**BRAIN INJURY**

**Formyl peptide receptor 1 signaling potentiates inflammatory brain injury**

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Acute brain insults elicit pronounced inflammation that amplifies brain damage in intracerebral hemorrhage (ICH). We profiled perihematomatous tissue from patients with ICH, generating a molecular landscape of the injured brain, and identified formyl peptide receptor 1 (FPR1) as the most abundantly increased damage-associated molecular pattern (DAMP) receptor, predominantly expressed by microglia. Circulating mitochondrial N-formyl peptides, endogenous ligands of FPR1, were augmented and correlated with the magnitude of brain edema in patients with ICH. Interactions of formyl peptides with FPR1 activated microglia, boosted neutrophil recruitment, and aggravated neurological deficits in two mouse models of ICH. We created an FPR1 antagonist T-0080 that can penetrate the brain and bind both human and murine FPR1. T-0080 attenuated brain edema and improved neurological outcomes in ICH models. Thus, FPR1 orchestrates brain inflammation after ICH and could be targeted to improve clinical outcomes in patients.

**INTRODUCTION**

Acute brain injuries encompass traumatic brain injury, acute ischemic stroke, and intracerebral hemorrhage (ICH). ICH is among the severest forms of acute brain injury, causing approximately 2.8 million deaths across the world annually (1). ICH survivors often suffer from disability due to irreversible neurological injury and lack of effective therapies (2). In ICH, rapid neural cell death and release of damage-associated molecular patterns (DAMPs) trigger neuroinflammation, which occurs within minutes to hours after ictus; activation of brain glia cells; infiltration of peripheral immune cells; and release of cytokotic factors, reactive oxygen species, matrix metalloproteinases, tumor necrosis factor–α (TNF-α), and other cytokotins within the brain (3, 4). Consequently, edema surrounding the hematoma is formed, leading to secondary brain ischemia. The perihematoma edema (PHE) exacerbates cell death, damages blood-brain barrier (BBB) integrity, and disrupts brain homeostasis after primary brain injury (5). Over time, brain inflammation disseminates to distal regions after ICH and contributes to long-term neurological deficits such as impaired cognition (6, 7).

Over decades, clinical trials targeting hematoma such as surgical evacuation, blood clot aspiration, and iron chelation have yet to produce substantial benefits for patients with ICH (8–10). Increasing clinical evidence demonstrate that PHE is associated with poor clinical outcomes and currently is considered a therapeutic target for ICH (5). However, attempts to attenuate brain edema via hyperosmolar therapy with tissue dehydrating agents (mannitol) or glyburide have yielded disappointing results on brain swelling (11, 12). Conversely, proof-of-concept studies suggest that blocking immune cells homing to the injured brain via sphingosine-1-phosphate receptor antagonist, fingolimod, may reduce PHE and confer benefits for patients with ICH (13). The definitive effects of this approach are currently being tested in a large international trial with siponimod (a selective sphingosine-1-phosphate receptor 1 modulator) (NCT03338998).

In ICH, as in many other acute brain injuries, the prevailing perception is that inflammation is triggered within the brain via cell death debris and further amplified by contribution from peripheral cells invading the central nervous system (CNS); however, this paradigm is derived primarily from animal studies with unknown relevance in patients. The emergency of patients with ICH management hinders brain sample access and research in comprehending the constellation of triggers, mediators, and key molecular events involved in the genesis and propagation of brain inflammation. As a result, limited attempts have been made to curb brain inflammation by precisely targeting brain intrinsic molecular pathways that cause edema and neurological defects. To this end, we performed an unbiased genome-wide transcript sequencing of human brain perihematomatous tissue surgically removed from patients with ICH. We identified formyl peptide receptor 1 (FPR1) as a master gene in triggering brain inflammation. We developed an FPR1 agonist and demonstrate its efficacy in preclinical models.

**RESULTS**

ICH increases the expression of FPR1 preferentially in microglia in patients with ICH

To probe the molecular events in brain edema after ICH, we performed transcriptome sequencing of 11 perihematomatous tissues from the basal ganglia region of patients with ICH who were subjected to craniotomy surgery for removing brain hematoma within 24 hours after ictus (fig. S1A). These tissues were obtained by neurosurgeons, not by biopsy. In contrast to a previous report of six healthy controls (phs000424.v8.p2), perihematomatous tissue developed a distinct gene expression pattern (Fig. 1, A and B). At 6 hours after ICH, a total of 1834 differentially expressed genes (DEGs) in the perihematomatous tissue were identified, and this difference gradually increased to 2330 in the elapsed period to 24 hours after ICH (Fig. 1C). The divergent gene profiles between control and patients with ICH

