Infarct size measurement

The brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and cresyl violet (Sigma, St. Louis, MO, USA) to detect infarct sizes at 3 d and 7 d, respectively, after occlusion. For TTC, brains were removed, cut into seven coronal slices and then immersed in a 2% TTC solution at 37°C for 20 minutes in the dark. The pale gray color indicates the infarct area, and a dark red color indicates normal tissues. For cresyl violet staining, brain sections
were treated with 0.5% cresyl violet staining solutions and rinsed with DDW and ethanol. Slices were photographed and analyzed with image analysis software (NIH ImageJ, Bethesda, MD) to calculate the infarct size. The infarct volume of each mouse was calculated by the following equation: (viable area of contralateral hemisphere—infracted area of ipsilateral hemisphere)/viable area of contralateral hemisphere.

2.4 | Behavior test

2.4.1 | NSS score

Neurological function was graded on a scale of 0-18 (normal score, 0; maximal deficit score, 18). The NSS included a composite of motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), reflex and balance tests. In the severity scores of injuries, one point is given for the inability to perform each test while one point is deducted for the lack of a tested reflex, and an overall composite score is given to determine the impairments. Higher score indicated severer injury.

2.4.2 | Rotarod test

The rotarod test was performed to assess loss of balance and sensorimotor coordination. Briefly, latency to fall on a rotating rod was recorded by a five-lane rotarod device (IITC Life Science). The rotating rod was placed horizontally, accelerating from 4 to 40 rpm for 5 minutes. The mice were trained for 3 days before the operation with 3 times a day, and each training lasts for 5 minutes with a 15-minutes training interval for rest. After 3 days of training, the mice that could not adhere to the fatigue rotating rod for 300 seconds were excluded. The mice were placed on the rod in turn, and the average time of latency to fall for three rounds of experiments was recorded.

2.4.3 | Grip strength test

Briefly, the test includes a spring balance coupled with a Panlab machine (LE902, Bioseb, USA) that is attached to a triangular steel wire. Animals were pulled by the tail followed by instinctive grasping of the spring by the animal. The Grip Strength Test measures the maximal peak force (g) of the rodent’s grip. The test was performed five times on the forepaws of mice. The maximal peak force during the five measurements was recorded. Neurobehavioral and histological studies were evaluated by an observer blinded to the experiments.

2.5 | Primary microglia culture and drug treatment

Primary microglia were isolated and purified from the mouse brain using P1-P2 pups. First, the cells from brain cortices were seeded into 75 cm² TC flasks and cultured for 14 d. The loosely adherent microglia was harvested from the culture medium after a slight shaking step. The unattached microglia were collected, centrifuged, and then re-plated onto plates at a density of $4 \times 10^5$/mL.

For drug administration, 15 μM RGFP966 was applied to the microglia culture medium 1 hour before adding 500 ng/μL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA). AG490 (10 μM; Santa Cruz, CA, USA), JSH-23 (10 μM; Selleck Chemicals, Houston, TX, USA), and 3-methyladenine (3-MA; 10 mM; Selleck Chemicals, Houston, TX, USA) were administered to primary cultured microglia 1 hour ahead of LPS stimulation. Cells were harvested for the following experiments at indicated time points.

