

# Simvastatin boosts neutrophil apoptosis and ameliorates secondary brain injury following intracerebral hemorrhage via LXA4/FPR2/p38 MAPK pathway

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## Research article

**Keywords:** Intracerebral hemorrhage, Statins, Neutrophils, Apoptosis, Lipoxin A4, Formyl-peptide receptor 2

**Posted Date:** November 23rd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1095461/v1>

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# Abstract

Statins, in addition to their well-known lipid-lowering effects, have also shown a wide range of neuroprotective effects in recent years. We previously found that simvastatin effectively attenuated intracerebral hemorrhage (ICH)-induced secondary brain injury in rats. This study aims to elucidate the underlying mechanism. The animal model was established in adult male Sprague–Dawley rats by an injection of autologous blood, then randomly treated with simvastatin or vehicle. Then, a series of experiments were conducted to investigate the involvement of lipoxin A4 (LXA4) / formyl-peptide receptor 2 (FPR2) / p38 MAPK signaling pathway in simvastatin-triggered neutrophil apoptosis. Results show that simvastatin significantly elevated the level of LXA4 (an endogenous FPR2 agonist) in plasma in early stage of ICH. Exogenous LXA4 administration effectively promoted circulating neutrophil apoptosis, reduced the neutrophil count in both peripheral blood and perihematoma area, as well as ameliorated neuroinflammation and brain injury after ICH, which is in line with the effect of simvastatin. Moreover, similar to simvastatin, the exogenous LXA4 markedly down-regulated the phosphorylation level of p38 and the Mcl-1/Bax ratio (the decreased ratio represents pro-apoptosis) in circulating neutrophils of ICH rat. Notably, all above effects of simvastatin on ICH were significantly abolished by Boc-2, a selective antagonist for FPR2. Moreover, simvastatin led to a similar reduction of Mcl-1/Bax ratio as SB203580 (p38 MAPK inhibitor), but it was abolished by P79350 (p38 MAPK agonist). Collectively, these results suggest that simvastatin boosts neutrophil apoptosis and alleviates subsequent neuroinflammation following ICH may via upregulating LXA4 in plasma through the FPR2/p38 MAPK signaling pathway.

## Introduction

Intracerebral hemorrhage (ICH) is a life-threatening illness of global importance, with a poor prognosis and few proven treatments. One in three patients die within the first month of onset, and survivors have varying degrees of residual disability and high risk of recurrent ICH.[1, 2] More and more studies have provided evidence for supporting the key role of neuroinflammation in secondary brain injury following ICH.[3] However, most of the current researches focus on the richest microglia in the central nervous system, while ignore the importance of peripheral inflammatory cells.

Recently, clinical reports indicate that early peripheral blood polymorph nuclear neutrophils (PMNs) elevation is closely related to the poor prognosis of patients with ICH, which is often accompanied by heavier brain edema and larger hematoma.[4–7] To understand the relationship between the elevated PMNs and poor prognosis among ICH patients, we performed a confirmatory experiment in a rat model of ICH.[8] The results show that the ICH rat showed a significant increase of PMNs in peripheral blood at an early stage, which is consistent with the clinical phenomenon. Interestingly, the increased PMNs count returned to normal level when pretreated with simvastatin. In addition, brain neutrophil-invading, neuroinflammation, brain edema and neurological dysfunction in ICH rats were markedly ameliorated after treated with simvastatin. Subsequent research reveals that, through breaking the balance between pro-apoptotic proteins and anti-apoptotic proteins in PMNs, simvastatin promoted the apoptosis of PMNs

and lowered the circulating PMNs count following ICH.[8] However, to date, the related signal pathways and molecular mechanisms of simvastatin in apoptotic regulation is not clear.

Lipoxin A4 (LXA4) is an important endogenous lipid that be synthesized by 5-lipoxygenase and exerts potent anti-inflammatory effect via the inhibition of PMNs infiltration and pro-inflammatory cytokines release.[9] In recent years, studies have reported that LXA4 exerts a notably anti-inflammatory neuroprotective effect by specifically activating its receptor formyl peptide receptor 2 (FPR2) in animal models of ischemia stroke and subarachnoid hemorrhage.[10, 11] LXA4 exerted these biological functions through down-regulating the activities of p38 mitogen-activated protein kinase (MAPK), which was mediated by FPR2.[11, 12] Recently, Gonzalez et al.[13] found that simvastatin can assist cyclooxygenase-2 (COX-2) to induce the conversion of arachidonic acid to LXA4 and exert anti-inflammatory effects. Furthermore, El Kebir et al.[14] found that LXA4 can effectively promote PMNs apoptosis and reduce subsequent PMNs infiltration around the respiratory tract, through accelerating the degradation of myeloid cell leukemia 1 (mcl-1), a key anti-apoptotic protein.

The above researches suggest that simvastatin may activate FPR2 by up-regulating the endogenous ligand LXA4, thus exerting pro-apoptosis and anti-inflammatory effects on rat of ICH. Therefore, we speculate that simvastatin promotes PMNs apoptosis may in part by regulating the LXA4/FPR2/p38 MAPK signaling pathway. Therefore, in this study, we investigated the involvement of LXA4/FPR2/p38 MAPK pathway with regard to the regulatory effects of simvastatin on peripheral PMN apoptosis and neuroinflammation in a rat model of ICH.

## **Materials And Methods**

### **Animals and ICH model**

The Institutional Animal Care and Use Committee at the Third Military Medical University approved this study (SCXK-PLA-20120011) and the procedures followed were in accordance with institutional guidelines. Two hundred and eighty-five adult male Sprague-Dawley rats (250–350 g) were used. ICH model establishment was refer to our previous studies.[15, 16] Briefly, nonanticoagulant whole blood was taken from the femoral artery of the rats. Then, the autologous blood was microinjected into the right caudate nucleus in 10 min. The coordinates is 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma. The sham groups received only needle injection.

### **Experimental protocol**

In the present study, the following three separate experiments were conducted:

#### **Experiment 1**

To determine simvastatin-induced changes of LXA4 levels in plasma after ICH, 24 rats were randomly divided into three groups: sham, ICH + Veh (ICH + saline), ICH + Simva (ICH + simvastatin). The levels of

LXA4 in plasma were assessed by Enzyme-Linked Immunosorbent Assays (ELISA) at 24hr and 72hr following blood injection, respectively (n = 4 per group, at each time point).

## **Experiment 2**

To validate the involvement of the LXA4/FPR2 pathway in simvastatin-mediated peripheral PMNs apoptosis promotion and subsequent neuroinflammation alleviation after ICH, we randomized 141 rats into the following five groups: Sham, ICH + Veh, ICH + LXA4 (an endogenous FPR2 agonist), ICH + Simva, ICH + Simva + Boc-2 (a selective FPR2 antagonist). Because our previous study has shown some corresponding data about the sham group, in present experiment, the sham group just undergo the measurements of PMNs count and proinflammatory factors protein level. Flow cytometric analysis of peripheral PMNs apoptosis (n = 6 per group), routine blood counts and immunofluorescence of MPO (n = 6 per group), Western blotting analysis of proinflammatory factors (n = 3 per group) were carried out 24hr after ICH induction. Brain water content measurement (n = 6 per group), Modified Neurological Severity Scores (mNSS, n = 6 per group) and corner test (n = 6 per group) were assessed at 1, 3 and/or 7 days after ICH.