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Fig. 1. The expression of FPR-1 and release of its mitochondria-derived ligands in brain and blood from patients with ICH. (A) A representative T2/Flair MR image denotes a hematoma and a perihematomal region in the right hemisphere of the brain from patients with ICH. (B) The cluster dendrogram of the gene expression profiles of basal ganglia tissue at 6 hours (n = 6) or 24 hours (n = 5) after ICH, compared to control (n = 6). (C) Comparisons of the DEGs in basal ganglia tissues among 6 and 24 hours after ICH and controls. Each number indicates the unique or common DEGs between comparisons. (D) Selected top 10 Gene Ontology enrichment in basal ganglia tissue at 6 and 24 hours after ICH versus control. (E) The expression profile of human DAMP receptors in basal ganglia tissue at 6 hours (n = 6) and 24 hours (n = 5) after ICH and control (n = 6). (F) Immunostaining and quantification of FPR1 expression in microglia (TMEM119+ cells), astrocytes (GFAP+ cells), and neurons (NeuN+ cells) in human perihematomal tissue at 6 hours after ICH and control. Scale bar, 20 µm. n = 10 per group, unpaired two-tailed t test. (G) Quantification of circulating mitochondrial N-formyl peptides (FPR1 ligands) in blood at 6 hours (n = 50), 24 hours (n = 80), and 72 hours (n = 36) after ICH in patients and controls (n = 47) by ELISA. One-way ANOVA. (H) Pearson correlation coefficient (r) and P value (P) between blood mitochondrial N-formyl peptides and relative PHE (PHE/hematoma) at 24 hours after ICH in log2 (n = 80). (I) Pearson correlation coefficient (r) and P value (P) between blood mitochondrial N-formyl peptides and modified Rankin score at 3 months after ICH in log2 (n = 30). Blood samples were collected at 24 hours after ICH. Data are presented as means ± SEM, **P < 0.01.
Peripheral emergence of mitochondria-derived N-formyl peptide and its association with expansion of brain edema in patients with ICH

After acute brain insult, dying cells release endogenous molecules such as mitochondrial DNA, S-100B, high mobility group box protein 1 (HMGB1) cytokines, and chemokines to the surrounding extracellular space and into the circulation (17–19). N-formyl peptides are ligands for FPR1 (20). We found that circulating mitochondrial N-formyl peptides remained at baseline concentration 6 hours after ICH. Concentrations of N-formyl peptides escalated to a peak at 24 hours and descended back to baseline at 72 hours (Fig. 1G). We noted a strong correlation between the dynamic concentrations of circulating mitochondrial N-formyl peptides and the progression of perihematomal edema after ICH (r = 0.52, P < 0.001; Fig. 1H). In addition, blood mitochondrial N-formyl peptide content after ICH also correlated to the modified Rankin scale (mRS) of patients with ICH at 3 months after disease onset (r = 0.51, P < 0.001; Fig. 1I).

FPR1 and N-formyl peptide interaction acts on microglia and dictates the magnitude of brain inflammation in ICH models

To dissect the role of FPR1 in ICH, we first assessed its expression in the perihematomal tissue of mice after ICH induced by autologous blood injection (21). To identify the major FPR1-expressing cells in the nascent PHE during the early stage of brain inflammation (5), we analyzed the expression of FPR1 in the perihematomal tissue at 0 and 6 hours after ICH. In brain intrinsic cells, the expression of FPR1 was primarily in microglia, whereas FPR1-positive astrocytes, neurons, neutrophils, and macrophages were minimal at 6 hours after ictus (Fig. 2A). This finding mirrors those in human (Fig. 1F).

Next, we generated Fpr1 knockout mice by CRISPR-cas9-mediated genome engineering (fig. S1, D to F). Fpr1 knockout did not affect immune cell genesis, brain homeostasis, or body weight of young adult mice (2 months old). However, spontaneous bacterial skin infections were more frequently found in older mice (8 months old) but not in young adult mice (2 to 3 months old) (fig. S1, G to J); this did not affect our experiments because only young mice were used. Upon autologous blood ICH induction, Fpr1−/− mice displayed reduced inflammatory profile compared to controls at 24 hours (Fig. 2, B and C). Enzyme-linked immunosorbent assay (ELISA) cytokine assay indicated a decrease in concentrations of interleukin-1β (IL-1β), IL-6, and TNF-α in the lysate of perihematomal tissue in Fpr1−/− mice compared to wild type at 24 hours after ICH (Fig. 2D).

The overwhelming expression of FPR1 on microglia points to their primary response to ICH injury and prime immune effectors (22). To test whether FPR1 ligands can activate microglia in vivo, we analyzed the phenotype of brain microglia after injecting an FPR1 agonist, N-formyl-I-methionyl-l-leucyl-phenylalanine (fMLF), into the striatum of the ipsilateral hemisphere of wild-type sham mice. We found that microglial expression of IL-1β, IL-6, TNF-α, and CD68 was increased in the ipsilateral hemisphere but not in the contralateral hemisphere (Fig. 2E). In addition, fMLF induced an up-regulation of inducible nitric oxide synthase (iNOS) and matrix metalloproteinase 9 (MMP9) in microglia (fig. S2, A and B). These results suggest that stimulation of FPR1 activates microglia predominantly in the ipsilateral hemisphere.

