Novel role of STAT3 in microglia-dependent neuroinflammation after experimental subarachnoid haemorrhage

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ABSTRACT

Background and purpose Signal transducer and activator of transcription 3 (STAT3) may contribute to the proinflammation in the central nervous system diseases by modulating the microglial responses. Thus, this study was intended to investigate the effect of STAT3 on microglia-dependent neuroinflammation and functional outcome after experimental subarachnoid haemorrhage (SAH).

Methods The SAH model was established by endovascular perforation in the mouse. Real-time PCR (RtPCR) and western blot were used to examine the dynamic STAT3 signalling pathway responses after SAH. To clarify the role of the STAT3 signalling pathway in the microglia-dependent neuroinflammation after SAH, the microglia-specific STAT3 knockout (KO) mice were generated by the Cre-LoxP system. The neurological functions were assessed by Catwalk and Morris water maze tests. Neuronal loss after SAH was determined by immunohistochemistry staining. Microglial polarisation status after STAT3 KO was then examined by RTPCR and immunofluorescence.

Results The STAT3 and Janus kinase-signal transducer 2 activated immediately with the upregulation and phosphorylation after SAH. Downstream factors and related mediators altered dynamically and accordingly. Microglial STAT3 deletion ameliorated the neurological impairment and alleviated the early neuronal loss after SAH. To investigate the underlying mechanism, we examined the microglial reaction after STAT3 KO. STAT3 deletion reversed the increase of microglia after SAH. Loss of STAT3 triggered the early morphological changes of microglia and primed microglia from M1 to M2 polarisation. Functionally, microglial STAT3 deletion suppressed the SAH-induced proinflammation and promoted the anti-inflammation in the early phase.

Conclusions STAT3 is closely related to the microglial polarisation transition and modulation of microglia-dependent neuroinflammation. Microglial STAT3 deletion improved neurological function and neuronal survival probably through promoting M2 polarisation and anti-inflammatory responses after SAH. STAT3 may serve as a promising therapeutic target to alleviate early brain injury after SAH.

INTRODUCTION

Subarachnoid haemorrhage (SAH) is a life-threatening disease, which accounts for the second mortality among the stroke population. Thirty per cent of victims develop a permanent disability, which severely affects patients’ quality of life. Recent evidence indicates the critical role of neuroinflammation in the pathogenesis of SAH. Neuroinflammation evolves with glial cell activation and the release of a variety of cytokines. Microglia, considered as the immune cells of the central nervous system (CNS), have both beneficially and detrimentally in CNS diseases. Microglia change morphology from ramified to amoeboid shape when activated and obtain bidirectional polarisation in response to the adverse stimuli with the release of distinct profiles of cytokines. The ‘classical’ M1 polarisation is related to the proinflammation and is regarded as the detrimental phenotype. The ‘alternative M2’ polarisation is responsible for anti-inflammation and contributes to tissue repair and recovery. The manipulation of microglial polarisation arises as an innovative approach to reverse the unfavourable inflammatory responses, which has been tentatively investigated in experimental SAH. Several mediators including mammalian target of rapamycin and tumor-specific glycoprotein-6 are evidenced contributing to the skewed M2 polarisation.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family and involves the proinflammatory responses in various CNS abnormalities, including traumatic brain injury, multiple sclerosis and glioma. Suppression of STAT3 signalling pathway activation decreases the infarct volume, number of apoptotic cells and restores the neurological deficits in ischaemic stroke. Recent studies imply that STAT3/Janus kinase-signal transducer 2 (JAK2) signalling pathway participates in the microglia-dependent neuroinflammatory responses, and this participation may relate...
to the modulation of the microglial polarisation. In SAH, activation of the STAT3/JAK2 signalling pathway contributes to early brain injury, cerebral vasculopathy and neurological deficit. The mediator erythropoietin suppresses the STAT3/JAK2 pathway activation, resulting in the reduction of M1-like inflammatory responses and the amelioration of brain injury after SAH. Therefore, we hypothesise that STAT3 may promote the proinflammation-related brain injury after SAH and the inhibition of STAT3 may reverse the unfavourable inflammatory responses and alleviate neurological deficits through the microglia-specific neuroinflammatory modulation.

STAT3 is crucial in early embryogenesis. Lack of STAT3 results in embryo lethality, even in the embryonic stem cells. The emergence of the Cre-loxP recombination system enables the investigation of STAT3 in adult tissues, which allows the ablation of specific genes in later life. The Cre-loxP recombination system is a site-specific recombinase technology allowing the manipulation of DNA in targeted cell types by deletions, insertions, translocations and inversions at specific sites. The applications of conditional STAT3 deletion are achieved in the cardio-vascular system by using the Cre-loxP recombination system.

In the present study, the temporal STAT3 activation status after SAH was assayed and the downstream factor changes of the STAT3 signalling pathway were investigated first. By generating microglia-specific STAT3 knock-out (KO) mice, we demonstrated the essential role of microglial STAT3 in the development of neurological deficits and neuronal loss after SAH. Finally, we provide a possible treatment strategy for the further treatment of SAH.

MATERIALS AND METHODS

Animals and SAH model

Male, wild-type C57BL/6, average weighing 25–30 g, were obtained from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Transgenic mice used in this study including B6.129S1-Stat3tm1Xyfu/J (homozygous STAT3lox/lox) and B6J. B6N(Cg)-Cx3Cr1tm1.1(cre)ung/J (homozygous Cx3Cr1Cre/Cre) were purchased from the Jackson Laboratory (JAX Stock #016923 and #25524; JAX, Maine, USA). SAH models were induced by the endovascular perforation as previously described. STAT3 conditional knockout mice were achieved with Cre-loxP system (figure 1).

See online supplementary material for full experimental methods (online supplemental material 1).

RESULTS

STAT3 signalling pathway activated after SAH

STAT3 is predominantly expressed and initially activated in microglia rather than astrocytes and neurons. Based on the pilot study, three brain areas, including the cortex adjacent to the perforated site (CAPS), M1 cortex and hippocampus, were chosen for the examination (n=3–4/group/time points). The STAT3 signalling pathway was activated acutely after SAH with the subsequent alteration of the downstream factors and related mediators. The activation of the STAT3 pathway was generally in the same pattern through the brain areas. STAT3 demonstrated significant upregulation in transcriptome within the first 3 days in SAH mice compared with the sham control (p<0.05 for all the three brain areas). The increased expression of upstream regulator JAK2 and downstream factor nuclear factor-kappa B (NF-κB) was appreciated. The pattern of NF-κB expression is consistent with our previous results of microglia accumulation from CAPS to motor area. The negative regulator suppressor of cytokine signalling-3 (SOCS3) was reactively increased as well. On day 10, Src showed a significant downregulation in SAH mice, which suggested the STAT3 signalling pathway responded to SAH injury through the negative modulation of Src path at the delayed stage (figure 2A). In protein expression, the levels of phosphorylated STAT3 and JAK2 increased within the first 3 days after SAH and then restored to the unphosphorylated status. The total STAT3 and JAK2 of SAH mice were comparable to the sham control (figure 2B,C). The clarification of the dynamics of STAT3 signalling pathway changes provided the fundamental understanding of how the STAT3 signalling pathway was involved and activated after SAH and the optimal time window for the targeted intervention in the subsequent experiments.