## **Experiment 3**

p38 MAPK, the potential downstream signaling pathway of FPR2-mediated PMNs apoptosis, was evaluated in PMNs isolated from circulating blood of ICH rats. First, 12 rats were randomly divided into four groups: ICH + Veh, ICH + LXA4 (an endogenous FPR2 agonist), ICH + Simva, ICH + Simva + Boc-2 (a FPR2 selective antagonist). Western blotting analysis for p38, pp38, Mcl-1, Bax and Mcl-1/Bax ratio (n = 3 per group) were conducted at 24hr after ICH. Second, another 12 rats were randomly divided as the following four groups: ICH + Veh, ICH + SB203580 (p38 MAPK pathway inhibitor), ICH + Simva, ICH + Simva + P79350 (p38 MAPK pathway agonist). Western blotting analysis for Mcl-1, Bax and Mcl-1/Bax ratio (n = 3 per group) were conducted at 24hr after ICH.

## **Drug Administration**

Firstly, the simvastatin was prepared as a 4mg/ml solution according to our previous study. [8] This simvastatin stock was stored at  $-80^{\circ}\text{C}$  and diluted with triple volume of sterile saline immediately before use. Animals received simvastatin injection (2 mg/kg/d, i.p) from five days before ICH until sacrificed.

## **Routine blood counts**

Routine blood counts were conducted as previously described.[8] First, 4 ml circulation blood were sampled from the heart of rat with the EDTA-anticoagulated tube. Then, give it a good shake. Next, 200  $\mu\text{l}$  of sample was moved to an eppendorf tube and tested on the bench-top analyzer (Hemavet 950, Shandong excellent science instrument co. LTD., CHN).

## **PMNs isolation**

As our previous described[8], the collected anticoagulated whole blood from rats was deal follow the instructions for density gradient centrifugation. Then, the PMNs cells were collected from the PMN-rich layer between Histopaque1083 and Histopaque1119. Next, the cells viability was examined with Trypan blue dye. The purity of isolated PMNs were tested with Wright-Giemsa staining. More than 95% of viability and purity were detected among these isolated PMNs.

### **PMNs apoptotic ratio detection**

The apoptotic ratio of PMNs were detected refer to the previous method.[8] The D-Hanks-washed cells were incubated on ice with 10  $\mu$ l propidium iodide (PI) solution and 5  $\mu$ l Annexin V-fluorescein isothiocyanate (FITC) solution for 15 min in the dark. Then, the apoptosis of PMNs were analyzed by the flow cytometry (BD LSRF Ortessa, USA).

### **Brain water content measurement**

Brain water content was examined in rats 24 h and 72 h after surgery, as previously described. [8] Brains were removed under deep anesthesia, and a coronal tissue was sliced (4 mm thickness) around the injection needle tract. Brain sections were divided into four parts: ipsilateral basal ganglia, ipsilateral cortex, contralateral basal ganglia, and contralateral cortex. The cerebellum was the internal control. Brain sample weights were determined immediately after removal and then drying for 24 h in a 100 °C oven. The brain water content (%) was calculated as (wet weight-dry weight)/wet weight  $\times$  100 %.

### **Neurological function assessment**

Neurological assessment was conducted with the modified Neurological Severity Score (mNSS) method and the corner test as previous.[17, 18] Briefly, the mNSS scale ranges from 0 to 18 (normal score, 0; maximal deficit score, 18). In corner test, each rat was leaded to a corner with 30° angle. Alternatively, the rat was allowed to turn left or right. After 10 times of repeated tests, the ratio of right turns was calculated by two blinded observers.

### **Cell counts**

Cell counting was performed on brain sections. Three high power images were taken around the hematoma using a confocal microscope (LSM-780; Zeiss). MPO-positive cells were counted by two researchers in a blinded manner on 1, 3, and 7 days after ICH. The counts were performed on four consecutive brain sections.

### **Western blot analysis**

Cell lysates were run on SDS/PAGE gels, and proteins were detected on nitrocellulose blots with enhanced chemiluminescence reagents (GE Healthcare). The following antibodies were used: TNF- $\alpha$ , diluted 1:500 (Beyotime, China); IL-6, diluted 1:500 (Beyotime, China); Mcl-1, p38 MAPK and phospho-p38 MAPK (1:1000, Cell Signaling, USA); Bax (1:500, Abcam, USA); complement C3 (1:500, Novus Biologicals,

USA); $\alpha$ -Tubulin (1:1000, Boster Biological Technology, China); GAPDH (1:1000, bs-2188R; BIOSS, China). Membranes were blocked in 5% nonfat milk that was dissolved in Tris-buffered saline solution for 1 h, followed by overnight primary antibody incubation[19].

## **Immunofluorescence**

Brains were removed after fixative perfusion and cryoprotected in 30% sucrose in phosphate buffered saline (PBS). Serial sections were cut on a freezing microtome, blocked, and incubated in the primary antibodies: MPO (1:100, Abcam, USA). Then, the sections were incubated with secondary antibody: Alexa Fluor 555-conjugated goat anti-rabbit IgG (H+L) (1:300, Beyotime, China) at 37°C for 3 hours. After washing, the sections were incubated with the appropriate fluorescent secondary antibody Alexa Fluor 555-conjugated goat anti-rabbit IgG (H+L) (1:300, Beyotime, China) and counterstained with DAPI.

## **Statistical analyses**

In present study, the values are given as the mean $\pm$ SD. Data were analyzed by one-way analysis of variance, followed by Scheffé's post hoc test. Differences were considered statistically significant at a P-value < 0.05.

# **Results**

## **Simvastatin significantly elevated the plasm level of LXA4 (an endogenous FPR2 agonist) at 24h after ICH**

To examine the potential correlation between simvastatin and FPR2 after ICH, we selected LXA4, an endogenous FPR2 agonist, as the detected object. ELISA was performed to determine the expression profile of LXA4 at 24h and 72h after ICH. As shown in Fig. 1, compared with both the sham and control groups, the plasm of ICH rats treated with simvastatin presented higher level of LXA4 at 24h after blood injection, but showed no significant difference at 72h. The result suggests that simvastatin has potential regulatory capacity on FPR2 through impacting LXA4 level change.

## **Boc-2, a selective FPR2 antagonist, weakened simvastatin-mediated peripheral PMNs apoptosis**

To determine the involvement of FPR2 in simvastatin-triggered PMNs apoptosis, we conducted the flow cytometry analysis with the using of exogenous LXA4 (as FPR2 agonist) and Boc-2 (as FPR2 antagonist) at 24h after ICH. In comparison with the control group, both the LXA4 and Simva groups displayed a higher apoptotic ratio of PMNs (Fig. 2A and B). The LXA4 group was more prominent. Notably, Boc-2, a selective FPR2 antagonist, markedly weakened simvastatin-inudced PMNs apoptosis (Fig. 2A and B).

## **Boc-2 crippled the inhibitory effects of simvastatin on PMNs count and PMNs brain-invasion**

Next, we investigated the role of FPR2 in the moderating effects of simvastatin on circulating PMNs count and PMNs infiltrating into brain after ICH. The routine blood test showed that ICH rats treated with

FPR2 agonist, LXA4, performed a lower PMNs count than the control groups at 24h after ICH (Fig. 3A and B). Interestingly, the simvastatin-treated animals showed a similar trend as the LXA4 group, but the inhibitory effects of simvastatin on PMNs count was balanced out by FPR2 antagonist, Boc-2 (Fig. 3A and B). As displayed in Fig. 3C and D, compared with the nontreated ICH group, exogenous LXA4 (as FPR2 agonist) administration significantly decreased the number of MPO (+) cells in the area around the hematoma at 24h post-ICH. Notably, the Simva-group presented a similar trend as the LXA4 group, but the inhibitory effects of simvastatin on PMNs invading into brain was reversed by Boc-2 (FPR2 antagonist).