2.6 | Western blot and immunoprecipitation

Protein from cultures or brain tissue was extracted and qualified using standard protocols described previously. Equal amounts of total protein from each sample were separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were probed with primary antibodies against HDAC3 (1:1000; Abcam, Cambridge, MA, USA), IL-1β (1:1000; Cell Signaling, Boston, MA, USA), IL-18 (1:500; Abcam, Cambridge, MA, USA), AIM2 (1:500; Cell Signaling, Boston, MA, USA), ASC (1:1000; AdipoGen, Santiago, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Bioworld, Minneapolis, MN, USA), STAT1 (1:1000, Cell Signaling, Boston, MA, USA), Tyr701-STAT1 (1:500, Cell Signaling, Boston, MA, USA), Ser727-STAT1 (1:500, Cell Signaling, Boston, MA, USA). The membranes were then submerged in solution containing HRP-conjugated secondary antibodies, and protein bands were visualized with an ECL kit (Millipore, Billerica, MA, USA). Image-Pro Plus (National Institutes of Health, USA) was employed to determine the intensities of the bands. Acetylated protein was immunoprecipitated with an Ac-lys antibody (Cell Signaling, Boston, MA, USA) and incubated with protein A/G agarose beads (Millipore, MA, USA) overnight at 4°C. After the beads were completely washed, proteins were eluted by boiling the samples in 2×SDS loading buffer. Then, the sample was further processed by western blot as described. Rabbit IgG and the antibody used in the immunoprecipitation assay served as negative controls.
2.7 | HDAC3 activity assay

Nuclear protein was extracted from the cultured microglia using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA). HDAC3 activity was quantified with HDAC3 Activity Fluorometric Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer’s instruction. Briefly, nuclear extraction samples were incubated with HDAC3 substrate and developer for indicated time durations. Then, each sample well and standard curve well was read at Ex/Em = 380/500 nm by TECAN spark (Austria).

2.8 | ELISAs for cytokines

Mouse IL-1β, IL-18, and IFN-β ELISA kits (Cusabio Biotech, Wuhan, China) were used to detect cytokine concentrations in supernatants of microglia cultures. Briefly, 100 μL of cultured media from different groups was added to each well of 96-well plates coated with anti-mouse cytokine antibody. The plates were incubated at 37°C for 90 minutes and then washed 5 times. Next, 100 μL of biotinylated cytokine-specific antibody was added to each well and incubated at 37°C for 60 minutes. Then, the plates were washed, treated with 100 μL of diluted streptavidin-HRP, and incubated at 37°C for 30 minutes. The color was produced by the addition of 100 μL of substrate solution for 10-15 minutes after washing. Finally, 100 μL of stop solution was added to terminate the reaction. Finally, the optical density at 450 nm was measured within 10 minutes.

2.9 | Immunofluorescence

Primary microglia were fixed with 4% PFA for 15 minutes. Brain sections from the indicated time points were exposed to 0.2% PBST (0.2% Triton X-100 in 0.01 mol/L PBS) for permeation and 0.2% BSA to exclude nonspecific staining. Subsequently, sections were incubated with the primary antibodies at 4°C overnight. For double immunofluorescence staining, AIM2 polyclonal antibody (1:200, Abcam, Cambridge, MA, USA), HDAC3 polyclonal antibody (1:500, Abcam, Cambridge, MA, USA), or TMEM119 (1:200, Abcam, Cambridge, MA, USA) were added to the sections, which were then washed with PBS, incubated with other primary antibodies against neuronal nuclei (NeuN) (1:500; Millipore, Billerica, MA, USA), glial fibrillary acidic protein (GFAP) (1:500; BD Biosciences, Sparks, MD, USA), and ionized calcium binding adaptor molecule 1 (Iba-1) (1:500; Abcam, Cambridge, MA, USA). Infarcted size of AIM2−/− group was detected by MAP2 stain (1:500, Millipore, Billerica, MA, USA). For ASC, caspase-1, AIM2 confocal staining, active caspase-1 were detected with a FLICA 660 caspase-1 assay kit according to the manufacturer’s instructions (ImmunoChemistry Technologies, Bloomington, MN, USA). After cells were labeled with FLICA, they were counterstained with AIM2 (1:200, Abcam, Cambridge, MA, USA) or ASC (1:1000; AdipoGen, Santiago, USA). Then, secondary antibodies (1:500, Invitrogen, USA) targeting their corresponding primary antibodies were applied for 2 hours at room temperature. The slides were completely rinsed with PBS, mounted, and covered. Images were captured with a fluorescence microscope (Olympus, Japan) or confocal laser-scanning microscope (Olympics FV10i, Japan). The percentage of cells was determined by counting three randomly selected fields across six slides of ipsilateral sides or cover-slips. Fluorescent-positive cells were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.10 | Isolation of adult primary microglia