FPR1 directs neutrophil brain homing and exacerbates injury of ICH brain

Having determined FRP1+ microglia as a prompt responder to ICH, we went on to investigate how FPR1 influences ICH in two rodent models (Fig. 3 and fig. S3). Compared to wild-type mice, Fpr1−/− mice had reduced perihematomal edema and diminished brain water content in the ipsilateral hemisphere (Fig. 3, A and B). Fpr1−/− mice displayed improved neurological outcomes at days 1 and 3 after ICH (Fig. 3C). In collagenase-induced ICH, FPR1 knockout mice similarly exhibited diminished PHE and improved neurological deficits (fig. S3, A to C).

To elucidate the mechanism underlying the detrimental role of FPR1 on ICH outcome, we quantified brain-infiltrating cells and found that Fpr1−/− mice had reduced neutrophil infiltration (reduced by 41% when normalized to wild-type mice), whereas counts of hematogenous monocyte-derived macrophages and T and B cells were not altered (Fig. 3D and fig. S4). The neutrophil counts of peripheral blood or femur bone marrow were similar between wild-type and Fpr1−/− mice under physiological conditions, whereas ICH resulted in an over twofold increase of blood neutrophil in wild-type and Fpr1−/− mice at 24 hours after ICH (fig. S2, C and D). The circulating neutrophil count per milliliter of blood was reduced by 18% in Fpr1+ ICH mice compared to wild-type ICH mice at 24 hours after ICH (fig. S2D). Because the counts of neutrophils are similar in bone marrow of Fpr1+ and wild-type mice, it is reasonable to infer that neutrophil generation is not involved in the reduced neuroinflammation seen in Fpr1−/− mice. The reduction of circulating neutrophils in Fpr1−/− mice after ICH may result from
the smaller ICH lesion in Fpr1−/− mice compared to wild-type mice, leading to dampened mobilization of peripheral neutrophils.

We then assessed the cytokine/chemokine profile of the perihematoma tissue lysates from wild-type and Fpr1−/− ICH mice to determine factors that drive neutrophil homing into the brain (Fig. 3E and fig. S2E). We found that IL-1α is the most abundant of 111 cytokines/chemokines at 6 hours after ICH (Fig. 3E; the top five cytokines/chemokines are shown), when neutrophils start to enter the injured brain (22). FPR1 knockout largely reduced the expression of IL-1α in perihematomatous tissue (Fig. 3E). The expression of IL-1β, IL-6, and TNF-α was relatively low in both wild-type and Fpr1−/− mice at 6 hours after ICH (fig. S2E). We further found that IL-1α was mostly expressed by microglia from perihematoma tissue in ICH mice compared to wild-type mice (fig. S5, A to C). In situ immunofluorescence staining showed that there was reduced cell loss in perihematoma tissue of mice treated with anti-mouse IL-1α monoclonal antibody (mAb) compared to controls (fig. S5, D and E).

**Design and characterization of BBB-permeable FPR1-selective antagonist T-0080 for clinical translation**

Presently, no BBB-permeable FPR1 antagonist exists. To create a selective FPR1 antagonist, we conducted computer-aided drug design using a virtual ligand screen based on compound docking to the binding pocket of human FPR1 as previously reported (25, 26). Among the 2,000,000 compounds screened, 18 hits were found. One compound showed the ability to inhibit fMLF-induced human FPR1 activation with a half-maximal inhibitory concentration of 6.7 μM (Fig. 4A, compound 1). We optimized compound 1 to improve its inhibitory activity and BBB permeability (referred to predicted brain/blood partition coefficient, “−3.0 to 1.2”) by modifying its branches noncovalently bound to FPR1. Last, we generated compound T-0080 (named using its testing order; fig. S6). T-0080 had an inhibitory concentration of 50 at 0.85 μM to human FPR1 when
treated with 10 nM fMLF (Fig. 4E). This compound is among the most potent FPR1 antagonist compared to prior reports (27). The mass spectrometry, artificial synthesis process, and binding of T-0080 to human FPR1 are shown (Fig. 4, B and C, and fig. S7A). At a concentration of 1 μM, T-0080 can inhibit fMLF-induced activation of human FPR1 (Fig. 4, D and E). Human and mouse FPR1 showed a conserved binding pocket and high homology (Fig. 4C and fig. S7, A and B). Our data showed T-0080’s ability of binding to and inhibiting mouse FPR1 at micromolar concentrations (fig. S7, C and D). In cultured mouse microglia, T-0080 inhibited FPR1-microglia–induced neutrophil migration and suppressed the production of IL-1β, IL-6, and TNF-α induced by exposure to FPR1 agonist fMLF (fig. S7, E to H). Such suppression was not evident after exposure to TLR4 agonist (lipopolysaccharide) (fig. S7, F to H). In addition, T-0080 showed no short-term toxicity in wild-type mice at a single dosage up to 2000 mg/kg given orally (fig. S7, I and J).

