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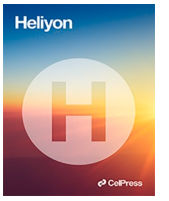

Research article

Low-dose IL-2 treatment confers anti-inflammatory effect against subarachnoid hemorrhage in mice

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ABSTRACT

Objective: Subarachnoid hemorrhage (SAH) was a stroke with high occurrence and mortality. At the early stage, SAH patients have severe cerebral injury which is contributed by inflammation. In this study, we aimed to explore the anti-inflammation effect of low-dose IL-2 in SAH mice.
Methods: The 12-week-old C57BL/6J male mice were conducted with SAH surgery (Internal carotid artery puncture method). Different dose of IL-2 was injected intraperitoneally for 1 h, 1 day, and 2 days after SAH. Single-cell suspension and flow cytometry were used for the test of regulatory T (Treg) cells. Immunofluorescence staining was used to investigate the phenotypic polarization of microglia and inflammation response around neurons. Enzyme-Linked Immunosorbent Assay (ELISA) was applied to detect the level of pro-inflammatory factors.
Results: Low-dose IL-2 could enrich the Treg cells and drive the microglia polarizing to M2. The level of pro-inflammatory factors, IL-1α, IL-6, and TNF-α decreased in the low-dose IL-2 group. The inflammation response around neurons was attenuated. Low-dose IL-2 could increase the number of Treg cells, which could exert a neuroprotective effect against inflammation after SAH.
Conclusion: Low-dose IL-2 had the potential to be an effective clinical method to inhibit inflammation after SAH.

1. Background

Subarachnoid hemorrhage (SAH) accounts for 5 % of stroke. Compared with other stroke types, the mean death age caused by SAH was 59 much younger than intracerebral hemorrhage at 73 and ischemic stroke at 81 [1]. Despite the occurrence of SAH increases with age, nearly half the patients were younger than 55 at their suffering from SAH [2]. The 85 % cause of SAH is intracranial aneurysm

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formation and rupture. Inflammation after the bleeding contributes to the severe headache which is the early typical symptom of SAH. Despite the limited treatments used by clinical neurologists, just as reducing intracranial pressure relieving cerebral vasospasm or even operating surgeries for critical rebleeding patients, researchers have made increasing attention to effective anti-inflammation therapies after acute SAH, which has performed considerable neurological benefits and would probably bring a new progression on the clinical therapies of SAH.

After SAH, patients often suffer from the initial bleeding, followed by the second brain injury of inflammation. The toll-like receptor (TLR)4 which is expressed on the surface of the microglia signaling pathway was proved to play an important role in SAH. Researchers found genetic depletion and deactivation of microglia showed obvious inhibition of cerebral-spreading inflammation after SAH [3]. In stroke and trauma brain injury, Microglia showed M1/M2 Phenotypic Polarization according to the different situations. In the M1 state, microglia tend to be pro-inflammatory by releasing cytokines, while M2 is anti-inflammatory. Microglia participated in the attenuation inflammatory process after SAH by activation of RARα Receptor [4].

Interleukin (IL)-2 could stimulate the T cells secreting some lymphokines, like IFN-γ, IL-4, IL-5, IL-6, TNF-β. Furthermore, IL-2 could induce the activation of killer cells, like CTL, NK, and LAK, secreting TNF-α and IFN-γ. IL-2 could also activate the macrophage, mediating immune response [5,6]. Researchers revealed that high-dose IL-2 had the potential possibility of immunotherapy for cancer and organ transplant [7,8] and infectious diseases [9,10].