Briefly, ipsilateral brain hemispheres of each group were dissected and quick transferred to cold HBSS. Single-cell suspensions were made using the Neural Tissue Dissociation Kit according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, we discard myelin debris through percoll (GE Healthcare) gradient centrifugation. CD11b-positive microglia were magnetically labeled with CD11b MicroBeads for 20 minutes (Miltenyi Biotec, Bergisch Gladbach, Germany), loaded onto a MACS Column and subjected to magnetic separation. To exclude the effect of macrophages, isolated cells were then stained with antibodies anti-CD45 (eBioscience, Frankfurk, Germany) and anti-CD11b (eBioscience, Frankfurk, Germany) in FACS buffer for 20 minutes at 4°C. Isoype antibodies were used as control. Cells were then washed two times, re-suspended in FACS washing buffer. We collected CD11bhigh CD45intermediate cells by flow cytometry (BD FACSaria II, Becton Dickinson, Franklin Lakes, NJ, USA) as isolated microglia samples.

2.11 | Q-PCR

Total RNA was extracted from the brain tissues using a commercial Trizol kit (Invitrogen, USA), and then RNA was reverse-transcribed into cDNA with a PrimeScript RT reagent Kit (Takara, Dalian, China). The quantitative experiment was completed using an ABI 7500 PCR instrument (Applied Biosystems, USA) through a SYBR green Kit (Applied Biosystems, USA), with the relative gene expression levels normalized to GAPDH. Primers were as follows:

IL-1β: Forward: ATTTGTCATAGCCCGCAGCT; Reverse: GCCTCATGGCCAATTTTCTC

AIM2: Forward: AAATGCTGTTGTTGACCGGC; Reverse: GAGTTGTCTTCGCGCAATCT

GAPDH: Forward: GCCAAGGCTGTTGGCGAAGGT; Reverse: TCTCCAGGGCGACGTCA
2.12 | Statistical analysis

Data are presented as the mean ± SD except for special indications. Comparisons between groups were performed by Student’s t test (single comparisons) or one-way analysis of variance (ANOVA) followed by a post hoc Scheffe test (for multiple comparisons). Two-sided \( P < .05 \) was considered significant. All analyses were performed using SPSS 18.0.

3 | RESULTS

3.1 | HDAC3 expression was induced in microglia/macrophage after ischemic stroke

In our previous study, we observed a substantial increase in HDAC3 expression in the early phases of experimental stroke.\(^5\) To further characterize the localization pattern of HDAC3 in the central nervous system, immunofluorescence was performed, and images were captured by Olympus microscopy. Minimal HDAC3-positive fluorescent signals were detected in intact microglia/macrophage. Interestingly, temporal analysis demonstrated that HDAC3 upregulation was detectable at 1 d, peaked at 3 d, and was gradually decreased at 7 d (Figure 1A,D). Specifically, HDAC3 was mainly concentrated in the nucleus 1 d following ischemia (Figure 1B) and gradually spread to cytoplasm 3 d following ischemia (Figure 1C). As shown in Figure S1, HDAC3 was widely distributed in the cerebral cortex (Figure S1A), lateral ventricles (Figure S1B), hippocampus (Figure S1C), and cerebellar cortex (Figure S1D). Double labeling of HDAC3 with NeuN (Figure S1F) and GFAP (Figure S1E) demonstrated that neurons and astrocytes were the dominant cells expressing HDAC3 protein in intact mouse brain. However, we did not observe specific HDAC3 enhancements at the ipsilateral penumbra area in astrocytes (Figure S1G) and neuron (Figure S1H). Therefore, our data indicated that HDAC3 expression was induced in microglia/macrophage after ischemic stroke.