Generation of microglia-specific STAT3 knockout (KO) mice

To confirm the efficiency of Cre-mediated deletion, we analysed the expression of STAT3 at the gene and protein levels. The significantly decreased STAT3 expression of mRNA and protein was defined in STAT3 KO mice compared with the control (n=3/group, p<0.005). The phosphorylation level of STAT3 and JAK2 was decreased as well. It was noted that there was still a certain amount of phosphorylated STAT3 and JAK2 within the first 3 days after SAH and then restored to the unphosphorylated status. The total STAT3 and JAK2 of SAH mice were comparable to the sham control (figure 2B,C). Other evidence shows that brain inflammation caused by tumour necrosis factor α (TNF-α)-induced interleukin (IL)-6 release in astrocytes was mediated by NF-κB but not by STAT3. Several studies found modulators can relieve brain inflammation of SAH that is through the microglia-mediated JAK–STAT3 signal pathway.

So the crosstalk of the JAK–STAT3 pathway in different brain cells during SAH needs further study to clarify. An overall downregulation of STAT3 signalling pathway was appreciated in STAT3 KO mice in the early phase after SAH. On day 5, SOCS3 and NF-κB showed the
Figure 1  Characterisation of microglia-specific STAT3 KO mice. (A) The schematic description of the breeding strategy. (B) PCR analysis of genotypes. STAT3\textsuperscript{flx} allele was electrophoretically separated and demonstrated as a 187 bp fragment. Wild-type STAT3 allele was 146 bp. Cx3Cr1\textsuperscript{Cre} fragment was 380 bp and Cx3Cr1\textsuperscript{−} was 302 bp. (C) Biochemical determination of STAT3 KO at the gene and protein levels. RNA and protein were extracted from the CAPS (C), M1 cortex (M) and hippocampus (H) of the SAH brain. RT-PCR examination was performed to determine the mRNA expression of STAT3 in STAT3 KO mice compared with the control. WB was performed to determine the STAT3/JAK2 phosphorylation status of STAT3 KO mice. (D) The STAT3 signalling pathway activation status of STAT3 KO mice at 1 and 5 days after SAH. The mRNA expression of the mediators involved in the STAT3 pathway is shown in the fold change in the CAPS, M1 cortex and hippocampus of STAT3 KO mice compared with the control. n=3 per group. Values are mean±SEM. *P≤0.05, **P0.01, ***P≤0.001. CAPS, cortex adjacent to the perforated site; JAK2, Janus kinase-signal transducer 2; KO, knockout; RT-PCR, real-time PCR; SAH, subarachnoid haemorrhage; STAT3, signal transducer and activator of transcription 3; WB, western blot.
Figure 2  STAT3 signalling pathway activated after experimental SAH. (A) RNA extract was prepared from CAPS, M1 cortex and hippocampus of SAH brain at 1, 3, 5 and 10 days. The mRNA expression of mediators involved in the STAT3 pathway after SAH was determined by RT-PCR and shown in the fold change compared with the sham-operated group. n=3–4/group/time point. Values were mean±SEM. *P≤0.05, **P≤0.01, ***P≤0.001. (B) STAT3/JAK2 phosphorylation status was detected by WB at 1, 3, 5 and 10 days. The levels of phosphorylated and total STAT3/JAK2 relative to internal control GAPDH are shown in the cortex adjacent to the perforated site (C), M1 cortex (M) and hippocampus (H), respectively, of SAH and sham-operated mice. n=3/group/time point. (C) The quantitative analysis of the pSTAT3/STAT3 and pJAK2/JAK of (B) to show the trend of pSTAT3/STAT3 and pJAK2/JAK in the SAH and sham groups. CAPS, cortex adjacent to the perforated site; JAK2, Janus kinase-signal transducer 2; NF-κB, nuclear factor-kappa B; RT-PCR, real-time PCR; SAH, subarachnoid haemorrhage; STAT3, signal transducer and activator of transcription 3; WB, western blot.
reversed upregulation, which suggested that microglia-specific STAT3 deletion had a critical effect on the related mediators over time (figure 1D).

**Microglial STAT3 deficiency ameliorated the neurological impairment after SAH**

Mice of both STAT3 KO and control group presented the SAH-like symptoms after the operation, which occurred mostly within the first 24–48 hours. Mortality was 7.55% in STAT3 KO mice and 14.29% in mice of the control group. The average body weight loss of STAT3 KO mice was 2.4 g, 9.85% of the original body weight, which was comparable to the control ones, which was 2.59 g, accounting for 10.37% of the original body weight. On day 10, body weight returned to the level of original status in the two groups (figure 3A).

To investigate the effect of microglial STAT3 ablation on the neurological function in SAH, we employed a battery of neurobehavioural tests (n=8–16/group). STAT3 KO mice exhibited an improved sensorimotor function with significantly higher mMSS scores compared with the control ones (group effect: p=0.015). The post hoc statistic tests indicated that the improvement was significant from day 1 to 3 after SAH. The sensorimotor function gradually recovered in both groups of mice in the delayed phase (figure 3B).

Gait analysis was performed by the Catwalk system. The data showed the gait parameters of the right front paws of mice, and the gait analysis of the other three paws presented similar results for the bilateral lesion. STAT3 KO mice showed the alleviated static gait deficits after SAH, compared with the control ones. The max contact area and max intensity of paws significantly increased in STAT3 KO mice (group effect: p<0.005, figure 3C). Other static gait parameters including mean intensity, max contact max intensity, max contact max intensity of paws increased as well in online supplemental figure 1A, which indicated microglial STAT3 deletion alleviated the muscle weakness and paw contracture after SAH. The dynamic gait function was not significantly improved in STAT3 KO mice (online supplemental figure IB), which suggested that the microglial STAT3 deletion might not affect the running speed and gait pattern after SAH.