Based on the different mechanisms of inhibiting inflammation, Treg cells were divided into $CD4^{+}CD25+$Tr, Tr1, and Th3. $CD4^{+}CD25+$Tr activated T cells expressing CTLA-4(cytotoxic T lymphocyte-associated antigen-4, CTLA-4), which way inhibited the immune response of T cells. Tr1 could secrete the IL-10 and Th3 secreted TGF-β to inhibit the proliferation and differentiation of T cells. Treg infiltrates the cerebral area rapidly after neuroinflammation happens, by secreting IL-10 and polarizing the microglia into a neuroprotective state. Researchers proved that Treg cell-derived osteopontin could promote white matter repair after ischemic stroke by mediating microglial activation [11]. In the injured spinal cord, researchers found that pyroptosis mainly happens in microglia and that treg transmitted exosome-miR-709 to microglia to inhibit pyroptosis [12].

In our literature, we focused on the anti-inflammation effect of low-dose IL-2 in mice after SAH, exploring the potential therapy value of it in SAH in clinical.

2. Materials and methods

2.1. Animals and experiment design

The 12-week-old C57BL/6J male mice (25 g ± 0.5 g) were purchased from the Shandong Experimental Animals Centre. The mice were raised in individual cages with unlimited water and food in a special experimental animal apartment with a 12-h light and dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of Shandong First Medical University and conducted following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

The arrangement of the whole experiment is shown in the picture below. Mice were divided into 5 groups. Sham group for the research of SAH surgery control (n = 4); SAH + vehicle group for the research of fake SAH surgery with solvent of target ingredient (normal saline) (n = 4); SAH + IL-2-0.1 μg group for the research of SAH surgery with 0.1 μg intraperitoneal injection per mouse, the same as the SAH + IL-2-0.2 μg group and SAH + IL-2-0.4 μg group. Mice were injected with IL-2 in intraperitoneal 1 h after SAH, and the injection was kept for five days, one time per day. (IL-2, MedChemExpress)

2.2. Mouse SAH model

The SAH surgery was performed in the way of intracranial endovascular perforation as before [13]. Before surgery, the animals were forbidden from food and water for 12 h. Mice were anesthetized with isoflurane (R510-22-10, Reward biotechnology company, Shenzhen, China) in a 30 % O_2/70 % N_2O mixture via a facemask of ether air anesthesia unit (R620-S1, Reward biotechnology company, Shenzhen, China). The animal was fixed on the operation desk in the supine position. The midline neck skin and the underlying muscle were incised for 2CM and the common carotid artery, external carotid artery, and internal carotid were recognized and separated. A blunted 4-0 nylon suture was inserted into the internal carotid by the way of the external carotid artery. Along with the internal carotid, the 4-0 nylon suture went 22-mm long and arrived at the branch of the internal carotid and middle cerebral artery with obvious resistance felt. Further 3 mm-long suture was inserted to puncture the vascular wall. The suture was laid in this way for 10 s and then the suture was withdrawn. At last, the external carotid artery was ligatured and the skin was sutured. In the sham group, the skin and the muscle were incised and the common carotid artery, external carotid artery, and internal carotid were recognized and separated. There was no further surgery with sham group animals. All the procedure was disinfected with iodophor disinfectant. This model surgery could result in the hemorrhage of Willis circle and the blood would go into subarachnoid space, which could imitate the condition of SAH in clinical. The evaluation of surgery was tested by the Bleed grade Score system 24 h later after surgery.

2.3. Single cell suspension and flow cytometry

Animals on the 5th day after SAH were sacrificed for peripheral blood collection from the heart to prepare the single cell suspensions. Briefly, 200 μL blood was obtained from a single animal in an anticoagulant tube coated with heparin and was lysed in the three-time volume of red blood cell lysis buffer (BD) for 10 min. After centrifuging with 300 g for 5 min, the supernatant was discarded and the cellular layer was resuspended with PBS. Cell density was controlled at 1×10^6/mL. Fluorophore-labeled antibodies(including

CD4 and CD25) were used following the manufacturer's instructions (ebioscience) [14]. BD FACS Calibur flow cytometer was applied.