### **The anti-inflammatory effect of simvastatin on ICH was slacked off by Boc-2**

Additionally, to determine the level change of some proinflammatory factors around the hematoma, we detected the protein expression of TNF- $\alpha$ , IL-6 and complement component C3 in acute stage of ICH. Western blotting analysis showed a lower expression level of all above chemokines in the LXA4-group than in the control at 24h after ICH (Fig. 4A-C), indicating FPR2 stimulation relieved the early neuroinflammatory response to ICH in some extent. Of particular note is that Simva-group performed a similar trend as the LXA4-group, but the anti-inflammatory effect of simvastatin on ICH was neutralized by Boc-2, a selective FPR2 antagonist.

### **The protective effect of simvastatin on brain edema and neurological deficits was abolished by Boc-2**

To further investigate the involvement of FPR2 in simvastatin-mediated brain protective effects on ICH, we measured the brain water content of animal models and calculated their neurological scorings. The brain water contents of ipsilateral basal ganglia and cortex in LXA4-group showed an obvious reduction when compared with the Veh-group at 24h and 72h after ICH (Fig. 5A, B). It is noticeable, animal models treated by simvastatin showed a similar trend as the LXA4-group, but the antiedema effect of simvastatin on ICH was weakened by Boc-2, a selective FPR2 antagonist. (Fig. 5A, B). Furthermore, as shown in Fig. 5C and D, ICH rats received the treatment of exogenous LXA4 (as FPR2 agonist) presented a lower neurological score in the mNSS test while a higher score during the corner test at day 7 after ICH.

### **Simvastatin exerted pro-apoptotic effect on PMNs in acute stage after ICH via LXA4/FPR2/p38 MAPK signaling pathway**

To elucidate the downstream signaling of FPR2-mediated PMNs apoptosis, we treated the animals with simvastatin, FPR2 agonist LXA4 and FPR2 antagonist Boc-2, p38 MAPK inhibitor SB203580 and p38 MAPK agonist P79350 analyzed the downstream proteins. 24 hours after ICH, PMNs were isolated from the circulating blood of rat, which then be detected by using western blotting analysis. As shown in Fig. 6A and B, FPR2 agonist LXA4 markedly downregulated the phosphorylation level of p38 than the control group. In addition, the expression of antiapoptotic proteins Mcl-1 was markedly inhibited by LXA4 (Fig. 6C), while the proapoptotic proteins Bax was significantly upregulated (Fig. 6D). Thus, rats treated with LXA4 displayed a lower Mcl-1 to Bax ratio (Mcl-1/Bax, plays a key role in affecting neutrophil survival), resulting in more PMNs apoptosis than the Veh-group. Of note, Simva-group performed a

similar trend of p38/Mcl-1/Bax level as the LXA4-group. However, co-treatment with Boc2 abrogated the pro-apoptotic effect of simvastatin on PMNs following ICH. Next, we further assessed the relation between p38 MAPK signal pathway and PMNs apoptosis. As displayed in Fig. 6F-I, SB203580 (p38 MAPK inhibitor) dramatically down-regulated Mcl-1 expression in PMNs while elevated Bax level after ICH, which leads to a lower Mcl-1/Bax ratio. Notably, ICH rats treated with simvastatin presented a similar effect on regulating Mcl-1/Bax balance as SB203580, but it was abolished by p38 MAPK agonist P79350.

## Discussion

In this study, we investigated the potential mechanism of simvastatin-induced PMNs apoptosis in rat model of ICH. The major findings of this study are as follows: (1) Simvastatin significantly elevated the level of LXA4 (an endogenous FPR2 agonist) in plasma after ICH; (2) Similar to simvastatin, exogenous LXA4 effectively promoted circulating PMNs apoptosis in early stage of ICH, and restricted the secondary brain injury triggered by brain-infiltrated PMNs; (3) Boc-2, a selective antagonist for FPR2, dramatically abolished the neuroprotective effects of simvastatin on ICH; (4) Further results suggest that the FPR2/p38 MAPK signaling pathway plays an important role in simvastatin-triggered PMNs apoptosis.

Statin is associated with improved outcome in patients with ischemic stroke.[20] In addition to the most familiar effect of cholesterol reduction, statins also display pleiotropic effects, like anti-inflammation, promoting neurogenesis and protecting blood-brain barrier.[21, 22] Thus, statins are attractive candidates for the development of a neuroprotective strategy. Over years, a series of retrospective cohort studies have reported that inpatient statin use is associated with improved outcomes following acute ICH.[23–27] Recently, accumulating evidence supports that higher PMNs count and neutrophil to lymphocyte ratio (NLR) at early stage of ICH leads to the poorer outcome.[4–7] Thus, in a proof-of-concept experiment in rat model of ICH, we found that simvastatin effectively decreased the peripheral PMNs count, NLR and neutrophil brain-invading, as well as attenuated brain edema and neurological deficits following ICH.[8] However, to date, the related molecular mechanisms of simvastatin involved in apoptotic regulation of PMNs is still unclear.

Lipoxins (LXs), lipids derived from arachidonic acid, exert anti-inflammatory effects by catalyzing arachidonic acid through different lipoxygenases through a cross-cellular pathway.[28] Several recent studies have reported that LXA4 plays a protective role in CNS injury like subarachnoid hemorrhage (SAH) and ischemic stroke.[10, 11] It has been reported in previous literature that LXA4 reduces PMNs infiltration in SAH rats by activating FPR2[11], which is consistent with our research results. However, the author only focused on the effect of LXA4 on microglial FPR2, and did not explore the effect and mechanism of LXA4 on PMNs surface FPR2. Recently, Gonzalez et al.[13] found that simvastatin can assist cyclooxygenase-2 (COX-2) to induce the conversion of arachidonic acid to LXA4 and exert anti-inflammatory effects. Consistently, in the present study, we found that Simvastatin significantly elevated the plasma level of LXA4 (an endogenous FPR2 agonist) at 24h after ICH. Similar to simvastatin, exogenous LXA4 effectively promoted circulating PMNs apoptosis in early stage of ICH, and restricted the secondary brain injury triggered by brain-infiltrated PMNs.

In recent years, studies have reported that LXA4 exerted the notably anti-inflammatory neuroprotective effect through down-regulating the activities of p38 mitogen-activated protein kinase (MAPK), which was mediated by FPR2.[11, 12, 29] In the present study, we found that Boc-2, a selective antagonist for FPR2, dramatically abolished the neuroprotective effects of simvastatin on ICH. In addition, FPR2 agonist LXA4 markedly downregulated the phosphorylation level of p38. Moreover, simvastatin led to a similar reduction of Mcl-1/Bax ratio as SB203580 (p38 MAPK inhibitor), but it was abolished by P79350 (p38 MAPK agonist). All these data suggest that the FPR2/p38 MAPK signaling pathway plays an important role in simvastatin-triggered neutrophil apoptosis.

## Conclusion

Collectively, we demonstrated that simvastatin-mediated PMNs apoptosis mainly through the FPR2/p38 MAPK signaling pathway via upregulating the plasma LXA4 level following ICH. To our knowledge, this study is the first to demonstrate the neuroprotective effect of LXA4 on ICH, which is characterized by cerebral edema relief, improved neurobehavioral function, reduced neutrophils aggregation and pro-inflammatory factors expression around the hematoma. Therefore, simvastatin or LXA4 may be a promising and safe therapeutic option in ICH. Large animal experiments and clinical trials are required to further explore the efficacy and safety in future study.

## Declarations

### Conflict of interest

The authors indicate no potential conflicts of interest.

### Ethics statement

All institutional and national guidelines for the care and use of laboratory animals were followed.

### Authors contribution

JZ, NH, XS and QC contributed to the implementation of the experiment. QC, HF and YW contributed to the design and paper writing.