In the training phase of Morris water maze tests, STAT3 KO mice showed remarkably decreased latency to find the escape platform (group effect: p=0.044), which indicated the improved learning ability after SAH. In the probe trial, STAT3 KO mice showed a remarkable amelioration of long-term memory deterioration, presenting significantly increased exploration in the target quadrant compared with the control ones (p=0.024). SAH mice of the control group failed to recall the memory of the position where the platform was previously placed after the interval. In addition, mice showed comparable swim speeds between the two groups, suggesting the equivalent swimming skills in the testing (figure 3D).
Microglial STAT3 deficiency contributed to the neuroprotection in SAH

To investigate if the microglial STAT3 ablation could positively affect neuronal survival in haemorrhage stroke, we subsequently determined the neuronal survival in STAT3 KO mice by using NeuN immunohistochemistry (STAT3 KO n=7, control n=8). In the NeuN immunohistochemistry, SAH mice showed significantly increased neuronal numbers after STAT3 deletion in CAPS and hippocampus on day 5 (p<0.05) (figure 4A,B). The results indicated that microglial STAT3 deficiency had a positive effect on neuroprotection in SAH, which was remarkable in the bleeding area.

STAT3 ablation primed microglia to M2 polarisation

To identify key factors alleviating the neurological impairment caused by depletion of microglial STAT3, we first examined the microglial reaction after STAT3 ablation. In iba1 immunohistochemistry (STAT3 KO n=7, control n=8), microglia demonstrated the decreased accumulation after STAT3 deletion in SAH, which was significant in the M1 cortex and hippocampus (figure 5A,B). We used immunofluorescence and confocal microscopy to determine and visualise the polarisation status and morphological changes of microglia (n=6). Microglia showed an

Figure 4  Microglial STAT3 deletion alleviated the neuronal loss after SAH. (A) Neuronal loss was detected by NeuN immunohistochemistry in the CAPS, M1 cortex and hippocampus of the brain. The representative NeuN labelled coronal brain sections were shown on day 5 after SAH. (B) NeuN-positive neurons were quantified in the aforementioned brain areas of STAT3 KO and control groups of mice. Bar=50 µm. STAT3 KO n=7, control n=8. Values are mean±SEM. *P≤0.05, **P≤0.01, ***P≤0.001. CAPS, cortex adjacent to the perforated site; KO, knockout; SAH, subarachnoid haemorrhage; STAT3, signal transducer and activator of transcription 3.

Figure 5  Stat3 deletion primed microglia to M2 polarisation. (A) Microglia was examined by iba1 immunohistochemistry in CAPS, M1 cortex and hippocampus of the brain. The representative iba1 labelled coronal brain sections were shown on day 1 after SAH. Bar=50 µm. (B) Iba1-positive microglia were quantified in the aforementioned brain areas of STAT3 KO and control groups of mice. (C) Microglial polarisation status was determined by immunofluorescence. The representative confocal microscopic images showed the visualisation of CD16/32 (M1, green), CD206 (M2, red) and DAPI (nuclei, blue) coexpression in CAPS on day 1 after SAH. The white arrows indicate the highly magnified view of the typical microglial processes. Bar=20 µm for all magnifications. (D) CD16/32-positive (M1) or CD206-positive (M2) cells were quantified in the aforementioned brain areas of STAT3 KO and control groups of mice at 1 and 5 days after SAH. n=5–8 per group. Values were the mean±SEM. *P≤0.05, **P≤0.01, ***P≤0.001. CAPS, cortex adjacent to the perforated site; KO, knockout; SAH, subarachnoid haemorrhage; STAT3, signal transducer and activator of transcription 3.
obvious reduction of M1 polarisation after STAT3 deletion with the decreased CD16/32 expression in SAH. Strikingly, M1-possessed microglia of STAT3 KO mice showed an early morphological transformation with the condensed processes. The CD206-positive M2 microglia appeared earlier in CAPS after STAT3 deletion (figure 5C and online supplemental figure 2). The quantification of M1 and M2 microglia ratio indicated that microglial STAT3 deletion suppressed M1 polarisation on day 1 after SAH, which was significant in CAPS and M1 cortex (p<0.05). The M2 polarisation was significantly promoted over all the brain areas (p<0.05). The suppression of M1 polarisation was still noted in the delayed phase of SAH on day 5 (p<0.005). The increase of M2 polarisation was significantly in the M1 cortex and hippocampus in this phase (p<0.05, figure 5D). Real-time PCR (RtPCR) measurement demonstrated identical results regarding the mRNA expression of microglial phenotype-specific markers (online supplemental figure 3). Thus, STAT3 deletion reduced the microglial responses toward M1 polarisation after SAH and triggered the early morphological transformation. STAT3 deletion primed microglia to M2 polarisation after SAH.

**Microglial STAT3 deficiency promoted the anti-inflammation after SAH**

The expression of microglia-related inflammatory cytokines after SAH was detected by RtPCR (n=3). The expression of anti-inflammatory factor-induced IL-4 increased significantly in the condition of microglial STAT3 deletion compared with the control in the early phase after SAH (p<0.05). The change of proinflammatory cytokine expression was not remarkable except in the hippocampus (figure 6A). On day 5 after SAH, IL-4 was continuously maintained at a higher level in STAT3 KO mice, especially in CAPS and hippocampus (p<0.05). The significant upregulating of transforming growth factor-β was also noted in the M1 cortex and hippocampus (p<0.05). The effect of microglial STAT3 deletion on proinflammatory cytokines was heterogeneous in this phase. IL-6 demonstrated the downregulation. However, TNF-α was observed upregulated in CAPS (figure 6B). Thus, the microglial STAT3 deletion suppressed the proinflammation in specific brain areas in addition to promoting the anti-inflammation after SAH. The alteration of cytokine expression after microglial STAT3 deficiency suggested a functional transition of microglia from M1 to M2 polarisation.

**DISCUSSION**

The activation of STAT3 was observed in a number of CNS diseases, such as traumatic brain injury, brain tumours and cerebral ischaemia.\(^{12,14,30}\) Especially recent studies found that several modulators can relieve early brain injury of SAH and act as an anti-inflammation medication through suppressing microglia-mediated JAK–STAT3 signal pathway.\(^{6,16,17}\) By using microglia-specific STAT3 KO SAH mice, the activation and biology effect of microglia-mediated JAK–STAT3 signal pathway was studied. In our study, the gene expression of STAT3 was consistent with STAT3/JAK2 protein level, upregulated immediately after SAH. As the disease progressed, STAT3 activation was alleviated at the late stage with the upregulation of the downstream regulators. The negative regulator SOCS3 showed temporal feedback with the upregulated expression to the STAT3 signalling modulation. The delayed downregulation of Src might be a considerable reason for neuronal apoptosis in the delayed phase. Determining the time course STAT3 signalling pathway activation status illustrated the dynamics of how the STAT3 pathway responded to SAH. However, the biology effect of the STAT3 pathway is controversial in some situations,\(^{28,31}\) and it is not
fully understood in SAH conditions at present. The present study also investigated the relationship between microglia-specific STAT3 KO and outcomes in SAH, thus giving new insights on the pathological mechanism and possible treatment strategy.