2.4. Immunofluorescence staining

The frozen cortex and hippocampus section slices of mice were collected for immunofluorescence staining after the SAH surgery on the 5th day. In brief, the animals were killed with an overdose anesthetic, following the first perfusion with cold saline solution to exclude the blood in an animal body and the second perfusion with 4 % formaldehyde buffered by saline solution for solidification of tissue all over the body. The solidified whole brains were soaked in 4 % paraformaldehyde for 24 h and then in 20 % sucrose/paraformaldehyde buffer for 72 h. Finally, the brains were stored in 30 % sucrose/PBS buffer for 72 h for use.

Serial brain slices (25 mm thickness) were produced from a freezing microtome and stored in a hyperosmolar sucrose solution in a frozen environment. Cortex and hippocampus section slices were picked out for the immunofluorescence staining. Firstly, the slices were rinsed in PBS and then the 1 % Triton X-100 was used for the permeabilization of the slices for 20 min. The prepared slices were blocked with 5 % donkey serum for 1 h and then the slices were incubated with the primary antibody (CD206, CD3, Iba1, NeuN) at 4 °C overnight, followed by the second antibody incubation for 1h at room temperature. After rinsing with PBS, the slices were attached to the glass slides and sealed with an agent containing DAPI after drying. The primary antibodies are as follows: rabbit anti-NeuN (Millipore, 1:200), rabbit anti-CD206 (Millipore, 1:200), rabbit anti-CD3 (Millipore, 1:200), rabbit anti-Iba1 (Millipore, 1:1000), Secondary antibody (Jackson Immuno Research Laboratories). The immunofluorescence staining images were obtained from a confocal microscope (Nikon). The positive cells in one field under the microscope were counted with NIH ImageJ software.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) analysis

The concentration of IL-1α, IL-6, and TNF-α in basal cortex tissues was quantified using specific ELISA kit for mice, following the manufacturer's instructions (mouse IL-1α, IL-6, TNF-α ELISA kit, Life Technologies, USA). In short, with IL-1α, IL-6, and TNF-α antibody-coated microtiter plate in an ELISA kit, the IL-1α, IL-6, and TNF-α in samples and standards would combine with the antibody pre-coated well. HRP-conjugated antibody standard-prepared was added into each well to bind the immobilized IL-1α, IL-6, and TNF-α on the plate. HRP reacted with the substrate for a while and then, the fluid acid of the substrate was added stopping the reaction. The wells in the plates were measured OD value (k = 450 nm). The standard curve was plotted based on the OD value to the concentration of standards, from which we could get the concentration of each sample which presented as IL-1α, IL-6, or TNF-α, ng/g tissue protein) (see Fig. 1).

3. Results

3.1. SAH model stability and grade scores

To investigate the anti-inflammation of low-dose IL-2, we first divided the mice into different groups with different low doses of IL-2. We divided the mice into 5 groups: Sham-operated group, sham-vehicle group, SAH + IL-2-0.1 μg group, SAH + IL-2-0.2 μg group and SAH + IL-2-0.4 μg group. Mice were injected with IL-2 in intraperitoneal 1 h after SAH. To evaluate the performance of SAH surgery, we recorded the bleeding clot and grading scores among different groups 24 h later after surgery. Grading scores came from the grading system which was a method to test the bleeding of subarachnoid hemorrhage [15]. In Fig. 2A, the harvested brains from different groups showed subarachnoid hemorrhage. The grading scores (Fig. 2B) among different groups showed no statistical significance among different groups ($P > 0.05$). That meant the SAH model we operated was stable and reliable.