### Funding

This work was supported by Grant Nos. 81701147(QW.C) from the National Natural Science Foundation of China.

## References

1. Cordonnier C, Demchuk A, Ziai W, Anderson CS: **Intracerebral haemorrhage: current approaches to acute management.** *Lancet* 2018, **392**(10154):1257–1268.

2. Xi G, Strahle J, Hua Y, Keep RF: **Progress in translational research on intracerebral hemorrhage: is there an end in sight?** *Prog Neurobiol* 2014, **115**:45–63.
3. Zhou Y, Wang Y, Wang J, Anne Stetler R, Yang QW: **Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation.** *Prog Neurobiol* 2014, **115**:25–44.
4. Lattanzi S, Cagnetti C, Provinciali L, Silvestrini M: **Neutrophil-to-Lymphocyte Ratio Predicts the Outcome of Acute Intracerebral Hemorrhage.** *Stroke* 2016, **47**(6):1654–1657.
5. Gusdon AM, Gialdini G, Kone G, Baradaran H, Merkler AE, Mangat HS, Navi BB, Iadecola C, Gupta A, Kamel H *et al*: **Neutrophil-Lymphocyte Ratio and Perihematoma Edema Growth in Intracerebral Hemorrhage.** *Stroke* 2017, **48**(9):2589–2592.
6. Morotti A, Phuah CL, Anderson CD, Jessel MJ, Schwab K, Ayres AM, Pezzini A, Padovani A, Gurol ME, Viswanathan A *et al*: **Leukocyte Count and Intracerebral Hemorrhage Expansion.** *Stroke* 2016, **47**(6):1473–1478.
7. Wang Z, Gong Q, Guo C, Luo Y, Chen L: **Neutrophil-to-lymphocyte ratio predicts hematoma growth in intracerebral hemorrhage.** *J Int Med Res* 2019, **47**(7):2970–2975.
8. Zhang J, Shi X, Hao N, Chen Z, Wei L, Tan L, Chen Y, Feng H, Chen Q, Zhu G: **Simvastatin Reduces Neutrophils Infiltration Into Brain Parenchyma After Intracerebral Hemorrhage via Regulating Peripheral Neutrophils Apoptosis.** *Front Neurosci* 2018, **12**:977.
9. Spite M, Serhan CN: **Novel lipid mediators promote resolution of acute inflammation: impact of aspirin and statins.** *Circ Res* 2010, **107**(10):1170–1184.
10. Ye XH, Wu Y, Guo PP, Wang J, Yuan SY, Shang Y, Yao SL: **Lipoxin A4 analogue protects brain and reduces inflammation in a rat model of focal cerebral ischemia reperfusion.** *Brain Res* 2010, **1323**:174–183.
11. Guo Z, Hu Q, Xu L, Guo ZN, Ou Y, He Y, Yin C, Sun X, Tang J, Zhang JH: **Lipoxin A4 Reduces Inflammation Through Formyl Peptide Receptor 2/p38 MAPK Signaling Pathway in Subarachnoid Hemorrhage Rats.** *Stroke* 2016, **47**(2):490–497.
12. Wu R, Zhou W, Chen S, Shi Y, Su L, Zhu M, Chen Q, Chen Q: **Lipoxin A4 suppresses the development of endometriosis in an ALX receptor-dependent manner via the p38 MAPK pathway.** *Br J Pharmacol* 2014, **171**(21):4927–4940.
13. Gonzalez-Herrera F, Cramer A, Pimentel P, Castillo C, Liempi A, Kemmerling U, Machado FS, Maya JD: **Simvastatin Attenuates Endothelial Activation through 15-Epi-Lipoxin A4 Production in Murine Chronic Chagas Cardiomyopathy.** *Antimicrob Agents Chemother* 2017, **61**(3).
14. El Kebir D, Jozsef L, Pan W, Wang L, Petasis NA, Serhan CN, Filep JG: **15-epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury.** *Am J Respir Crit Care Med* 2009, **180**(4):311–319.
15. Tan Q, Chen Q, Niu Y, Feng Z, Li L, Tao Y, Tang J, Yang L, Guo J, Feng H *et al*: **Urokinase, a promising candidate for fibrinolytic therapy for intracerebral hemorrhage.** *J Neurosurg* 2017, **126**(2):548–557.
16. Chen Q, Shi X, Tan Q, Feng Z, Wang Y, Yuan Q, Tao Y, Zhang J, Tan L, Zhu G *et al*: **Simvastatin Promotes Hematoma Absorption and Reduces Hydrocephalus Following Intraventricular**

- Hemorrhage in Part by Upregulating CD36.** *Translational stroke research* 2017, **8**(4):362–373.
17. Liew HK, Pang CY, Hsu CW, Wang MJ, Li TY, Peng HF, Kuo JS, Wang JY: **Systemic administration of urocortin after intracerebral hemorrhage reduces neurological deficits and neuroinflammation in rats.** *J Neuroinflammation* 2012, **9**:13.
  18. Hua Y, Schallert T, Keep RF, Wu J, Hoff JT, Xi G: **Behavioral tests after intracerebral hemorrhage in the rat.** *Stroke* 2002, **33**(10):2478–2484.
  19. Chen Q, Tang J, Tan L, Guo J, Tao Y, Li L, Chen Y, Liu X, Zhang JH, Chen Z *et al*: **Intracerebral Hematoma Contributes to Hydrocephalus After Intraventricular Hemorrhage via Aggravating Iron Accumulation.** *Stroke* 2015, **46**(10):2902–2908.
  20. Amarenco P, Bogousslavsky J, Callahan A, 3rd, Goldstein LB, Hennerici M, Rudolph AE, Silllesen H, Simunovic L, Szarek M, Welch KM *et al*: **High-dose atorvastatin after stroke or transient ischemic attack.** *N Engl J Med* 2006, **355**(6):549–559.
  21. Chen Q, Zhang J, Feng H, Chen Z: **An update on statins: Pleiotropic effect performed in intracerebral hemorrhage.** *Atherosclerosis* 2019, **284**:264–265.
  22. van der Most PJ, Dolga AM, Nijholt IM, Luiten PG, Eisel UL: **Statins: mechanisms of neuroprotection.** *Prog Neurobiol* 2009, **88**(1):64–75.
  23. Flint AC, Conell C, Rao VA, Klingman JG, Sidney S, Johnston SC, Hemphill JC, Kamel H, Davis SM, Donnan GA: **Effect of statin use during hospitalization for intracerebral hemorrhage on mortality and discharge disposition.** *JAMA Neurol* 2014, **71**(11):1364–1371.
  24. Witsch J, Al-Mufti F, Connolly ES, Agarwal S, Melmed K, Roh DJ, Claassen J, Park S: **Statins and perihemorrhagic edema in patients with spontaneous intracerebral hemorrhage.** *Neurology* 2019, **92**(18):e2145-e2149.
  25. Chen PS, Cheng CL, Chang YC, Kao Yang YH, Yeh PS, Li YH: **Early statin therapy in patients with acute intracerebral hemorrhage without prior statin use.** *Eur J Neurol* 2015, **22**(5):773–780.
  26. Pan YS, Jing J, Wang YL, Zhao XQ, Song B, Wang WJ, Wang D, Liu GF, Liu LP, Wang CX *et al*: **Use of statin during hospitalization improves the outcome after intracerebral hemorrhage.** *CNS Neurosci Ther* 2014, **20**(6):548–555.
  27. Chung CM, Lin MS, Liu CH, Lee TH, Chang ST, Yang TY, Pan KL, Lin YS: **Discontinuing or continuing statin following intracerebral hemorrhage from the view of a national cohort study.** *Atherosclerosis* 2018, **278**:15–22.
  28. Fu T, Mohan M, Brennan EP, Woodman OL, Godson C, Kantharidis P, Ritchie RH, Qin CX: **Therapeutic Potential of Lipoxin A4 in Chronic Inflammation: Focus on Cardiometabolic Disease.** *ACS Pharmacol Transl Sci* 2020, **3**(1):43–55.
  29. Lv W, Lv C, Yu S, Yang Y, Kong H, Xie J, Sun H, Andersson R, Xu D, Chen B *et al*: **Lipoxin A4 attenuation of endothelial inflammation response mimicking pancreatitis-induced lung injury.** *Exp Biol Med (Maywood)* 2013, **238**(12):1388–1395.