**FIGURE 1** HDAC3 expression was induced in microglia/macrophage after ischemic stroke. Double-labeling of HDAC3 with Iba-1 in intact mouse brain and ipsilateral penumbra areas at 1 d, 3 d, and 7 d after ischemic stroke (A). Confocal images showed cellular distribution pattern of HDAC3 in microglia at 1 d and 3 d following ischemia (B & C). Quantitative graph represented HDAC3/Iba-1 double positive cells at different time points (D). \(* P < .05\), \(** P < .01\) versus the 1 d group, Scale bar = 20 μm, N = 3 mice per time point.
3.2 | HDAC3i protected against ischemic brain injury in vivo

We next investigated whether RGFP966 (a selective HDAC3 inhibitor) protects the brain against ischemic injury induced by 60 minutes of MCAO. Infarct sizes at 3 d and 7 d were detected with TTC staining (Figure 2A) and cresyl violet staining (Figure 2B), respectively. As shown in Figure 2C, infarct sizes were smaller in the RGFP966 group than in the vehicle group (3 d: 24.15% ± 7.06% in the RGFP966 group versus 34.48% ± 6.73% in the vehicle group, \( P < .05 \); 7 d: 24.76% ± 4.88% in the RGFP966 group versus 33.30% ± 5.61% in the vehicle group, \( P < .05 \)). rCBF as measured by Doppler laser flowmetry showed no significant differences between these two groups. The neurological performance of each mouse at the indicated time points was evaluated by the NSS score and the Rotarod test. As shown in Figure 2D, RGFP966 alleviated neurological deficits at 24 hours (\( P < .05 \)), 48 hours (\( P < .05 \)), and 7 d (\( P < .05 \)) after reperfusion based on the NSS score. Consistent with the histological data, HDAC3i mice also showed improved motor functions in Rotarod test, as indicated by increased latency to fall down at 24 hours (\( P < .05 \)) and 6 d (\( P < .05 \)) after reperfusion time.

3.3 | LPS evoked HDAC3 and AIM2 inflammasome activation in primary cultured microglia

Then, we cultured primary microglia to study HDAC3 in vitro. The HDAC3 level increased immediately and profoundly in LPS-treated microglia, with the peak time point at 8 hours (2.2 folds of control, \( P < .05 \)) (Figure 3A,B). Additionally, LPS evoked AIM2 and IL-18 levels in microglia (Figure 3A). We also observed that HDAC3 activity increased after LPS-treatment with a peak at 12 hours (3.1 folds of control, \( P < .05 \)) (Figure 3C). The elevation of HDAC3 expression and activity inspired us to investigate the role of HDAC3 in the regulation of microglia after stroke.

FIGURE 2 | HDAC3i protected against ischemic brain injury in vivo. MCAO was induced in C57BL/6J mice, and RGFP966 was intraperitoneally injected at the onset of reperfusion. Representative TTC staining (A) and cresyl violet staining (B) of brain sections at 3 d and 7 d after MCAO, respectively. Infarct volumes at 3 d and 7 d were calculated (C). N = 8-12 mice per group. The neurological performance of mice in each group at different time points was recorded by the NSS score (D) and Rotarod test (E). N = 10-15 mice per group. *\( P < .05 \) versus the vehicle group. Data are presented as the mean ± SD, except data of Rotarod test are presented as the mean ± SEM.
We further delineated the AIM2 inflammasome complex and active caspase-1 with the FLICA 660-YVAD-FMK probe, which specifically binds the active caspase-1 enzyme and excites in the far-red spectrum within cells. As depicted in Figure 3D, foci of active caspase-1 staining were located beside the nucleus upon LPS stimulation. AIM2 was diffusely distributed in the cytoplasm and nucleus, whereas ASC was mainly concentrated in the nucleus. Active caspase-1 could merge with AIM2 and ASC, which strongly suggested that AIM2, ASC, and caspase-1 may form a functional inflammasome complex in LPS-stimulated microglia.