To examine its accessibility to brain, we measured the pharmacokinetics of T-0080 in the plasma and brain tissue of mice from 5 min to 6 hours after intraperitoneal administration at 5 mg/kg. We found that T-0080 had a stable molecular structure in plasma and brain tissue in mice (Fig. 4F). The peak concentration of T-0080 was found at 5 min after intraperitoneal administration (Fig. 4G).
T-0080 had a brain plasma ratio at 0.3 to 1.2 within 6 hours after intraperitoneal injection (Fig. 4G), indicating that T-0080 could cross the BBB (brain plasma ratio of 0.3 to 0.6) in vivo.

**T-0080 attenuates brain injury in two experimental models of ICH**

We evaluated the effects of T-0080 on brain injury and inflammation with two mice models of ICH, induced by autologous blood or collagenase injection (Fig. 5 and fig. S8). Mice were injected with T-0080 (5 mg/kg daily) or vehicle intraperitoneally, 1 hour after ICH in the two models. Administration of T-0080 reduced PHE size (reduction by 35%) and total brain water content (reduction by 2.7%) at day 1 (Fig. 5, B and C); neurological outcomes were improved from day 1 to day 14 after ICH in treated wild-type mice (Fig. 5D). The total number of brain-infiltrated neutrophil was also reduced by 47 and 54% at days 1 and 3 after ICH (Fig. 5E). After receiving T-0080 at 24 hours after ICH, mice had reduced neuro deficits from day 3 to day 14 (fig. S8A). Hematoma absorption was not altered in mice that received T-0080 injection starting from 1 hour after ICH (fig. S8, B and C). In the ICH mouse model induced by collagenase injection, T-0080 had similar effects on brain injury and neutrophil infiltration (fig. S8, D to G). Together, these findings showed that T-0080 had therapeutic effects in vivo in ICH preclinical models.

**DISCUSSION**

By profiling brain tissues surgically removed from patients with ICH, we were able to compile the emergent molecular landscape in the acute phase after ictus. ICH swiftly and profoundly alters the transcriptomic profile of the affected brain regions. Specifically, most genes and signaling pathways engaged in cell death and immune response became up-regulated, reaching maximum expression in tissue surrounding the hematoma in as early as 6 hours, and such alterations persisted throughout the formation of brain edema and subsequent expansion. This landscape shows that brain insults by hematoma after ICH provoke immune reactions by up-regulating DAMP receptors FPR1, TLR1, TLR2, P2RX4, and P2RX6. The role for aforementioned receptors has been described in sterile inflammatory conditions including systemic inflammatory response syndrome, hepatic injury, renal ischemia/reperfusion injury, neuropathic pain, and renal cancer (28–32). However, generalization of other findings...
Infiltrates, especially of neutrophils, and neurological deficit exhibitous ligands of FPR1 after ICH. A large number of patients with ICH mirrors the release of endogenous mitochondrial N-formyl peptides from blood mitochondria-derived N-formyl peptides could also activate microglia cells in vivo (fig. S9). The elevated blood mitochondria-derived N-formyl peptides could also activate circulating FPR1+ immune cells such as neutrophils and monocytes. In addition, exogenous bacterial N-formyl peptides may activate FPR1+ cells after ICH because they release cytotoxic factors such as reactive oxygen species and matrix metalloproteinase 9, which exacerbate cell death and BBB breakdown in ICH (33). Our investigation has revealed that microglia attract neutrophils to perihematomatous tissues via FPR1-mediated expression of IL-1α. Different from previous findings of sterile inflammatory conditions in the periphery such as focal hepatic necrosis, pulmonary fibrosis, and breast cancer (20, 34, 35), this study offers a deep mechanistic insight into central nervous system inflammation, in which brain-resident microglia are the predominant cellular component expressing FPR1 shortly after ICH in humans and mice. Switching on FPR1 leads to microglial production of IL-1α that guides neutrophils into perihematomatous tissues and exacerbates PHE. Therefore, multiple lines of solid evidence collected from both patients with ICH and two ICH models point toward FPR1 as a locus of secondary injury in ICH and suggest that the mechanism of action relies on microglia’s ability to boost cellular infiltrates and the focal inflammatory milieu (fig. S9).

In addition to microglia, FPR1 is also expressed on neurons, astrocytes, and other invading immune cells, albeit in much lower amount, in the brain shortly after ICH onset. It is possible that the gross effects of FPR1 on the clinical manifestation of ICH and benefit of T0080 may also be achieved, at least in part, via its effects on these cell types. Besides IL-1α, CCL5, and CCL12, other immune modulatory proteins were also reduced in peripheral naïve immune cells and reduced in Fpr1−/− mice after ICH (36). A recent study shows that FPR1 promotes transcriptional activity of IL-1α in ICH (40). The elevated blood mitochondria-derived N-formyl peptides could also activate circulating FPR1+ immune cells such as neutrophils and macrophages. In addition, exogenous bacterial N-formyl peptides may activate FPR1+ cells after ICH because they release cytotoxic factors such as reactive oxygen species and matrix metalloproteinase 9, which exacerbate cell death and BBB breakdown in ICH (41). Thus, FPR1+ cells may synergistically act on brain injury with other factors such as thrombin after ICH.