3.2. Enrichment of Tregs after low-dose IL-2 injection

After the injection of different low-dose of IL-2 in intraperitoneal at 1 h after SAH surgery, peripheral blood of different groups was prepared for flow cytometry test of Tregs on the 5th day after SAH surgery. The enrichment of Tregs was calculated with the ratio of the

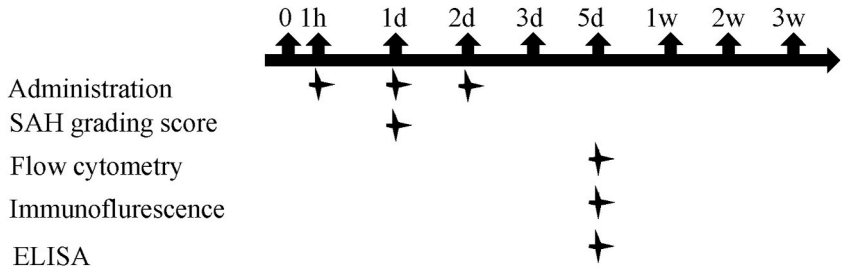

Fig. 1. Experiment arrangement. All mice were conducted with SAH surgery or sham-operated surgery. Different dose of IL-2 was injected intraperitoneally at 1 h, 1 day, and 2 days after surgery. SAH grading score was tested one 1day after surgery and other detections including flow cytometry, immunofluorescence, and ELISA were carried out on the 5th day after surgery.

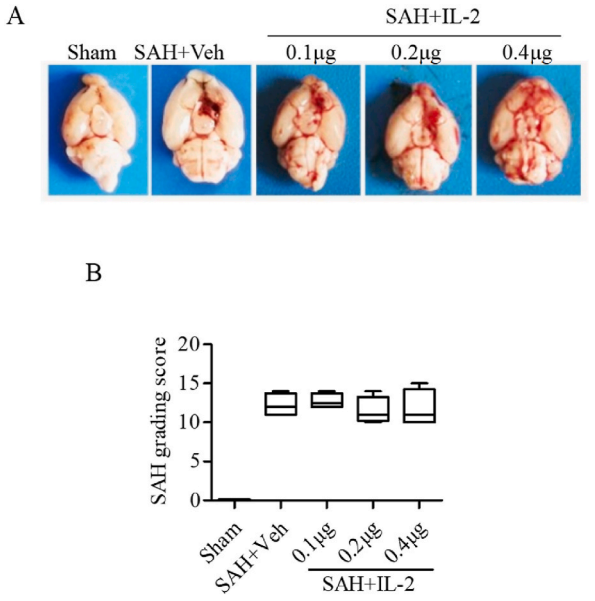

Fig. 2. SAH animal model surgery and the grade scores in sham, vehicle, and different doses of IL-2 group at 24 h after surgery. (A) Representative image of SAH mouse brains. (B) The grading performance after SAH surgery. (n = 4/group).

number of $CD4^+CD25^+$ to all lymphocytes. As shown in Fig. 3A and B, $CD4^+CD25^+$ T cells increased greatly in SAH + IL-2-0.4 μg group compared with other groups ($P < 0.05$). The 0.4 μg of IL-2 was the most effective dose to up-regulate the Tregs population among the three doses of groups. Based on this result, we chose a dose of 0.4 μg IL-2 injection as our low-dose IL-2 treatment dose.

3.3. IL-2 treatment directed the microglia into the M2 state

Immunofluorescence staining was used to test the microglia activation polarization. CD206 was applied to label M2 polarization of microglia and Iba-1 was utilized to mark microglia. The brain slices of the cortex and hippoCA1 regions were harvested on 5day after SAH surgery from the sham-operated group, SAH + PBS group, and SAH-IL-2 group. As in Fig. 4A, the representative images of immunofluorescence staining showed Iba-1+ cells increased in the SAH + IL-2 group and SAH + PBS group, which meant microglia gathered after SAH surgery. Compared with the SAH + PBS group, the activated microglia in the cortex and hippoCA1 regions were