3.4 | RGFP966 inhibited the AIM2 inflammasome in primary cultured microglia

Our previous proteomic study addressed the suppressive effects of HDAC3i on multiple inflammatory cytokines in microglia. We supposed that HDAC3i may protect against ischemic injury though modulating inflammatory response. 15 μM RGFP966 could exert efficient inhibitory function of HDAC3 (14% of LPS treatment group, *P < .05) (Figure S2A) by HDAC3 specific activity kit. Thus, we used 15 μM RGFP966 to study the role of HDAC3 in vitro.

Pretreatment with RGFP966 significantly reduced the protein expression of ASC and AIM2. This trend was profound at 12 and 24 hours after LPS treatment (Figure 4A-C). Perturbed secretion of IL-1β and IL-18 was also observed when RGFP966 was incubated with cells for 12 hours (IL-1β: *P < .05; IL-18: *P < .05) and 24 hours (IL-1β: *P < .05; IL-18: *P < .05) (Figure 4E,F). By immunostained with AIM2, microglia showed branched processes radically oriented to a small elliptical soma at rest. Upon LPS stimulation, microglia were converted into enlarged cell bodies with a ramified appearance. Strikingly, RGFP966 partially draws activated microglia back to their quiescent morphology (Figure 4D).

Taken together, these data indicated that RGFP966 negatively regulated the AIM2 inflammasome in primary cultured microglia.

3.5 | RGFP966 decreased inflammasome expression in an experimental mouse model of stroke

To further dissect how the inflammasome is regulated during acute ischemic stroke, we utilized a well-established MCAO model. Then, we detected ipsilateral levels of AIM2 inflammasome complexes and inflammatory cytokines at...
24 hours after stroke by western blotting. AIM2 and ASC were significantly increased in the MCAO+Veh. group compared with the sham group. A massive decrease was observed in the MCAO+RGFP966 group regarding AIM2 (0.60-fold of the Veh. group, \( P < .05 \)) and ASC (0.43-fold of the Veh. group, \( P < .05 \)) (Figure 5A,C). In accordance with the in vitro results, the levels of the inflammatory cytokines IL-1\( \beta \) and IL-18 were reduced in response to RGFP966 treatment (Figure 5A,B). To detect AIM2 inflammasome components in microglia after stroke, we prepared single-cell suspension and gated CD11b\(^{\text{high}} \) CD45\(^{\text{intermediate}} \) as microglia in situ (Figure 5F) by flow cytometry. Then, RNA was extracted from the sorting microglia and inflammasome component level was measured by Q-PCR. We found that AIM2 and IL-1\( \beta \) were significantly increased in the MCAO+Veh. group compared with the sham group, while RGFP966 treatment significantly decreased their expressions (Figure 5D,E).

### 3.6 AIM2 inflammasome played an essential role in ischemic stroke

Next, we addressed the functional roles of AIM2 in ischemic stroke by employing AIM2 knockout mice. As shown in Figure 6A,B, an approximately 10.64% reduction in infarct size in the AIM2\(^{-/-} \) group compared with the WT group following 48 hours of reperfusion was observed \( (P < .05) \). Approximately 8.33% mice in the AIM2\(^{-/-} \) group and 14.29% mice in the WT group died within 7 d after...
reperfusion. Neurological performances at the indicated time points were evaluated by the NSS score and grab strength as depicted in Figure 6C,D. AIM2−/− mice exhibited a better performance than did the WT group at 2 d ($P < .05$) and 3 d ($P < .05$) after stroke onset by NSS score. Consistent with the NSS results, the grab strength of the upper limbs in the AIM2−/− group was stronger than that in the WT group (1 d: $P < .05$; 2 d: $P < .05$). Then, we compared microglia proliferation in AIM2−/− group with its wide-type littermates by immunostaining with TMEM119 and Iba-1. As shown in Figure 6E,F, the amount of TMEM119/Iba-1-positive cells was decreased in AIM2−/− group at 48 hours after stroke ($P < .05$).