The Cre-loxP recombination system has been particularly useful in neurosciences targeting the various cell types and complex neural circuits of the brain which integrate to form cognition and behaviours. Studies by Tsien et al have applied the Cre-loxP recombination system in the adult mouse forebrain, in which the various type of neurons are evidenced to be postmitotic. Analyses of tissue-specific STAT3-deficient mice indicate that STAT3 involves a variety of biological functions including cell growth, suppression and induction of apoptosis, and cell motility. In this study, we first demonstrated the microglia-specific STAT3 knockout on mice by using this technique. The dispersion of the genotypes of the F2 hybrids is basically aligned to the expectation according to the principles of Mendel’s genetics. The behaviours and social performance of STAT3 deficient mice indicate that STAT3 plays a critical role in the regulation of neuro-immune system and complex neural circuits of the brain which integrate to form cognition and behaviours.

The present study found that the microglial STAT3 deletion significantly improved neurological function and neuronal survival in SAH. The ablation of the inflammation-related STAT3 pathway might largely blunt the unfavourable neuroinflammatory responses and thus ameliorate the neuronal damage. For investigating the protective mechanism of microglial in SAH, our previous study found that the reactive immune cells in SAH mice brain were largely from resident microglia pool rather than infiltrating macrophages by using Cx3crl1+/- Ccr2RFP/RFP transgenic mice. A significantly continuous (p<0.001) accumulation of microglia (Iba1+ cells) in CAPS was found after SAH. While at the M1 cortex and hippocampus, microglia showed an acute and temporary accumulation within the first 3 days after SAH. Moreover, we also found the microglia reserved as M1 microglia in the early stage of SAH, which acts as a proinflammation function and the morphology, are dendritic. While in the late stage of SAH, microglia transfer to M2 microglia, which were amoeboid/bushy ‘activated’ morphology connected with anti-inflammation effect. In the present study, STAT3 deletion reduced the microglial responses with the decreased accumulation in the early phase after SAH. In brief, the CD16/32+ and CD206+ cells in STAT3 KO mice are more reminiscent of the activated microglia/macrophage morphology, which shows that STAT3 KO can reverse or induce polarisation microglia to M2 state. M2 microglia shows more neuroprotective characteristics, which is consistent with the neurobehavioural test and cytokine test that STAT3 KO can suppress the inflammation in SAH. The finding that the inhibition of STAT3 pathway activation ameliorated the M1-like microglial polarisation was also reported in other published data. Microglia after STAT3 deletion demonstrated curved and circled processes rather than the remarkably decreased processes compared with the control. The morphological transformation did not limit the process motility, which was regarded as the dynamic surveillants of brain parenchymal.

The critical role of the STAT3 signalling pathway in the mediation of the function-related microglial polarisation was also evidenced by the expressed cytokine profile. The microglial STAT3 deletion upregulated the expression of IL-4 dominant anti-inflammatory cytokines after SAH, which implied the protective function in neuroinflammation. Although the increase of TNF-α was observed in D5 after microglial STAT3 deletion, the overall cytokine level tends to be anti-inflammatory. Also, TNF-α has been reported that in brain inflammation, microglia can be activated by IL-6, which is released from TNF-α-sensitive brain pericytes cooperating with IκB-NF-κB and JAK–STAT3 pathways, and the changes in TNF-α level in different time and brain area in SAH need further study to detect. STAT3 signalling pathway contributed to the acute M1 related proinflammation, which might contribute to the early brain injury in SAH. The STAT3 deletion primed the microglia-dependent neuroinflammation to M2 directed anti-inflammation. Indeed, we could not exclude the effect of infiltrating peripheral immune cells. However, the proportion of the peripheral immune cells was relatively small compared with the resident microglia, especially at the early stages after SAH. The microglial STAT3 ablation-induced anti-inflammatory responses ameliorated neurological impairment and neuronal loss. This neuroprotection might be the result of microglia–neuron interactions. However, the direct effect of neuronal STAT3 on neuronal survival was not investigated in this study. Future studies might make use of the Cre-LoxP system to create the neuronal STAT3 deficiency model to address this issue.

CONCLUSIONS

In conclusion, the STAT3 signalling pathway played a critical role in the mediation of the function-related microglial polarisation, which might be prone to an M1 proinflammatory direction. The microglia-specific STAT3 deletion alleviated
the unfavourable neuroinflammation and enhanced the anti-inflammatory function in experimental SAH probably by triggering the microglial transition from M1 to M2 polarization. The results suggested that microglia-specific STAT3 deletion might have a positive effect on the functional outcome after SAH. It might provide us a potential therapeutic approach aiming at the modulation of neuroinflammation for SAH.

Contributors ZV and GKDW designed the experiments; ZV, JC, HL and SYEL conducted the experiments; GL and WY advised on the design and supervised the study together with GKDW; ZV, JC and GKDW drafted the manuscript; all authors contributed to the revision of and approved the manuscript.

Funding The study was partly supported by the Direct Grant for Research, the Chinese University of Hong Kong (grant number 2016.112).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved by the Animal Experimentation Ethics Committee, the Chinese University of Hong Kong (reference number 19-108-GRP).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Experiment data are available upon reasonable request.

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REFERENCES
SUPPLEMENTAL MATERIAL

SAH model establishment

Male, wild type C57BL/6, average weighing 25–30g, were obtained from the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Transgenic mice used in this study including B6.129S1-Stat3<sup>tm1Xyfu</sup>/J (homozygous STAT3<sup>flox/flox</sup>) and B6J.B6N(Cg)-Cx3Cr1<sup>tm1.1(cre)Jung</sup>/J (Homozygous Cx3Cr1<sup>Cre/Cre</sup>) were purchased from the Jackson Laboratory (JAX Stock #016923 and #25524, JAX®, ME, USA). All of the animals were housed in a temperature-controlled environment, with a 12-hour light-dark cycle and free access to food and water. The mice expressing STAT3<sup>flox/flox</sup> Cx3Cr1<sup>cre/-</sup> were selected for subsequent experiments. The littermate carrying STAT3<sup>flox/flox</sup> Cx3Cr1<sup>-/-</sup> was used as control; the sham model was operated in the same procedures of SAH except without filament perforation of intracranial vascular. The procedures involving animals and their care were conducted under the approval of the Ethics Committee of the Chinese University of Hong Kong.