Lack of effective therapies for ICH has prompted us to pursue the translational potentials of our findings. This motivation is further boosted by our observation that circulating mitochondria-derived ligands for FPR1 N-formyl peptide are strongly correlated with expansion of brain edema of a large number (80 patients) of

Fig. 5. Administration of T-0080 attenuates brain injury and neuroinflammation in ICH mice. (A) Schematic of experimental T-0080 administration, brain injury, and neuroinflammation assessment in ICH mice. (B) MR images (7 T) and quantification of lesion volume and PHE volume at 24 hours after ICH (n = 15 mice per group). Unpaired two-tailed t test. (C) Brain water content at 24 hours after mouse ICH model treated with T-0080 or vehicle (n = 15 mice per group). Unpaired two-tailed t test. (D) Neurological scores of ICH mice treated with T-0080 or vehicle at days 1, 3, 7, and 14 after ICH (n = 15 mice per group). Two-way ANOVA. (E) The number of brain-infiltrating neutrophils at days 1 and 3 after ICH (n = 15 mice per group). Two-way ANOVA. Data are presented as means ± SEM. *P < 0.05 and **P < 0.01.
patients with ICH. We screened 2 million compounds and ultimately created a BBB-permeable FPR1 antagonist, T-0080. The properties of T-0080, such as being BBB permeable and being able to effectively bind to and inhibit both human and mouse FPR1 at micromolar concentrations, compared to existing compounds (42) make it a tool drug for the preclinical study of ICH. In both autologous and collagenase models for ICH, T0080 administration reduced neutrophil and BBB leakage and improved neurological deficits. These results not only support the critical role for FPR1 in ICH but also suggest that T0080 is a viable drug foundation for ICH.

An alternative approach is to block the downstream factors of FPR1 such as IL-1α. A clinical trial blocking IL-1 receptor is ongoing with the aim to reduce inflammation and edema in patients with ICH (NCT03737344). In addition, in context with preliminary and yet promising means to intervene brain inflammation through blocking cell egress to the CNS as with fingolimod and/or siponimod, this work offers a new approach to tune brain edema within the brain. Combination of the two approaches may offer more benefits for patients (3, 7).

This study has several limitations. First, the expression profile of FPR1 in human perihematomal tissues at the later stage of ICH is still unclear, as brain tissues around hematoma can only be acquired within 24 hours after ICH onset. Second, FPR1-expressing myeloid cells from periphery such as neutrophils and monocytes can be recruited into injured brain after ICH. Whether FPR1 directly regulates these infiltrating FPR1-expressing cells in ICH injury requires further investigation. Third, assessment of the binding of T-0080 to human or mouse FPR1 is needed to better understand the pharmacological effects of T-0080 on ICH injury.

The characterization of the molecular landscape of brain injury after ICH and the identification of FPR1 as a prominent regulator for inflammatory cascade fundamentally increase our understanding of propagation of neuroinflammation. The screening and subsequent creation of the FPR1 antagonist T-0080 equipped with BBB permeability opens a potential therapeutic avenue not only for ICH but also for other acute brain injuries such as traumatic brain injury and acute ischemic stroke.

**MATERIALS AND METHODS**

**Study design**

The goal of this study was to investigate the molecular mechanisms governing inflammatory brain injury after ICH. To this end, this study used two experimental ICH models in wild-type and Fpr1−/− mice. Power analysis was performed to determine sample size in these experiments involving patients with ICH, ICH mice, and in vitro cell cultures. We collected brain tissues (n = 11) and blood samples (n = 80) from patients with ICH within 72-hour onset, as well as blood samples from healthy individuals (n = 47). We included all patients with ICH in which the mRS score was available in 3 months follow up (n = 30). Bulk RNA-seq was performed to analyze the gene expression profile of perihematomal area in patients with ICH. Blood mitochondrial N-formyl peptides were assessed by ELISA. To determine the role of FPR1 in ICH injury, no less than six mice were included per experimental group for the assessment of neurological deficits, brain edema and hematoma size, immune cell infiltration, cytokine/chemokine expression in the brain, and the effectiveness, pharmacokinetics, and toxicity of T-0080 in vivo. In experiments that use culturing cells to measure neutrophil migration and cytokine expression in microglia, at least six samples were included in each treatment group. Experimental subjects were assigned randomly to all experimental groups. The exact values of sample size (n) are provided in figure legends. Experimental groups, data collection, and data analysis were blinded by using different investigators or masking sample labels. Sample exclusion was done as a result of mouse death after surgery. All experiments presented were successfully reproduced for at least three times.