3.7 RGFP966 failed to exhibit extra protective effects in AIM2−/− stroke mice

To address the causative relationship that HDAC3 inhibitor is protective through downregulating AIM2, we separated the AIM2 −/− mice into AIM2−/− +Vehicle group
and AIM2−/− +RGFP966 group. In AIM2−/− stroke mice, treatment with RGFP966 failed to reduce additional infarct volume compared to vehicle group 48 hours after stroke (Figure 7A,B). In behavior tests, RGFP966 treatment in AIM2−/− group did not improve neurological performances compared to vehicle group, which further confirmed our hypothesis that HDAC3 inhibitor RGFP966 could ameliorate ischemic brain damage by downregulating the AIM2 inflammasome (Figure 7C,D).

3.8 | RGFP966 suppressed AIM2 probably via modulating the acetylation and phosphorylation of STAT1

To further establish the fundamental regulatory mechanisms of AIM2 in the inflammatory response, we applied 3-MA (a PI3K and autophagy inhibitor), AG490 (a JAK/STAT pathway inhibitor), and JSH-23 (an NF-κB nuclear translocation inhibitor) to cultured primary microglia. As shown in Figure 8A, LPS lost the ability to initiate AIM2 expression in cells exposed to AG490 (0.77-fold of the LPS+RGFP966 group, \( P < .05 \)). The amplitude of AG490 inhibition was stronger than that of JSH-23 (0.97-fold of the LPS+RGFP966 group, \( P > .05 \)). Then, we detected activation of the JAK/STAT pathway in primary cultured microglia and in a mouse stroke model. Additionally, 3-MA elevated AIM2 (1.3-fold of the LPS+RGFP966 group, \( P < .05 \)) expression, which was perturbed by RGFP966.

Tyrosine 701 and serine 727 of STAT1 were phosphorylated upon 2 and 4 hours LPS stimulation while the expression of STAT1 remained stable. Nevertheless, RGFP966 did not impair STAT1 phosphorylation at 2 hours but significantly attenuated the phosphorylation of STAT1 4 hours upon LPS stimulation (Tyr701: 0.23-fold of the LPS group, \( P < .01 \); Ser727: 0.32-fold of the LPS group, \( P < .01 \)) (Figure 8B,D,E). Furthermore, we explored STAT1 activation at 6 and 24 hours after MCAO. Consistent with the in vitro data, phosphorylation (Tyr701 and Ser727) of STAT1 was significantly attenuated by RGFP966 at 24 hours after reperfusion (Figure 8C,D,E).

It has been reported that STAT1 acetylation may subsequently diminish STAT1 tyrosine phosphorylation and activation.\(^\text{18}\) Then, we suspected whether direct acetylation of

FIGURE 6 The AIM2 inflammasome played an essential role in ischemic stroke. Representative brain sections stained for MAP2 (A), showing a reduction in the infarct size in the AIM2−/− group (B). Neurological performance of the WT and AIM2−/− knockout groups at different time points was recorded by the NSS score and grab strength (C&D). N = 6-12 mice per group \( *P < .05 \) versus WT littermates. (E&F) The number of TMEM119/Iba-1 positive cells was decreased AIM2−/− group at 48 hours after stroke, scale bar = 20 μm, N = 3 mice per group.
STAT1 protein could interfere with STAT1 activation. Using co-IP, we detected enhanced STAT1 acetylation after incubation with RGFP966 for 2 hours (1.62-fold of the LPS group, \(P < .05\)), which was 2 hours ahead of STAT1 phosphorylation inhibition (Figure 8F,G). Overall, our data suggested that RGFP966 suppressed AIM2 expression, probably by regulating STAT1 acetylation and phosphorylation. Fundamentally, these data suggested that RGFP966 negatively regulated the AIM2 inflammasome by enhancing the acetylation of STAT1.