Mice were anesthetized and fixed in a supine position in advance, and all the surgical operations were through a surgical microscope. Firstly, make a 1-cm incision in the midline of the neck, dissecting the left common carotid artery (CCA), left external carotid artery (ECA) and left internal carotid artery (ICA). The ECA was ligated as far cranially as possible, the occipital artery was exposed and coagulated to avoid bleeding. Two 1.5-cm-length 5–0 silk sutures were used for filament fixation. Temporarily block the blood of ECA with a microclip, and make an incision on the ECA for filament insertion then a 20-mm-long blunted 5–0 monofilament nylon suture was inserted from ECA to the lumen of ICA continue to the intracranial vessels. The vessel was perforated at the bifurcation of the middle cerebral artery (MCA) where the resistance was encountered. Then immediately withdrawn the filament to introduce the bleeding into
subarachnoid space. The sham model was operated in the same procedures except without filament perforation [21]. The mice were maintained at 37 °C throughout the operation and recovery. Ointment protecting vision was applied to their eyes. Buprenorphine was injected intraperitoneally (i.p.) twice a day for consecutive 3 days for analgesia. Bodyweight was evaluated on day 1, 3, 5, and 10 after SAH induction for the wellbeing of mice.

**Establishment of STAT3 conditional knock-out mice model**

The Cre-LoxP system was utilized. Mice with a STAT3 deletion in microglia were generated by crossing mice with the floxed STAT3 alleles with mice expressing Cre under the control of the Cx3Cr1 promoter. Homozygous \( \text{STAT}^3_{\text{flox/flox}} \) mice were mated to homozygous Cx3Cr1\(^{\text{Cre/Cre}} \) mice to generate the hybrid mice of the first generation carrying Cx3Cr1-Cre and heterozygous STAT3 (F1: \( \text{STAT}^3_{\text{flox/+}} \times \text{Cx3Cr1}_{\text{Cre/+}} \)). The F1 mice were then crossed with the homozygous \( \text{STAT}^3_{\text{flox/flox}} \). The hybrids of the F2 generation was expected to disperse the genotypes into four types, which were \( \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{Cre/-}}, \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{-/+}}, \text{STAT}^3_{\text{flox/+}} \times \text{Cx3Cr1}_{\text{Cre/-}}, \) and \( \text{STAT}^3_{\text{flox/+}} \times \text{Cx3Cr1}_{\text{-/+}} \). The F2 mice expressing \( \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{Cre/-}} \) (\( \text{Cx3Cr1}_{\text{Cre/-}} \) as a heterozygote, means Cx3Cr1 positive cell have dominant Cre thus can work in the Cre-LoxP system and can cooperate with LoxP to specific deplete the STAT3 in Cx3Cr1-Cre positive cells.) were selected for subsequent experiments. The littermate carrying \( \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{Cre/-}}, \) was used as control. Genotype assay was described in the online-only Data Supplement.

**Generation of microglia specific STAT3 knockout mice**

To investigate the role of STAT3 in microglia-dependent neuroinflammation in SAH, we generated transgenic mice in which STAT3 was deficient in a microglia-specific manner. The genotypes of F1 and F2 generation (\( \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{Cre/-}}, \text{STAT}^3_{\text{flox/flox}} \times \text{Cx3Cr1}_{\text{-/+}}, \text{STAT}^3_{\text{flox/+}} \times \text{Cx3Cr1}_{\text{Cre/-}}, \text{STAT}^3_{\text{flox/+}} \times \text{Cx3Cr1}_{\text{-/+}} \)).
CX3CR1\textsuperscript{cre/−}, and \textit{STAT3}\textsuperscript{flax/+ CX3CR1\textsuperscript{−/−}} were determined by PCR. Theoretically, each of the four phenotypes of F2 generation accounted for one-fourth of the total number of the F2 generation. Mice carrying \textit{STAT3}\textsuperscript{flax/flax CX3CR1\textsuperscript{cre/−}} indicated the complete Cre-mediated deletion of STAT3 occurred in microglia at the DNA level, which was employed for the subsequent SAH induction. The littermates carrying \textit{STAT3}\textsuperscript{flax/flax CX3CR1\textsuperscript{−/−}} presenting two alleles of \textit{STAT3}\textsuperscript{flax} however without the expression of Cre recombinase were used as the control group (Figure 2A). PCR determination of genotypes demonstrated that transgenic mice of F1 generation carrying \textit{STAT3}\textsuperscript{flax/+ CX3CR1\textsuperscript{cre/−}} demonstrated the heterozygous expression of one 187-bp \textit{STAT3}\textsuperscript{flax} allele and a 146-bp wild-type STAT3 allele, as well as one allele of 380-bp CX3CR1\textsuperscript{cre} and a 302-bp CX3CR1\textsuperscript{−}. The genotyping assay of F2 hybrids was generally consistent with the theoretical hypothesis (Figure 2B). Total of 548 transgenic mice of F2 mice were bred. \textit{STAT3}\textsuperscript{flax/flax CX3CR1\textsuperscript{cre/−}}, \textit{STAT3}\textsuperscript{flax/flax CX3CR1\textsuperscript{−/−}}, \textit{STAT3}\textsuperscript{flax/+ CX3CR1\textsuperscript{cre/−}}, \textit{STAT3}\textsuperscript{flax/+ CX3CR1\textsuperscript{−/−}} and, \textit{Stat3}\textsuperscript{+/+ CX3CR1\textsuperscript{−/−}} (wild-type) were presented in F2 generation, accounting for 20.26%, 27.01%, 25.73%, 26.64%, and 0.36% of the total number respectively. Both F1 and F2 offspring were born alive and capable of normal fertility. It was noted that 2 out of the total F2 hybrids were found with cataracts, which was excluded from the subsequent experiments. Otherwise, mostly transgenic mice grew healthy and behaved normally through the close monitor. There was no evidential preponderance of gender distribution among F2 hybrids (Supplementary Table VI).

\section*{Statistical analysis}

All the data were expressed as mean ± SEM. Statistical analyses were performed by IBM SPSS 23.0 software. For the cross-sectional assessment cohort, statistical analyses are done by two-way ANOVA to assess the treatment effect across 4 time points. The independent t test was used to determine the significance of the between-group comparison. The equality of error
variance was tested as appropriate. P<0.05 after Bonferroni adjustment for multiple comparisons was considered statistically significant.