**Human brain tissue and blood samples**

Collection of human samples was performed according to protocols approved by the institutional review board of Tianjin Medical University General Hospital (Tianjin, China), Tianjin Huanhu Hospital (Tianjin, China), or the Third People’s Hospital of Datong (Shanxi, China). Written informed consent was obtained from each patient or legal surrogate. In the RNA-seq of human brain, 17 ICH cases of brain basal ganglia tissues were included (6 controls: 55 ± 2.6 years, 5 males and 1 female; 11 ICH: 57.3 ± 3.3 years, 9 males and 2 females, means ± SEM; P = 0.233, unpaired two-tailed t test). The ICH cases were acquired from patients with ICH requiring craniotomy surgery to evacuate the hematoma within 24 hours after onset. The sequencing data of six control basal ganglia samples were from previously published resources at NCBI SRA database (phs000424.v8.p2). For the immunostaining of human brain tissues, 10 cases of normal postmortem or ICH human brain basal ganglia sections were included (10 controls: 54.8 ± 3.6 years, 10 ICH: 56.7 ± 3.1 years, means ± SEM; P = 0.3787, unpaired two-tailed t test). Inclusion criteria of controls and patients with ICH were aged 18 years and older. Patients with ICH had a preliminary intracerebral hematoma volume of 50 to 100 ml at basal ganglia measured by computerized tomography (CT). Exclusion criteria of enrolled control and ICH brain cases include tumor, autoimmune diseases, preexisting brain disease and infection, and concomitant use of immunosuppressive or immune-modulating therapies.

Human blood samples were obtained from 47 healthy controls and 80 patients with ICH (controls: 56.7 ± 1.9 years, 27 males and 20 females; ICH: 60.5 ± 1.2 years, 46 males and 33 females, means ± SEM; P = 0.177, unpaired two-tailed t test). All enrolled patients agreed to participate in follow-up mRS scoring [a commonly used stroke ordinal outcome scale with scores ranging from 0 (no symptoms at all) to 6 (dead)] at 3 months after ICH onset. Several inclusion and exclusion criteria were designated for patients with ICH. Enrolled patients with ICH were aged 18 years and older with a preliminary intracerebral hematoma measured by CT. Exclusion criteria include patients with tumor, autoimmune disease, preexisting brain disease and infection, and concomitant use of immunosuppressive or immune-modulating therapies. Control subjects were chosen based on standardized inclusion and exclusion criteria. Inclusion criteria were as follows: (i) subjects were 18 and older; (ii) normal basic laboratory tests; and (iii) normal neurological function on neurological examination. Exclusion criteria were as follows: (i) neurological or psychiatric disorders; (ii) past history of tumor; (iii) past history of drug or alcohol abuse; and (iv) past history of medications including CNS stimulants, antiepileptic drugs, cortisone, and insulin treatment.

**Mice**

Ten- to 12-week-old male mice were used in this study. C57BL/6 mice were purchased from Charles River Laboratories. Fpr1−/− mice...
were made from Model Animal Research Center of Nanjing University. 

**The DEGs and gene ontology analysis**

For data quality control, the sequencing data were filtered with SOAPnuke (v1.5.2) (43) by (i) removing reads containing sequencing adapter; (ii) removing reads whose low-quality base ratio (base quality less than or equal to 5) is more than 20%; and (iii) removing reads whose unknown base (“N” base) ratio is more than 5%; thereafter, clean reads were obtained and stored in FASTQ format (a common format for genome sequencing data). The clean reads were mapped to the reference genome (the Human Genome 19) using HISAT2 (v2.0.4) (44). The clean reads were assembled separately using the Trinity method for each library. Gene expression was measured by counting the number of reads for each gene and was normalized to reads per kilobase of transcript per million mapped reads (FPKM). To compare gene expression variation between two samples, an analysis method based on Poisson distribution was used as previously reported (45), and the false discovery rate (FDR) was preset as less than 0.05 at the beginning of the statistical analysis. Besides the gene’s FDR value, we also calculated the fold changes in gene expression between samples based on their FPKM value. The smaller the FDR value and (or) the greater the multiple of difference, the greater the difference of gene expression. In our analysis, DEGs between two samples are defined as genes with FDR ≤ 0.001, and the fold difference is more than two times. For insight into the change of brain phenotype, gene ontology analysis was performed at https://david.ncifcrf.gov/ after loading the list of DEGs between two groups. Gene ontology terms with a Benjamini and Hochberg (BH)–corrected P value <0.05 were considered significant.