## DISCUSSION

In this current study, we initially found HDAC3 expression increased in microglia/macrophage of mouse experimental stroke model. The AIM2 inflammasome was spatiotemporally regulated by RGFP966 in primary microglia and in a mouse model of stroke. Both RGFP966-treated and AIM2-deficient mice were highly resistant to ischemic challenge. RGFP966 failed to confer extra protective effects in AIM2−/− stroke mice. RGFP966 enhanced STAT1 acetylation and subsequently attenuated STAT1 phosphorylation. Taken together, these data led us to conclude that RGFP966 protected against ischemic injury by regulating AIM2 inflammasome. These findings may provide a novel avenue for therapeutic strategies against stroke in the future.

HDAC3 is the most widely expressed HDAC in the brain.\(^{19,20}\) In our study, we detected the accumulation of HDAC3 in microglia/macrophage after stroke. Our data showed HDAC3 occurred prominently in microglia nuclei at 1 d and 7 d after stroke, which is consistent with the previous study in spinal cord injury.\(^{21}\) However, it was also expressed in cytoplasm 3 d after stroke. Unlike other Class I HDACs, HDAC3 contains both nuclear export signal (180-313 aa in the central portion) and the nuclear localization signal (312-428 aa in the C-terminal),\(^{22}\) which indicated that HDAC3 may shuttle between the cytoplasm and nucleus by itself. However, the molecular events that initiate the shuttling after stroke are not clear. Emerging evidences suggested that cytoplasmic translocation of HDAC3 may be related with inflammatory molecular including IκBα\(^{23}\) and STAT3.\(^{24}\)

Over the past few years, pharmacological manipulations with nonspecific HDAC inhibitors have shown severe dose-limiting toxicities when translated to clinical trials.\(^{25}\) Thus, accurately understanding the biological effects of specific HDACs could be beneficial to the development of more specific and well-tolerated therapeutic strategies. In neurological diseases, the critical roles of HDAC3 in Huntington disease\(^1\) and Alzheimer disease\(^26,28\) have been well established. HDAC3 was reported to participate in dopaminergic neuronal cell death\(^29\) and embryonic neurogenesis.\(^{30}\) A previous study stated that inhibition of HDAC3 may contribute...
to the neuronal survival elicited by preconditioning and that peripheral injection of RGFP966 at 24 and 6 hours prior to MCAO reduced the infarct volume.15 In our study, stroke mice exhibited a smaller infarct volume when receiving RGFP966 immediately after MCAO. Until now, the anti-inflammatory activities of HDAC3i in ischemic stroke have not been reported. Our previous in vitro study described HDAC3 inhibition largely suppressed inflammatory gene expression in cultured microglia.4 This anti-inflammatory repertoire of HDAC3 has been reported in macrophages3,31,32 and rheumatoid arthritis fibroblast-like synoviocytes.33 Here, we confirmed that RGFP966 suppressed the production of inflammasome-associated cytokines in primary microglia and stroke models, through which RGFP966 conferred the protection.

By sensing cytoplasmic DNA, AIM2 assembles the ASC through its pyrin domain and facilitates caspase-1 activation and IL-1β/IL-18 maturation.34 Apart from free dsDNA, offending agents such as LPS, IFN-γ, and IFN-β could also provoke AIM2 activation.35,36 The most prominently held opinion regarding AIM2 expression in the central nervous system was that it was confined to developing neurons.12,37 The neuronal distribution pattern of AIM2 endowed these neurons with diverse functionalities, including regulating neuronal morphology and pyroptotic neuronal cell death. Consistent with our study, expression of AIM2 was detected in highly purified primary murine microglia.36 Thus, it is anticipated that AIM2 is required for microglial activation and inflammation. Available evidence suggested that AIM2−/− mice exhibited reduced