**Real-time reverse transcription polymerase chain reaction**

Mice were cardinally perfused with sterile saline followed by RNALater Solution at each time point. The RNA was extracted via using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and PureLink™ RNA Mini Kit (Thermo Scientific. Waltham, MA, USA) according to the manufacturer's instructions. For mRNA expression, 1μg of total RNA was reverse-transcribed into cDNA by using high capacity reverse transcriptase (Applied Biosystems, Carlsbad, California, USA).

Real-time reverse transcription polymerase chain reaction (PCR) was performed to determine the activation status of the STAT3 signaling pathway, biochemical characterization of STAT3 deletion the expression of M1/M2 related microglial markers and inflammatory cytokines. The primer were designed by using AlleleID 6.0 software (AlleleID®, PREMIER Biosoft. USA). The primers sequences were listed in Supplementary Table I-II. A 10-μL total PCR reaction mixture with SYBR Green master premix Ex Taq (Takara, Japan) was utilized to perform the amplification reaction by QuantStudio 12 Flex Real-Time PCR System according to the manufacturer’s instruction (ABI 7500, Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with the cycling conditions as follow: 50 °C, 2 min, 95°C, 10 min, 40 cycles of 95°C, 15 s; 60°C, 1 min. Differential gene expressions were calculated using the 2-ΔΔCT method with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control. The expression levels of the genes were reported as fold changes compared with the sham group.

**Western blotting**
Western blotting was performed to examine the activation status of STAT3 and JAK2 at the protein level. Mice were cardinally perfused with sterile saline at each time point. Brain tissue was lysed and extracted by Radioimmunoprecipitation assay (RIPA) buffer (Beyotime, # P0013B) with Pro tease Inhibitor (Roche, # 5892970001), Phosphatase Inhibitor (Thermo Scientific, #78420) and Phenylmethylsulfonyl fluoride (PMSF) (Beyotime, # ST506). 20 μg total proteins from each sample were separated on Tris-polyacrylamide gel by electrophoresis and blotted onto nitrocellulose membranes (GE Healthcare). Membranes were blocked by 5% non-fat milk for 1 hour at room temperature. Then the membranes were incubated with primary antibodies p-STAT3 (1:1000; Cell Signaling Technology, #9145S), STAT3 (1:5000; Cell Signaling Technology, #8768S), p-JAK2 (1:1000; Cell Signaling Technology, #3771S), JAK2 (1:2000; Cell Signaling Technology, #3230S), and GAPDH (1:5000; Cell Signaling Technology, #5174S) overnight at 4°C. After the thorough wash, the membranes were incubated with the secondary antibody (1:5000; Cell Signaling Technology, #7074) for 1 hour at room temperature. The blotting was visualized by the enhanced chemiluminescence (ECL) plus detecting reagent and exposed onto x-ray films. Image J was used to quantitatively analyze the western blot results, and Grapad Prism 7.00 was used for the statistical analysis.

Genotype assay

The 2 mm tip of the tail was collected for genotyping. Genomic DNA was extracted by QuickExtract™ DNA Extraction Solution (Lucigen, #QE09050) according to the manual. PCR was employed for the genotyping. Two different pairs of primers were routinely used to detect the genotypes of every litter of transgenic mice. The primer sequences were listed in Supplementary Table III. 2 μl DNA templates were used for PCR examination. The amplification was performed in 20 μl volume system by using 2×Power Taq PCR MasterMix (BioTeke, # PR1702) at the...
following conditions: 4 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C (denaturation), 45 seconds at 63°C (annealing), 30 seconds at 72°C (elongation), and 5 minutes at 72°C (reading). PCR products were separated on a 1.5% agarose gel, stained with Ethidium bromide (EtBR), and photographed for analysis. STAT3\textsuperscript{flox} was amplified in a 187-bp fragment. STAT3\textsuperscript{+} was amplified in a 146-bp fragment. Cx3Cr1\textsuperscript{cre} was amplified in a 380-bp fragment. Cx3Cr1\textsuperscript{-} was amplified in a 302-bp fragment.

### Neurobehavioral tests

The Mouse Motor and Sensory Scale (mMSS) was employed to evaluate the sensorimotor deficits of SAH mice before the operation and at day 1, 3, 5 and 10 after SAH induction as published previously \(^3\)\(^5\). The sensorimotor function was graded on a scale of 5-27 (27 as normal score and 5 as maximal deficits score). The scale was a composite of the motor (0-12) (spontaneous activity, the symmetry of limb movements, climbing, balance) and sensory (5-15) (proprioception, vibrissae, visual, olfactory and tactile responses) (Supplementary Table IV). In terms of the severity of the injury, the lower score indicated the more serious brain injury symptoms and vice versa.

Gait analysis was performed by computer-assisted Catwalk XT (Noldus Information Technology, Wageningen, Netherlands) test (Datto et al., 2016). The catwalk system consisted of a glass walkway with an internal light source, an inverted camera, and computer software. The light through the glass plate could be reflected when the animal’s paws were in contact with it. The images of footprints were captured by the inverted camera and then converted into digital signals by the software. Mice were trained for 7 days before the experiment to ensure the mice reaching the same criteria walking unforcedly through the walkway without interruptions or...
hesitation. Gait assessments were conducted at days 1, 3, 5 and 10 after SAH. In a darkened environment, the footprints along 1.3-meter-long glass plate were recorded simultaneously when mice walked through the walkway. A large amount of spatial and temporal gait parameters related to individual paws were generated and analyzed (Supplementary Table V).

Mice were then trained to learn to escape on hidden platform conditions, depending on the spatial cues. The platform was placed in the center of one quadrant of the tank and submerged 2 cm beneath the water surface. The platform remained in the same position throughout the training trials. The Intertrial interval (ITI) is 30 seconds to diminish the quadrant preference. Briefly, mice were placed facing the tank wall on a hidden condition from pseudo-randomly selected 4 quadrants Northwest (NW), Southwest (SW), Southeast (SE), and Northeast (NE). Mice were allowed to swim freely for maximal 60 seconds to find the platform, followed a 5 seconds rest on the platform as a trial. On D1 post- SAH, mice were trained for 5 days with 4 trials per day. Escape latency, moved distance and swimming velocity of each training trial were recorded and analyzed. The shorter latency indicated better spatial learning ability.