## Figures

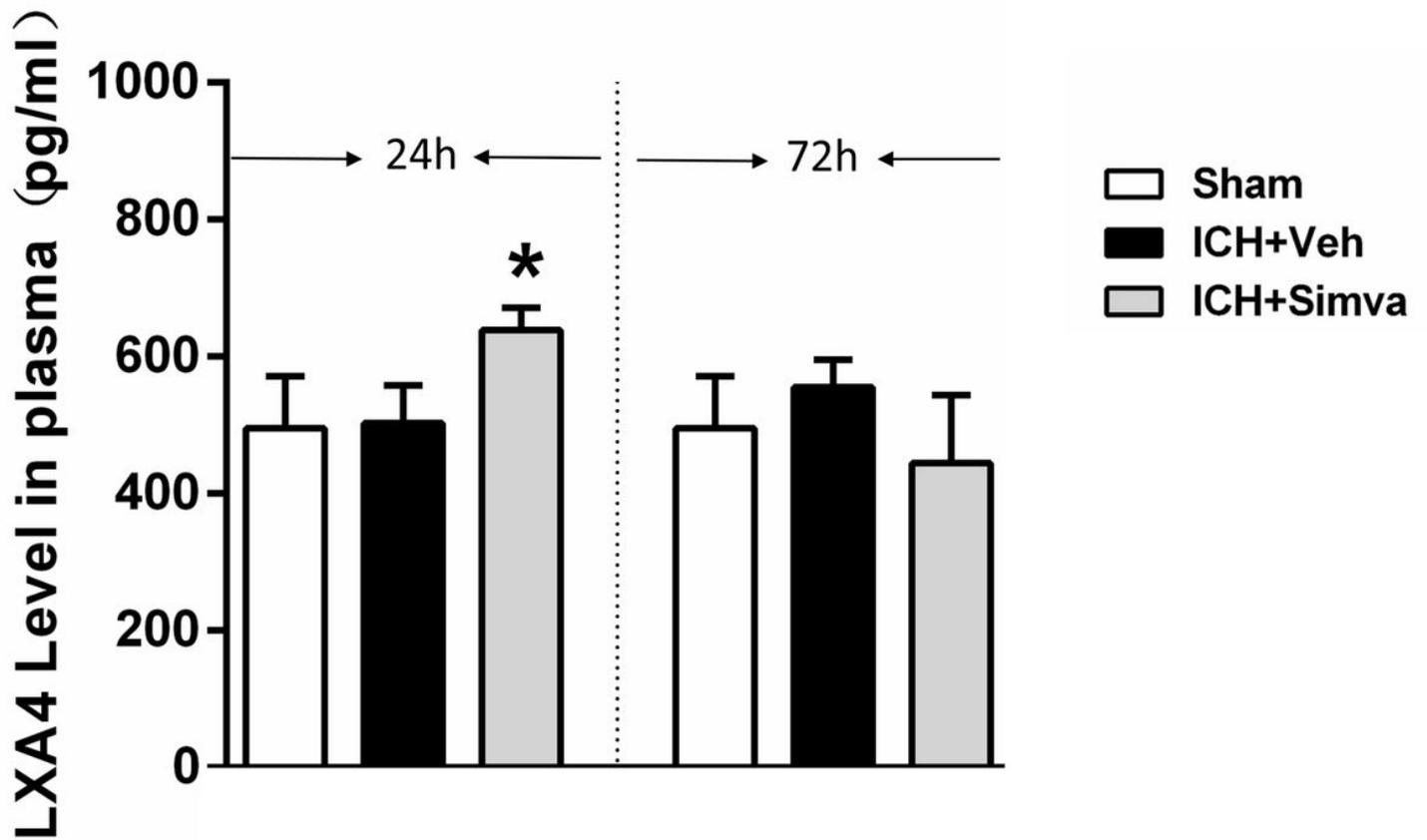
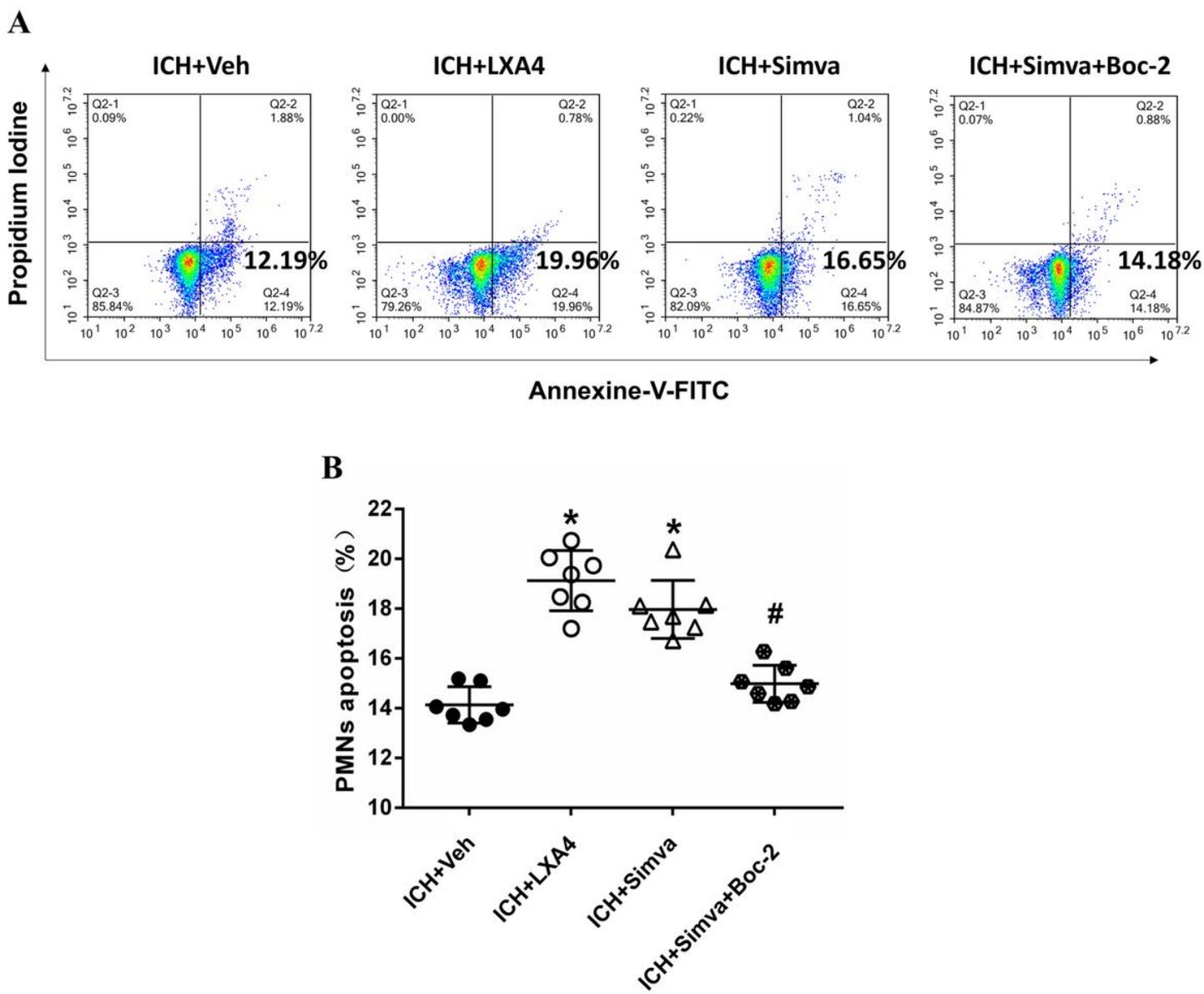