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**FIGURE 8** RGFP966 modulates the acetylation and phosphorylation of STAT1. 3-Methyladenine (3-MA,10 mM), AG490 (10 µM) and JSH-23 (10 µM) were applied to primary microglia one hour ahead of LPS stimulation. Representative western blotting (B) and quantitative analysis of AIM2 (A) indicated that AIM2 expression evoked by LPS could be blocked by AG490. The levels of phosphorylated Tyr701 and Ser727 on STAT1 at 2 and 4 hours after LPS treatment were detected by western blotting (B) and quantified (D&E). Representative images (C) and statistical analysis (D&E) showing phosphorylation of STAT1 at 6 and 24 hours after MCAO. STAT1 acetylation was measured by immunoprecipitating Ac-lys and blotting for STAT1 (G). Bar graph of STAT1 acetylation in microglia incubated with RGFP966 or LPS (F). N = 3 independent repeats for cell study. N = 3 mice per group for the animal study. *P < .05 versus vehicle group.
Our data confirmed that AIM2−/− inhibited microglia infiltration 48 hours after stroke and protected against ischemic brain injury.

In this study, we reported that HDAC3 could regulate the AIM2 inflammasome in vivo and in vitro. Currently, the precise molecular mechanisms that govern AIM2 inflammasome activity are still poorly understood. Studies reported AIM2 subjected to the regulation of type I IFNs, which, in turn, drive AIM2 inflammasome activation. Chen et al. reported that the impact of Hdac3 deletion on the LPS-induced gene suppression was largely dominated by an impaired IFN-β response. Therefore, it is possible that RGFP966 inhibited AIM2 expressions through suppressing IFN-β response. We also confirmed that RGFP966 decreased supernatant IFN-β concentrations of LPS-treated microglia (data did not show). IFN-β may provide a feedback loop to activate STAT1/STAT2 through interacting with IFNAR and promote IFN-dependent gene expressions. In our study, AG490 interfering with the JAK/STAT pathway diminished AIM2 expression. RGFP966 decreased the phosphorylation of STAT1 in cultured microglia and experimental stroke, which partially explain why RGFP966 could modulate AIM2 activation. The notion that HDAC facilitates inflammatory gene expression through histone acetylation at the 5′ end of coding regions seems counterintuitive. Actually, lysine acetylation of transcriptional regulators may explain why HDACi causes prominent inflammatory gene depression. Our data demonstrated that LPS-induced acetylation of STAT1 was enhanced by RGFP966. Evidence from previous studies reported that STAT1 acetylation is necessary for its subsequent dephosphorylation. Thus, we surmised that RGFP966 may inhibit STAT1 activation through directly enhancing acetylation of STAT1.

It has been reported that the AIM2 inflammasome could be delivered to autophagosomes for degradation in a p62-dependent manner. It was also observed that 3-MA particularly abolished AIM2 inhibition by RGFP966 in our study, indicating plausible autophagosome regulations between RGFP966 and AIM2. It bears noting that HDAC3 inhibition to a single mechanism would be unreasonable. Regardless, in this study, we considered that STAT1 could be a potent target of HDAC3 in regulating the AIM2 inflammasome.

5 | CONCLUSIONS

Our data demonstrated an essential role of HDAC3 in the deployment of AIM2 inflammasome activation after ischemic stroke, which could be of therapeutic relevance for the treatment of ischemic injury in the foreseeable future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

M.-J. Zhang and Y. Xu designed the research; Q.-C. Zhao and M.-X. Xia analyzed the data; J. Chen, M.-X. Xia, X. Cao, Y.-T. Chen, Y. Liu, and Q.-C. Zhao performed the research; M.-J. Zhang wrote the article; Z.-Q. Yuan and X.-Y. Wang provided technological support for the experiments and revised the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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