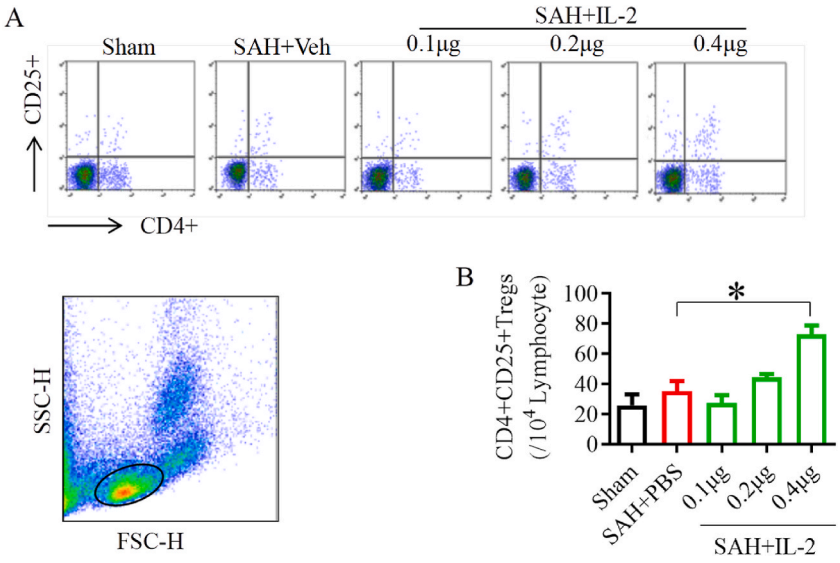

Fig. 3. Low-dose IL-2 treatment expands the Treg population 5 days after SAH. Mice were injected with different doses of IL-2 intraperitoneally at 1 h after SAH surgery. (A) Representative $CD4^+CD25^+$ flow cytometric plots in peripheral blood from different groups. (B) The ratio of $CD4^+CD25^+$ to lymphocyte 5 days after SAH. The dose of IL-2-0.4 μg was the most effective in expanding the Tregs population. (n = 4/group).

polarized more into the M2 state (CD206+) in the SAH + IL-2 group. The histogram in Fig. 4B and C showed the percentage of CD206+ in all the microglia in a field in the cortex and hippoCA1 regions in different groups. After SAH surgery, the activated microglia in the cortex and hippoCA1 areas were polarized into M2 state partially and this was greatly enhanced by IL-2.

3.4. Anti-inflammatory effect of IL-2 5 days after SAH

ELISA kit was used to test the level of IL-1α, IL-6, and TNF-α in basal cortex. Basal cortex tissues were harvested on 5 days with an injection of different low doses of IL-2 in the intraperitoneal at 1 h, 1 day, and 2 days after SAH surgery. The concentration of IL-1α, IL-6, and TNF-α was tested and compared among different groups. We found the level of IL-1α (Fig. 5A), IL-6 (Fig. 5B), and TNF-α (Fig. 5C) after SAH surgery increased compared with the sham-operated group ($p < 0.05$), but was counteracted by IL-2 ($p < 0.05$). This result meant low low-dose IL-2 could inhibit the inflammation after SAH surgery.

Researchers have found that anti-$CD3^+$ monoclonal antibodies could effectively induce tolerance in animal trials [16]. CD3 reacted with T cell receptor, combined which consisting the complex of T receptor. We use CD3 to label the activated T cells. We conducted the immunofluorescence staining. The brain slices from the basal cortex were prepared for immunofluorescence staining of $CD3^+$ for activated T and NeuN + for neurons.

As shown in Fig. 6A, we found a large amount of T cells were activated which infiltrated around Neurons in SAH groups, especially in the SAH + PBS group, which meant inflammation happening in this region.

Compared with the sham-operated group, the ratio of $CD3^+$ NeuN+ and NeuN+ (Fig. 6B) and the mean fluorescence intensity of $CD3^+$ (Fig. 6C) were statistically increased in the SAH + PBS group ($p < 0.001$). While the results in the SAH + IL-2 group decreased ($p < 0.001$). The inflammation after SAH was inhibited by low-dose IL-2.