**CRISPR-Cas9–mediated generation of Fpr1−/− mice**

Fpr1−/− mice (C57BL/6 background) were generated using the CRISPR-Cas9 system from Model Animal Research Center of Nanjing University (Nanjing, China). Briefly, single-guide RNAs (sgRNAs) were designed and synthesized as follows (5′–3′): CATGATGGAATGTCCGGCAG; AAGTCCTTCACACTCAAGCT; CTTCAATTGAATGTCCGGCAG; AAGTCCTTCACACTCAAGCT; CTTCAATTGAATGTCCGGCAG. sgRNAs and Cas9 mRNA were microinjected into fertilized embryos of C57BL/6 mice. The embryos were transferred to surrogate mice to obtain F0 offspring. F0 mice were genotyped and were backcrossed with background C57BL/6 mice to obtain F1 heterozygotes. All mice were genotyped at 2 weeks after birth. PCR was performed for genotyping of Fpr1+/−, Fpr1−/−, and wild-type mice, to verify the knockout of Fpr1 in genome. The primer sequence is 5′-TAACCCATCTGTGGCCTTGC-3′ and 5′-GGATGACACAGTGGCATAGCCTCA-3′ with a 0.5-kb PCR product for Fpr1+/− or Fpr1−/− mice. An additional primer sequence of 5′-TAACCCATCTGTGGCCTTGC-3′ and 5′-GGATGACACAGTGGCATAGCCTCA-3′ with a 0.75-kb PCR product for Fpr1+/− or Fpr1−/− mice was also used. Fpr1+/− mice were backcrossed to the C57BL/6 background for 8 to 12 generations and were housed in the pathogen-free facility for breeding and experimental studies.

**ICH models**

ICH was induced in mice by injection of autologous blood or collagenase as previously described (21, 46). Mice were anesthetized using 1 to 3% isoflurane inhalation and fixed on a stereotactic frame. A burr hole was drilled on the right side of the skull at 2.3 mm lateral to midline, 0.5 mm anterior to the bregma. For the autologous blood model, 30 μl of nonheparinized autologous blood was withdrawn from the angular vein. The first 5 μl of blood was injected at...
parameters were as follows: repetition time (TR) = 4500 ms, echo time (TE) = 10 ms, flip angle = 25°, FOV = 32 × 32 × 16 mm³, and image matrix = 256 × 256. The volumes were manually outlined and calculated by multiplying the sum of the volume by the distance between sections (0.5 mm) using medical image processing, analysis, and visualization (MIPAV) software. PHE volumes were calculated as total lesion volume minus hematoma volume. MRI data were analyzed by two investigators blinded to experimental groups.

Quantification of reactive oxygen species generation in the mouse brain after ICH through live bioluminescence images was captured using the in vivo imaging systems (IVIS) Spectrum (Caliper LifeSciences) after intraperitoneal injection of Luminol (200 mg/kg; Sigma-Aldrich, A8511). Signal intensities from the brain were defined and measured in the efficiency mode with the IVIS Spectrum. Data were collected as photons per second per square centimeter using Living Image software (Caliper Life Sciences).

**Brain water content assessment**

Brain water content was measured at day 1 after ICH, as previously described (49). Briefly, without perfusion, brain tissue was removed and divided into three parts: the ipsilateral hemisphere, contralateral hemisphere, and cerebellum. The brain tissues were weighed to measure wet weights and then dried for 24 hours at 100°C to obtain dry weights. The following formula was used to calculate the brain water content: (wet weight − dry weight)/wet weight × 100%.

**Flow cytometry**

Single-cell suspension of blood or brain tissues was prepared and stained with fluorochrome-conjugated antibodies as we previously described (48, 50, 51). Briefly, red blood cells were eliminated from blood samples using a lysing buffer (BD Biosciences) before antibody staining. Brain tissues were digested with 1% collagenase (Sigma-Aldrich) at 37°C for 30 min, and then myelin sheath was removed by density gradient centrifugation in 30% Percoll (Sigma-Aldrich) at 700g for 10 min. All antibodies were stained at 4°C for 30 min following their instruction. For intracellular antigen staining, cells were fixed and permeated with a fixation/permeabilization solution kit (BD Biosciences). Antibodies were directly labeled with one of the following fluorescent tags, unless stated otherwise: fluorescein isothiocyanate, phycoerythrin, PerCP-Cy5.5, or allophycocyanin. Antibodies used were as follows: anti-human CD16 (BD Biosciences, 550868; 1:100), anti-human CD11b (BD Biosciences, 550019; 1:100), anti-human CD45 (BD Biosciences, 557748; 1:100), anti-mouse CD45 (BioLegend, 103108; 1:100), anti-mouse Ly-6G (BioLegend, 127616; 1:100), anti-mouse F4/80 (BioLegend, 123114; 1:100), anti-mouse CD11b (BD Biosciences, 553311; 1:100), anti-mouse CD3e (BD Biosciences, 553066; 1:100), anti-mouse CD19 (BD Biosciences, 557655; 1:100), anti-mouse IL-1β (eBioscience, 12-7114-82; 1:100), anti-mouse IL-6 (BioLegend, 504503; 1:100), mouse TNF-α (BioLegend, 506303; 1:100), mouse CD206 (BioLegend, 141704, 1:100), anti-mouse glial fibrillary acidic protein (GFAP; BD Biosciences, 561483; 1:100), anti-mouse NeuN (Abcam, ab190195; 1:100), anti-mouse CD68 (BioLegend, 137013; 1:100), anti-mouse IL-1α (BD Biosciences, 559810; 1:100), anti-mouse FPR1 (Antibody Online, AA160-210; 1:100), and anti-rabbit AF488, AF546, or AF647 (Invitrogen, A-21206, A-10040, or A-27040, respectively; 1:1000). Common isotype controls were purchased from BD Biosciences.