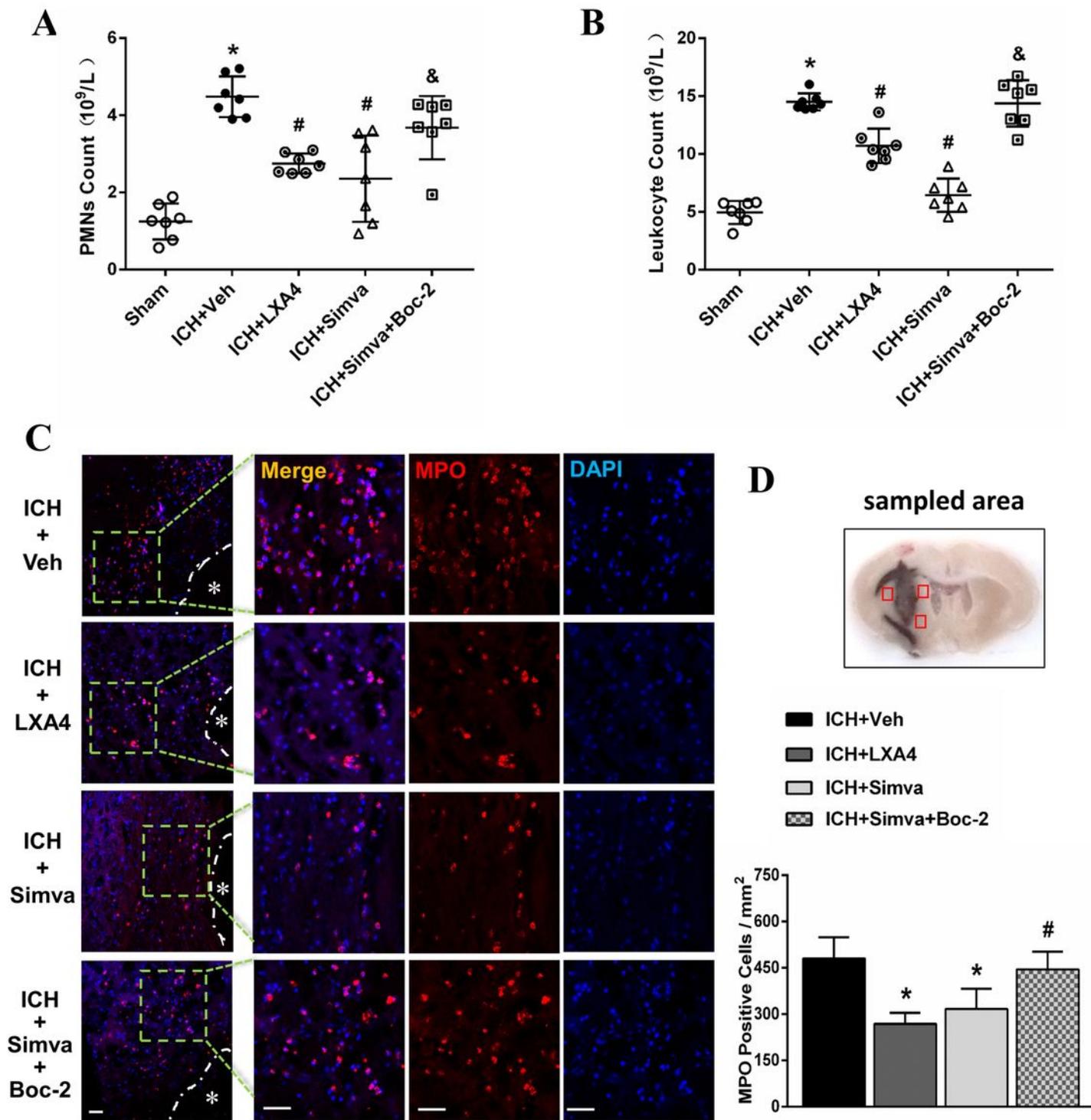
Figure 1

Simvastatin significantly elevated the plasma level of LXA4 (an endogenous FPR2 agonist) at 24h after ICH. ELISA analysis of LXA4 level in rat plasma at 24h and 72h after ICH. Values are expressed as mean±SD, n=4 per group; \*P<0.05 ICH-Simva versus ICH-Veh group. ICH, intracerebral hemorrhage; Simva, simvastatin; Veh, vehicle.



**Figure 2**

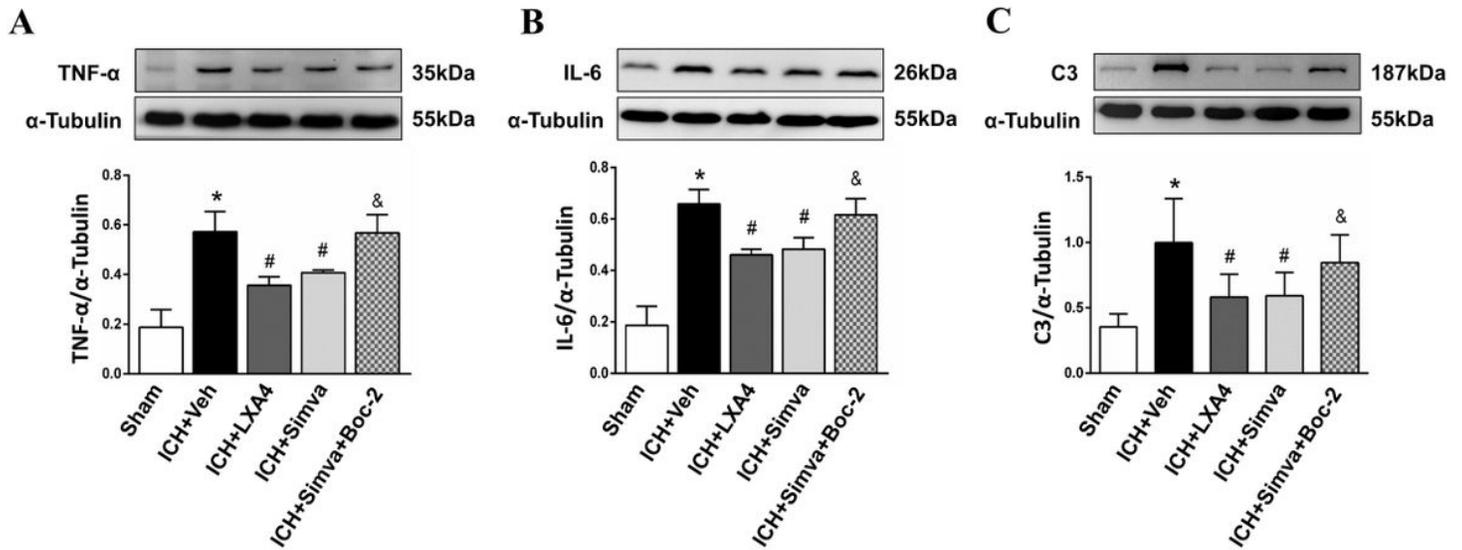
Boc-2, a selective FPR2 antagonist, weakened simvastatin-mediated peripheral PMNs apoptosis. (A) Representative flow cytometric dot plots showing circulating PMNs apoptosis, which freshly isolated from ICH rat. Annexin V+ and PI- cells were considered early apoptotic cells (lower right quadrant). (B) Flow cytometric analysis of peripheral PMNs in the ICH rat treated with vehicle, LXA4, Simva and Simva+Boc-2 at 24h after ICH. The apoptotic ratio was calculated from the ratio of apoptotic cells to total cells counted. Values are expressed as mean±SD, n=7 per group; \*P<0.05 versus ICH+Veh group; #P<0.05 versus ICH+Simva group. PMNs, polymorph nuclear neutrophils.