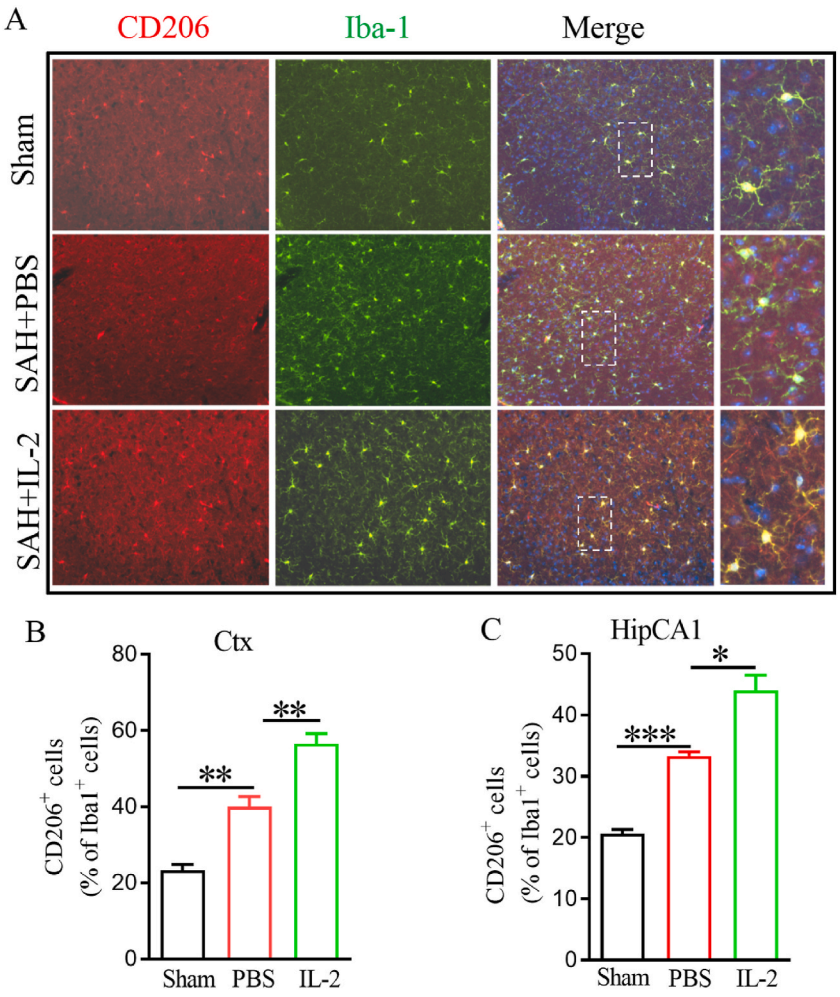

Fig. 4. The impact of IL-2 on microglia in cortex and hippoCA1 regions 5 days after SAH. (A) The representative images of microglia immunofluorescence staining in the cortex. (B) The representative images of microglia immunofluorescence staining in hippoCA1. (C) The percentage of CD206+ in all the microglia in a field among different groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

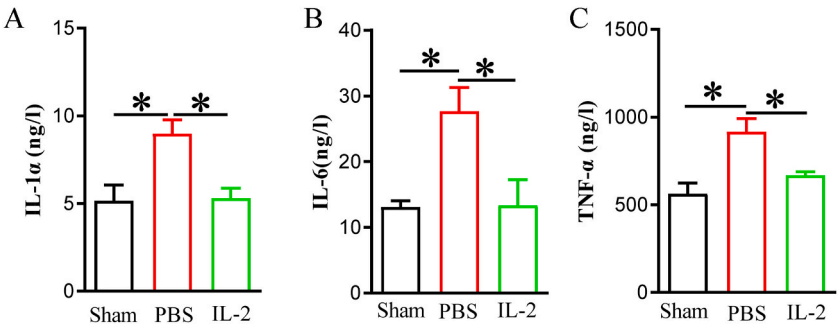

Fig. 5. The expression of inflammatory factors among groups 5 days after SAH in basal cortex tissues. (A) The concentration of IL-1α. (B) The concentration of IL-1α; (C) the concentration of TNF-α. *$p < 0.05$ Low-dose IL-2 treatment attenuated the infiltration of activated T cells in basal cortex tissues 5 days after SAH.