To detect the memory function, a probe test was conducted 24 h after the last training trial. The platform was removed from the tank during the probe test, and the mouse was allowed to swim freely for 60 seconds. The time mice spent in platform quadrant and platform area and the distance in platform quadrant was recorded of probe trial. After removal from the tank, mice were manually dried with paper towels and warmed under an infrared light before placed back to home cages. All tests were performed at roughly the same time every day to minimize the variability in performance due to time of day.
**Immunohistochemistry**

Immunohistochemistry (IHC) was performed to examine neurons and microglia. The 5 µm paraffin brain sections were used. After a xylene/ethanol dewax-rehydration series, the microwave antigen retrieval was performed in citrate buffer for 20 minutes. Endogenous peroxidase activity was quenched with 0.3% Hydrogen peroxide (H$_2$O$_2$) after primary antibodies incubation. The brain sections were then incubated for 1 hour with blocking buffer comprising 2.5% goat serum, 1% Bovine serum albumin (BSA) and 0.1% Triton-100. The primary antibody NeuN (1:400; Millipore, clone A60, #MAB337) or Iba1 (1:200; Abcam, #ab5076) was applied subsequently at 4 °C overnight. Envision+System-Horseradish peroxidase (HRP) secondary antibody was applied for 1 hour at room temperature. Diaminobenzidine (DAB) was utilized for visualization of colorimetric reaction. Three random fields were examined on CAPS, Hippocampus (The CA1 region of the hippocampus was selected for analysis), and M1 cortex respectively of each mouse under Microscope (Leica) at 40X magnification. The define of neuronal loss was used cell counting methods, NeuroN+ cells in 3 continue filed of one location were recorded. The survived neurons were quantified by Image-Pro software.

**Immunofluorescence**

Immunofluorescence was utilized to define the microglial polarization after SAH. Mice were cardinally perfused with saline followed by 10% buffered formalin at each time point. The brain samples were immersed in the gradient concentration of sucrose solution from 15% to 30% for dehydration, followed by embedding in the Optimal cutting temperature (OCT) compound for cryosection. The frozen sections were immunolabeled with primary antibodies including CD 68 (1:400; Biorad, clone FA-11, #MCA1957), CD16/32 (1:200; BD Biosciences, #553141), and
CD206 (1:500; R&D, MMR, #AF2535), at 4 °C overnight. The sections were subsequently stained
with fluorescence-conjugated secondary antibodies, including Donkey anti Rat Donkey DyLight
680 IgG H+L (1:200; Invitrogen, #SA5-10030), anti-Goat Alexa Fluor® 647 IgG H+L (1:200;
Invitrogen, #A21447), and Donkey anti-Rat Alexa Fluor® 488 IgG H+L (1:200; Invitrogen,
#A21208), at room temperature for 2 hours. After washed thoroughly with Phosphate-buffered
saline (PBS) buffer, slides were then mounted with 4′,6-diamidino-2-phenylindole (DAPI)
(Abcam, #ab104139). Immunofluorescent images were acquired by using confocal microscope
(Zeiss, # LSM880). Quantification of M1/M2 microglial phenotype were performed on three
randomly selected high power microscopic fields across three sections.

Supplemental tables
**Primer sequences for the factors of STAT3 signaling pathway**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5' to 3')</th>
</tr>
</thead>
</table>
| STAT3 | Forward: GAACCTCCAGGACGACTTTGA  
Reverse: GCTCACTCAAAATGCTTCTCC |
| SOCS3 | Forward: ACCAGCGCCACTTCTTCACG  
Reverse: GTGGAGCATCATACTGATCC |
| JAK2 | Forward: GCAGCAAGCATGATGAGTC  
Reverse: CAACTGCTTAGCCACCTCA |
| NF-Kb | Forward: AACACTGGAAGCAGGATGA  
Reverse: CTGGCGGATGATCTCCCTTCTC |
| Src | Forward: CGGTTACATCCCAAGCAACTA  
Reverse: TGTGGTCTCCTACTCCTCCTCA |
| GAPDH | Forward: GAGAGTGTTTCCTCGTCCCG  
Reverse: ACTGTGCCGTTGAATTTGCC |

**Supplementary Table I.** Primer sequences for the factors of STAT3 signaling pathway.
### Primer sequences for M1/M2 microglial markers and cytokines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 markers</td>
<td></td>
</tr>
<tr>
<td>CD16</td>
<td>Forward: TTCTGCTGCTGTTGGCTTTTGTC&lt;br&gt;Reverse: GGGTTGTGGGTCCTTCCTCGC</td>
</tr>
<tr>
<td>CD32</td>
<td>Forward: GCCGTGCTAAATCTTGGCTGTGCTG&lt;br&gt;Reverse: TGTCAGTGTACCGTGTTCTTCC</td>
</tr>
<tr>
<td>CD86</td>
<td>Forward: GCAGCAGGACTTGAACAAC&lt;br&gt;Reverse: TTGTAATGGGCACGGCAGA</td>
</tr>
<tr>
<td>M2 markers</td>
<td></td>
</tr>
<tr>
<td>CD206</td>
<td>Forward: GTCAGAACAGACTGCCTGGA&lt;br&gt;Reverse: AGGGATCGCCTGTTCCTCCAG</td>
</tr>
<tr>
<td>CD163</td>
<td>Forward: CACTGGCTCTGCTATTACCG&lt;br&gt;Reverse: TTCTTTGTCGGCTCTGGTTGTC</td>
</tr>
<tr>
<td>TREM2</td>
<td>Forward: TGGTGAGGAGTGGCTGGAGGAC&lt;br&gt;Reverse: AGGTGGTGGGAAGAGGTGC</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: GCTGGAGTCACAGAAGGAGTGGC&lt;br&gt;Reverse: GCCATAACGCACATGCTTTGCGGA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: GACGTGGAAGTGGGCAGAAGAG&lt;br&gt;Reverse: TGCCACAAGCAGAATGAGA</td>
</tr>
<tr>
<td>Anti-inflammatory cytokines</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Forward: GAGACTTTTCGGGCTTTTCG&lt;br&gt;Reverse: TGCTTTTAGGGCTTCCAGGA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward: CGAGGCAGATTTGCAGGTA&lt;br&gt;Reverse: CGGCTGGACTGTTGTGACT</td>
</tr>
<tr>
<td>Internal control</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: GAGAGTGTTTCTCCTGTCCTCG&lt;br&gt;Reverse: ACTGTGCCGTTGAAATTTGCC</td>
</tr>
</tbody>
</table>

Supplementary Table II. Primer sequences for M1/M2 microglial markers and cytokines.
### Primer sequences for transgenic mice genotyping

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3^{lox} or STAT3^{+}</td>
<td>Forward: TTG ACC TGT GCT CCT ACA AAA A</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCC TAG ATT AGG CCA GCA CA</td>
</tr>
<tr>
<td>Cx3Cr1^{cre} or Cx3Cr1^{+}</td>
<td>Cx3cr1 forward: CCT CAG TGT GAC GGA GAC AG</td>
</tr>
<tr>
<td></td>
<td>Cre forward: GAC ATT TGC CTT GCT GGA C</td>
</tr>
<tr>
<td></td>
<td>Common reverse: GCA GGG AAA TCT GAT GCA AG</td>
</tr>
</tbody>
</table>