**Figure 3**

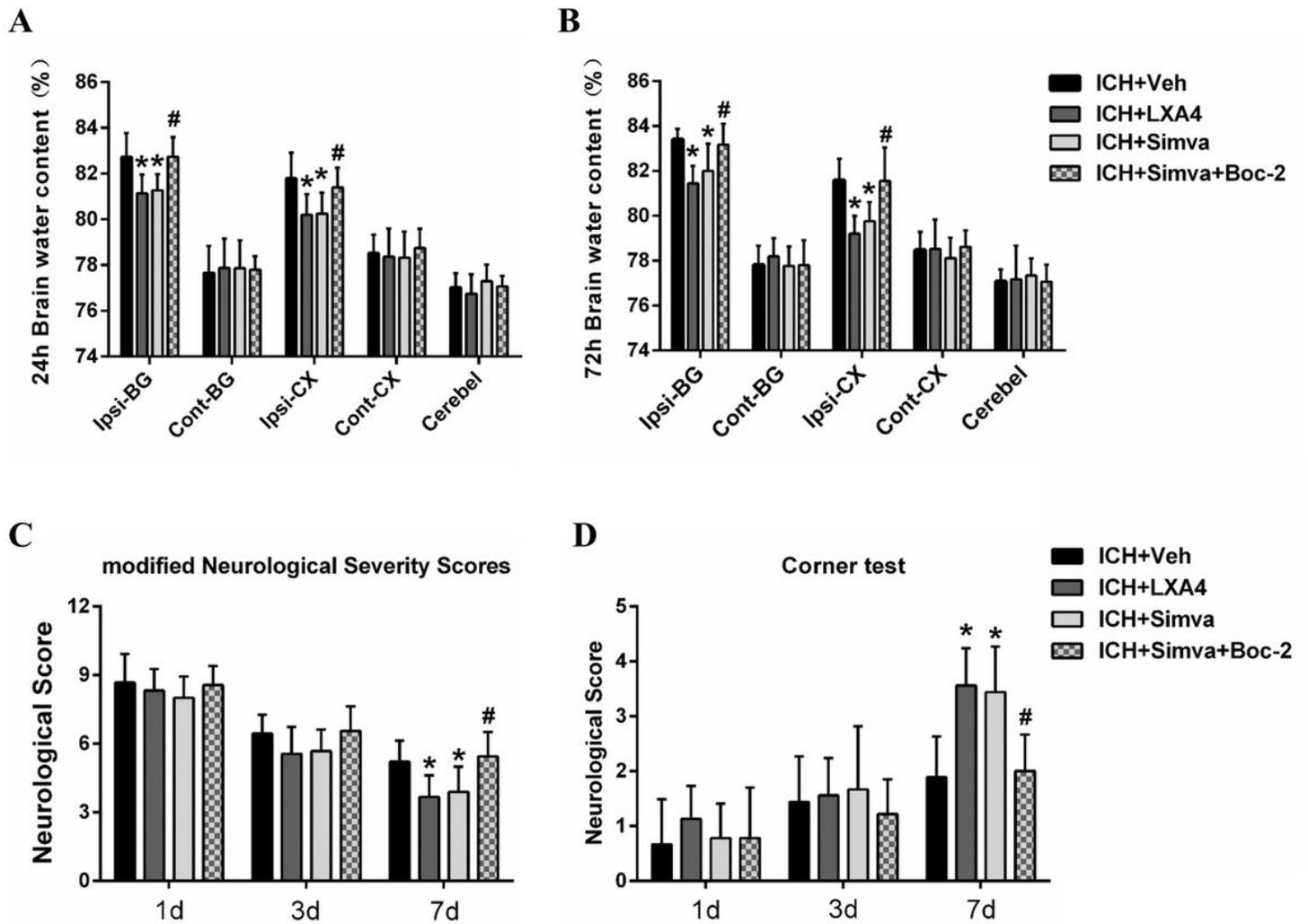
Boc-2, a selective FPR2 antagonist, crippled the inhibitory effects of simvastatin on peripheral PMNs count and PMNs brain-invasion. Blood routine analysis of peripheral PMNs count (A), leukocyte count (B) at 24h after ICH. Values are expressed as mean $\pm$ SD, n=7 per group; \*P<0.05 versus sham group; #P<0.05 versus Veh group; & P<0.05 versus Simva group. (C) Representative immunofluorescence images of MPO positive in perihematomal area at 24h after ICH. (D) MPO (+) cells count around hematoma after ICH.

Values are expressed as mean±SD, n=6 per group; \*P<0.05 versus Veh group; #P<0.05 versus Simva group.