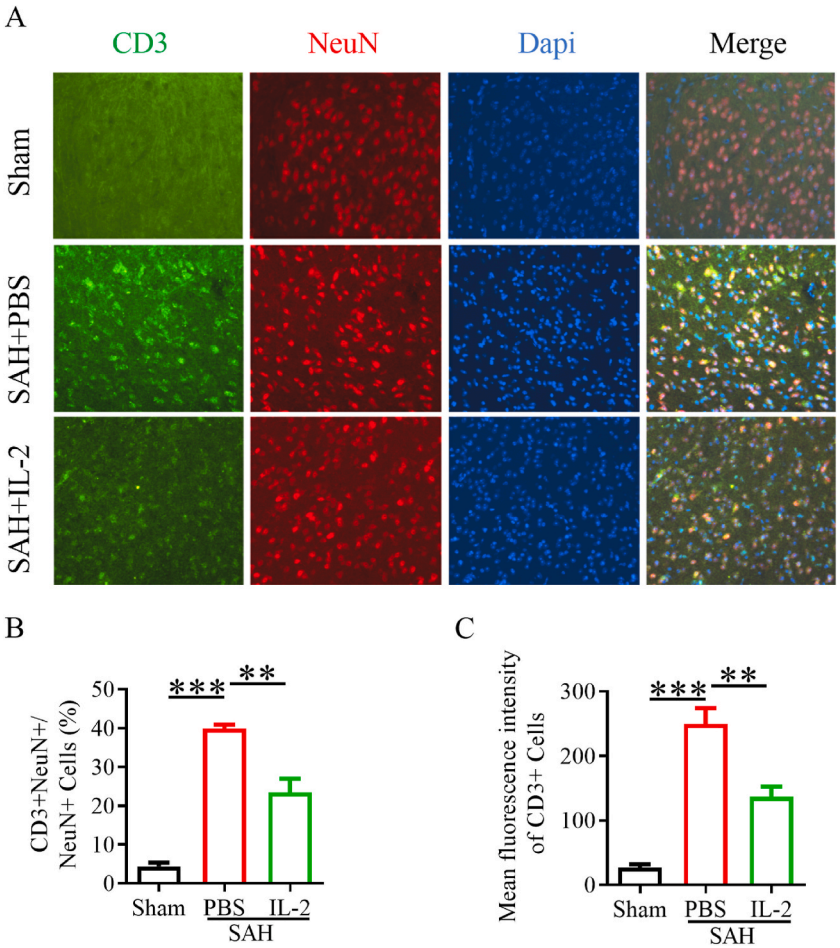

Fig. 6. The infiltration of $CD3^+$ cells in basal cortex 5 days after SAH. (A) the representative images of immuno-fluorescence staining of CD3, NeuN, and DAPI. (B) the percentage of CD3+NeuN+ in NeuN + cells. C. the mean fluorescence intensity of $CD3^+$. *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$.

4. Discussion

We performed stable SAH surgery on mice to research the anti-inflammatory effect of low-dose IL-2 on SAH. We found the number of Treg cells ($CD4^+CD25^+$) increased in the IL-2 0.4 μg group than in IL-2-0.1 μg or IL-2-0.2 μg, based on which we chose IL-2 0.4 μg to carry out our following researches. We found microglia tended to M2 phenotypic polarization after 3 times intraperitoneal injections of IL-2-0.4 μg 5 days after SAH surgery. What's more, low-dose IL-2 could also exert anti-inflammation by decreasing the IL-1α, IL-6, and

TNF-α and attenuating infiltration of $CD3^+$ cells.