**Supplementary Table III.** Primer sequences for transgenic mice genotyping.
<table>
<thead>
<tr>
<th>Function</th>
<th>Motor (5 minutes open field)</th>
<th>Activity</th>
<th>No movement</th>
<th>Moves, no walls approached</th>
<th>1-2 walls approached</th>
<th>3-4 walls approached</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limb symmetry (suspended by tail)</td>
<td>Left forelimb, no movement</td>
<td>Minimal movement</td>
<td>Abnormal forelimb walk</td>
<td>Symmetrical extension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Climbing (on inverted metal mesh)</td>
<td>Fails to hold</td>
<td>Hold &lt; 4 seconds</td>
<td>Holds, no displacement</td>
<td>Displaces across mesh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Balance</td>
<td>Falls &lt; 2 seconds</td>
<td>Falls &gt; 2 seconds</td>
<td>Holds, no displacement</td>
<td>Displaces across rod</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensory</th>
<th>Proprioception (cotton tip to both sides of neck)</th>
<th>No reaction</th>
<th>Asymmetrical head turning</th>
<th>Symmetric head turning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vibrissae (cotton tip to vibrissae)</td>
<td>No reaction</td>
<td>Asymmetrical head turning</td>
<td>Symmetric head turning</td>
</tr>
<tr>
<td></td>
<td>Visual (tip toward each eye)</td>
<td>No reaction</td>
<td>Unilateral blink</td>
<td>Bilateral blink</td>
</tr>
<tr>
<td></td>
<td>Olfactory (lemon juice on tip)</td>
<td>No sniffing</td>
<td>Brief sniff</td>
<td>Sniff &gt; 2 seconds</td>
</tr>
<tr>
<td></td>
<td>Tactile (needle stick to palm)</td>
<td>No reaction</td>
<td>Delayed withdrawal</td>
<td>Immediate withdrawal</td>
</tr>
</tbody>
</table>

* Mouse Motor and Sensory Scale is was combined from 2 prior scales: Garcia (1995) and Crawley (1999&2000)

**Supplementary Table IV.** Mouse Motor and Sensory Scale (mMSS). mMSS was employed to evaluate the sensorimotor function of SAH mice. Sensorimotor function was graded on a scale of 5-27 (27 as normal score and 5 as maximal deficits score). The scale was a composite of motor (0-12) (spontaneous activity, symmetry of limb movements, climbing, balance) and sensory (5-15) (proprioception, vibrissae, visual, olfactory and tactile responses) (Du et al., 2016)
### Definitions of gait parameters

<table>
<thead>
<tr>
<th>Gait parameters</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Static gait parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Max Contact Area (cm²)</td>
<td>The maximum area of the left hind paw that comes into contact with the glass plate</td>
</tr>
<tr>
<td>Max Intensity</td>
<td>The maximum intensity of a paw</td>
</tr>
<tr>
<td>Mean Intensity</td>
<td>The mean intensity of the complete paw</td>
</tr>
<tr>
<td>Max Contact Max Intensity</td>
<td>The maximum intensity of the paw at Max Contact</td>
</tr>
<tr>
<td>Max Contact Mean Intensity</td>
<td>The mean intensity of the paw at Max Contact</td>
</tr>
<tr>
<td><strong>Static gait parameters</strong></td>
<td></td>
</tr>
<tr>
<td>Run Duration (s)</td>
<td>The duration in seconds of the recorded run</td>
</tr>
<tr>
<td>Stride Length (cm)</td>
<td>The distance between successive placements of the same paw</td>
</tr>
<tr>
<td>Stand (s)</td>
<td>The duration in seconds of contact of a paw with the glass plate</td>
</tr>
<tr>
<td>Swing (s)</td>
<td>The duration in seconds of no contact of a paw with the glass plate</td>
</tr>
<tr>
<td>Swing Speed (mm/s)</td>
<td>The speed of a paw during Swing phase. Swing speed = Stride length / Swing</td>
</tr>
<tr>
<td>Step Cycle (s)</td>
<td>The time in seconds between two consecutive initial contacts of the same paw. Step Cycle = Stand + Swing</td>
</tr>
</tbody>
</table>

* The mean values of each parameters during a complete trial are used for the analysis

**Supplementary Table V.** The definition of gait parameters in Catwalk gait analysis.
Supplementary Table VI. Genotype analysis and sex distribution of F2 transgenic hybrids crossed by STAT3\(^{\text{flox/flox}}\) Cx3Cr1\(^{-/-}\) and STAT3\(^{\text{flox/+}}\) Cx3cr1\(^{\text{cre/-}}\) mice.
Supplementary Figure I. The time-course gait analysis of STAT3 mice after SAH. A, The static gait parameters included the Mean intensity, Max contact max intensity, and Max contact mean intensity of paws were assessed. B, The dynamic gait parameters included Run duration, Stride length, Stand, Swing, Swing speed, and Step cycle. Microglial STAT3 deletion significantly improved the static rather than dynamic gait function in SAH. n=8-16 per group. Values were the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Supplementary Figure II. Immunofluorescent examination of M1/M2 microglial polarization in STAT3 KO mice after SAH. The representative confocal microscopic images showed the visualization of CD16/32 (M1, Green), CD206 (M2, red) and DAPI (Nuclei, blue) co-expression in M1 Cortex and hippocampus of STAT3 KO and control groups of mice at 1 day after SAH. n=5-6 per group. Bar=20μm.
**Supplementary Figure III.** Real time PCR analysis of microglial polarization in STAT3 KO mice after SAH. **A-B.** The M1 microglial markers including CD 16, CD32, and CD86, and the M2 microglial markers including CD 206, CD163, and TREM2 were detected at 1 and 5 days after SAH. The mRNA expression of M1/M2 microglial markers was shown in the fold change compared to the control. n=3 per group. Values were the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Supplementary Figure IV. The NeuN IHC assessment of neuronal loss in the CAPS, M1 cortex and hippocampus after experimental SAH. (A, C, E)- Time course representative NeuN staining of coronal brain sections in the CAPS, M1 cortex and Hippocampus of SAH and Sham mice. Bar=50µm. (B, D, F)- Quantification of neuronal loss in CAPS, M1 cortex and Hippocampus of SAH and Sham mice (n=6-7/group/time point). Values were the mean ± SEM. *P≤0.05, **P≤0.01, ***P≤0.001.