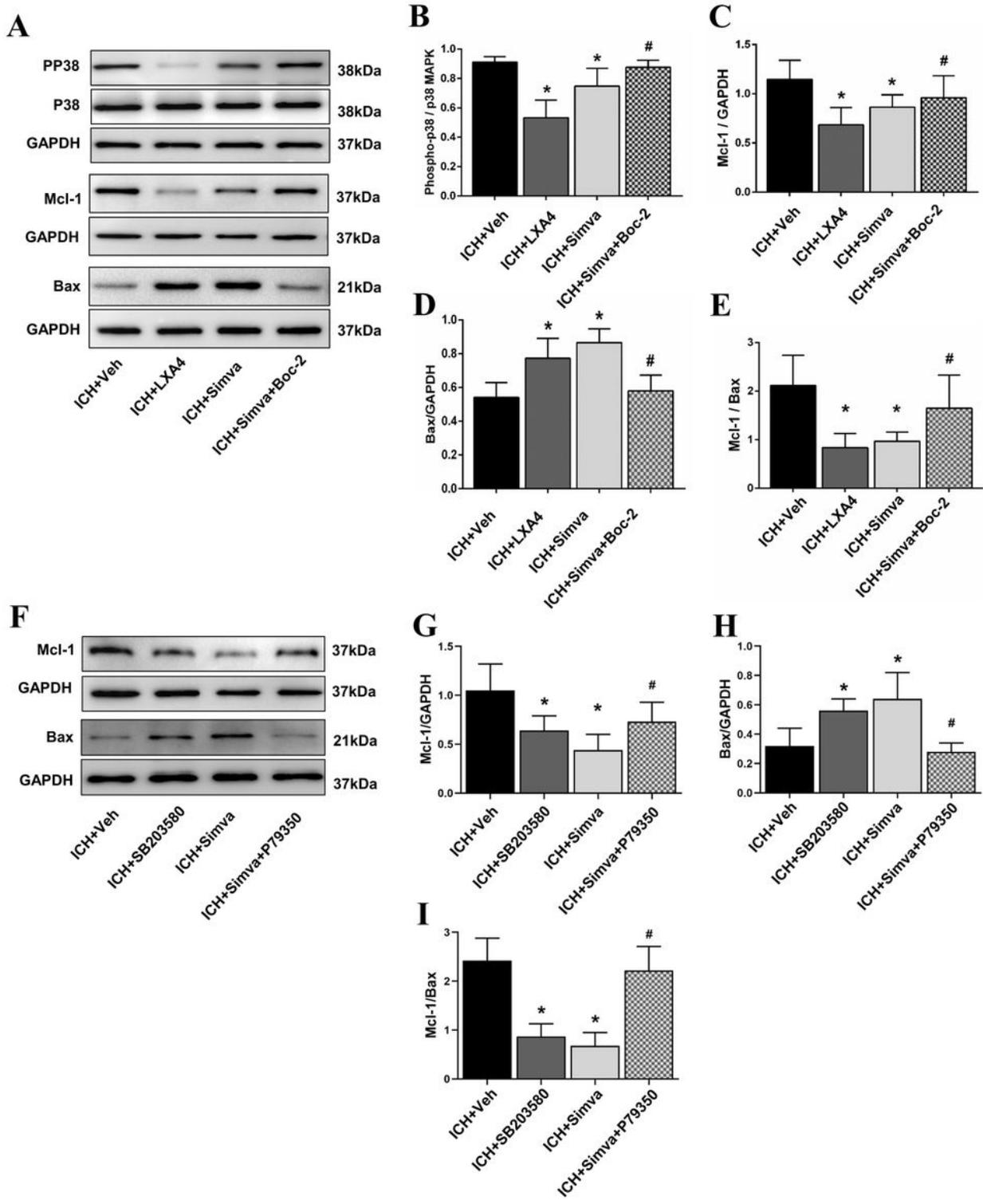
**Figure 4**

Boc-2, a selective FPR2 antagonist, counteracted the anti-inflammatory effect of simvastatin on ICH rat. Western blot analysis showing inflammatory relevant protein levels of TNF-α (A), IL-6 (B) and complement component C3 (C) in perihematomal area at 24h after ICH. Values are expressed as mean±SD, n=3 per group; \*P<0.05 versus Sham group; #P<0.05 versus Veh group; &P<0.05 versus Simva group.



**Figure 5**

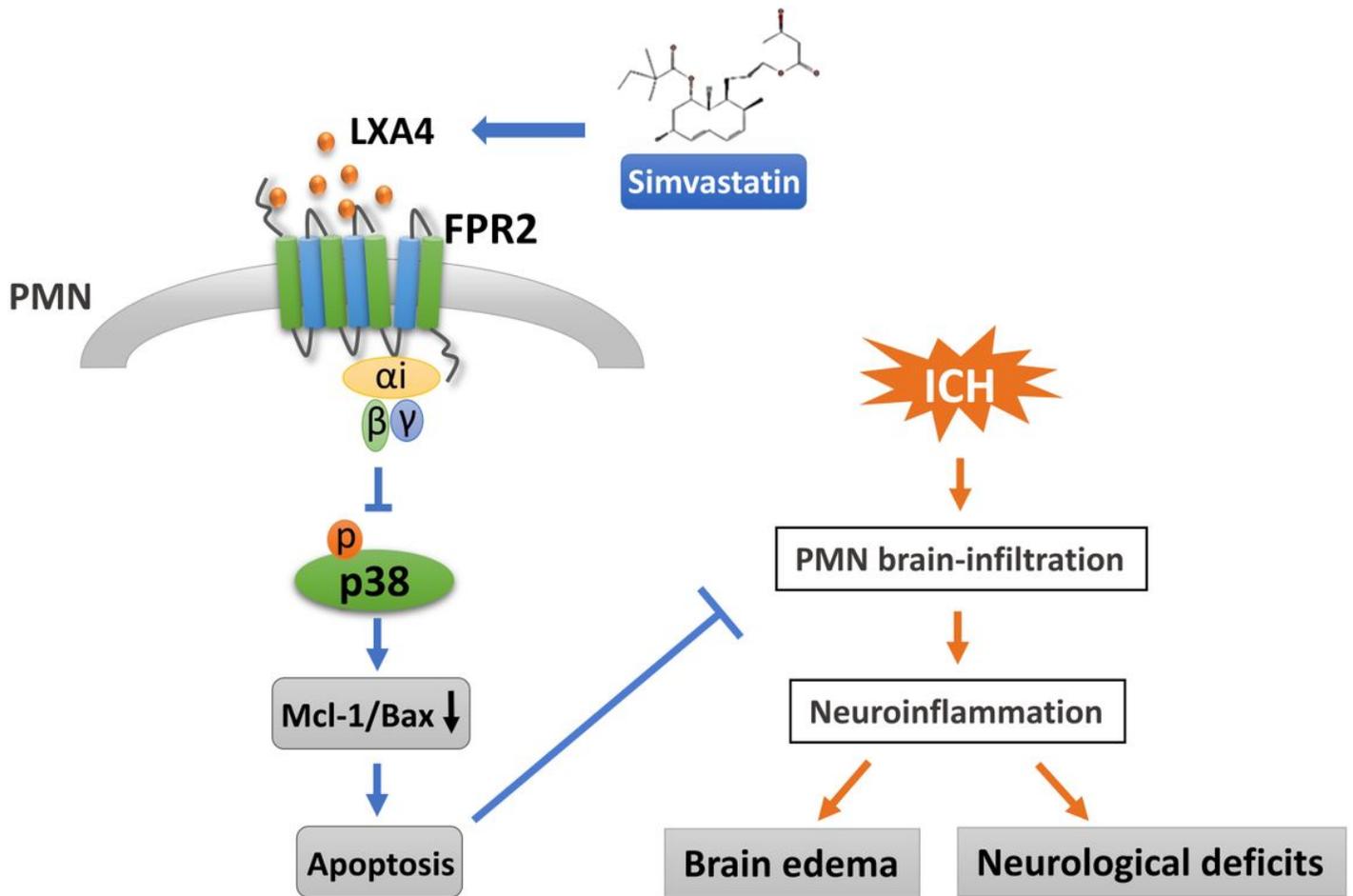
The protective effect of simvastatin on brain edema and neurological deficits was abolished by Boc-2. Result of brain water content at 24h (A) and 72h (B) after ICH. Values are expressed as mean±SD, n=6 per group; \*P<0.05 versus Veh group; #P<0.05 versus Simva group. Ipsi-BG, ipsilateral basal ganglia; Con-BG, contralateral basal ganglia; Ipsi-CX, ipsilateral cerebral cortex; Con-CX, contralateral cerebral cortex; Cerebel, cerebellum. Neurological score by using mNSS (C) and Corner Test (D) at the day1, 3 and 7 after ICH. Values are expressed as mean±SD, n=6 per group; \*P<0.05 versus Veh group; #P<0.05 versus Simva group.



**Figure 6**

FPR2/P38 MAPK signaling pathway play a key role in simvastatin-induced PMNs apoptosis in acute stage after ICH. Representative images (A, F) and quantitative analysis of pp38/p38 ratio (B), anti-apoptotic protein Mcl-1(C, G), pro-apoptotic protein Bax (D, H), and Mcl-1/Bax ratio (the decreased ratio represents pro-apoptosis) (I) in the isolated peripheral PMNs at 24 h post ICH. Values are expressed as

mean±SD, n=3 per group; \*P<0.05 versus Veh group; #P<0.05 versus Simva group. SB203580, p38 MAPK inhibitor; P79350, p38 MAPK agonist.



**Figure 7**

Schematic for the proposed mechanisms. Simvastatin-triggered LXA4 upregulation in plasma selectively activated G protein coupled receptor FPR2 on peripheral PMNs at early stage of ICH, by which inhibited the phosphorylation of P38 MAPK and resulted in Mcl-1/Bax ratio decline, which then promoted more circulating PMNs apoptosis and less PMNs infiltration into brain, and finally attenuated ICH-mediated neuroinflammation and brain injury.