Regulatory T (Treg) cells were the typical immunosuppressive cells in the body [17]. In obvious research on cancers, one of the suppressive immune mechanisms of Treg cells was the consumption of IL-2. A high dose of IL-2 could neutralize the suppressive function of Treg [18] by inhibiting the effector T cell proliferation and activation. IL-2 was often regarded as a pro-inflammatory cytokine in some research [19]. In our research, we applied the low-dose IL-2(0.4ug) treatment for the gathering of Treg cells to suppress the inflammation after SAH in mice. Tregs were initially defined as $CD4^+$ T cells with high expression of CD25 (IL-2 receptor α chain). Based on the previous research and our research, we concluded that high-dose and low-dose administration of IL-2 could have different effects on effector T cells, resulting in different immunity effects.

In 1976, in the cell culture supernatant, Morgan found a substance later named TCGF (T cell growth factor) which could stimulate the growth of thymocytes and accelerate and preserve the long-term growth of T cells. In 1979, TCGF was renamed Interleukin-2(IL-2). IL-2 was secreted by $CD4^+$ T cells, $CD8^+$ T cells, some T-tumor cell lines, and gene-edit cells. The effect of proliferation of IL-2 was weak on T cells in the rest state, while the pro-growth effect was enhanced greatly after the target cells were stimulated and activated by mitogen [20]. Microglia were the rapid response cells in the brain after cerebral injury. The activated microglia were polarized into two forms, M1 for pro-inflammatory and M2 for anti-inflammatory. The M1 microglia could deteriorate the injury by secreting pro-inflammatory factors, like IL-1α, IL-6, and TNF-α. On the contrary, M2 microglia exerted neuroprotection by releasing anti-inflammatory factors [21,22]. Research has proved that activation of the RARα receptor could attenuate the inflammation by promoting the M1-to-M2 phenotypic polarization of microglia after SAH [4]. In our research, we found more M2 microglia (CD206+) appeared and that IL-1α, IL-6, and TNF-α decreased after administration of low-dose IL-2, which meant the neuroprotective function. While we didn't investigate the underlying mechanism. Investigations of $CD3^+$ over the past 20 years have shown that anti-CD3 antibodies could induce tolerance. In our research, low-dose IL-2 could effectively decrease the $CD3^+$ around neurons in the basal cortex after SAH, which showed the anti-inflammatory effects of low-dose IL-2. Other scientists designed a combination treatment with anti-CD3 antibody (CD3-Ab) and IL-2/anti-IL-2 monoclonal antibody complex (IL-2 complex) to inhibit atherosclerosis [23], which was consistent with our results.

Despite the anti-inflammation of low-dose IL-2, there was no underlying mechanism investigated in our study, which would be our focus of issue. What's more, we applied 0.4-μg as our target dose. More different doses of IL-2 were needed to be detected to find the optimal dose.

In our results, we found that low-dose IL-2 could effectively inhibit inflammation after SAH. We concluded that low-dose IL-2 had the potential to be a treatment for SAH in clinical.

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Data availability statement

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

Ethics approval

The study was approved by the ethics committee of Zhongshan Hospital (No. ZSH102349).

CRediT authorship contribution statement

Jia Liu: Writing – original draft. **Biao Qi:** Writing – original draft. **Yanrong Ye:** Writing – original draft. **Yun Shen:** Funding acquisition, Formal analysis. **Yufu Lin:** Resources, Project administration. **Yabo Chen:** Resources, Project administration, Methodology. **Shan Ding:** Visualization, Validation, Supervision. **Jun Ma:** Data curation, Conceptualization. **Shaozhuang Chen:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shaozhuang Chen reports financial support was provided by Fujian Natural Science Foundation(No. 2023J011697). Shaozhuang Chen reports financial support was provided by Xiamen Natural Science Foundation(No. 3502Z202373086). Shaozhuang Chen reports was provided by Xiamen Medical and Health Science and Technology Project (No. 3502Z20194027). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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