**Neuroimaging**

The 7-T small-animal magnetic resonance imaging (MRI) scans (Bruker) were used as previously described (48). T2-weighted imaging (T2) was performed to assess total lesion volume. The setup parameters were as follows: repetition time (TR) = 4500 ms, echo time (TE) = 65.5 ms, field of view (FOV) = 28 × 28 mm², image matrix = 256 × 256, and 0.5-mm slice thickness. Susceptibility-weighted imaging (SWI) was used to measure hematoma. The setup parameters were as follows: TR = 30 ms and TE = 10 ms, flip angle = 25°, FOV = 32 × 32 × 16 mm³, and image matrix = 256 × 256. The volumes were manually outlined and calculated by multiplying the sum of the volume by the distance between sections (0.5 mm) using medical image processing, analysis, and visualization (MIPAV) software. PHE volumes were calculated as total lesion volume minus hematoma volume. MRI data were analyzed by two investigators blinded to experimental groups.

**In vivo antibody or drug administration**

Anti-mouse IL-1α mAb was purchased from BioLegend (503206). Mouse immunoglobulin G2a (IgG2a; Sigma-Aldrich, PP102) was used as the isotype control antibody. For in vivo blockage of IL-1α in the brain, 1 μg of anti–IL-1-α mAb was injected into mouse brain by intracerebroventricular injection as previously described (47). In preclinical studies, ICH mice were given T-0080 (5 mg/kg daily) twice a day by intraperitoneal injection, and the first dose is at 1 or 24 hours after ICH onset.

**Neurological function assessment**

Neurological function assessment was performed by two investigators who were blinded to the treatment groups. The modified Neurological Severity Score, corner turn test, and rotarod test were conducted to evaluate neurodeficits of ICH mice at defined time points, as we previously described (48).

**Modified Neurological Severity Score**

In the neurologic deficit scoring system, mice were evaluated for motor function (muscle and abnormal movement), sensory function (visual, tactile, and proprioceptive), and reflexes (pinna, corneal, and startle reflexes). The range of scores is from 0 to 18, defined as follows: a score of 13 to 18 indicates severe injury, 7 to 12 indicates moderate injury, and 1 to 6 indicates mild injury.

**Corner turn test**

To evaluate sensorimotor and postural asymmetries, a corner turn test was performed in our study. Mice could proceed into a corner with an angle of 30° and then must turn right or left. Each mouse repeated this procedure 10 times with an interval of at least 30 s between trials. The percentage of ipsilateral turns was then calculated.

**Rotarod test**

The rotarod test was performed to evaluate motor coordination and balance. Mice were trained for 1 week before ICH induction. At indicated time points after ICH, mice were placed on a rotarod apparatus. The rotating rod has a 3-cm diameter with a non-slippery surface. The rod was 30 cm in length and placed at a height of 20 cm from the base. Each mouse was placed on the rod at a speed of 4 rotations per minute (rpm), which accelerates over the course of 300 s to 40 rpm. The duration of each mouse on the rod was recorded automatically. Each mouse was tested in three consecutive trials with an interval of 15 min in between. The results were calculated as the average of three trials.

**Neuroimaging**

The 7-T small-animal magnetic resonance imaging (MRI) scans (Bruker) were used as previously described (48). T2-weighted imaging (T2) was performed to assess total lesion volume. The setup parameters were as follows: repetition time (TR) = 4500 ms, echo time (TE) = 65.5 ms, field of view (FOV) = 28 × 28 mm², image matrix = 256 × 256, and 0.5-mm slice thickness.
measurements were performed on a FACS Aria III (BD Bioscience) and analyzed using Flowjo 7.6 software (Informer Technologies).

**Immunostaining and histology**
Paraffin-embedded brain tissue from patients with ICH or mice was cut into 5-μm continuous coronal sections. Sections were permeabilized and incubated with blocking solution consisting of 5% donkey serum and 0.3% Triton X-100, followed by incubation with primary antibodies or Western blotting with secondary antibodies at room temperature for 2 hours. Then, the slices were washed with PBS three times before being incubated with Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole (Abcam). Antibodies used were as follows: anti-human transmembrane protein 119 (TMEM 119) (Abcam, ab185333; 1:100); anti-human FPR1 (BioLegend, 391602; 1:100); anti-human GFAP (Abcam, ab53554; 1:100); anti-human microtubule-associated protein 2 (MAP2) (Boster, bs-1369R; 1:100); anti-human/mouse FPR1 (FabGennix, FPR1-101AP; 1:100); anti-human NeuN (Abcam, ab104224; 1:1000); anti-human/mouse caspase 3 (Cell Signaling Technology, 9661S; 1:1000); anti-mouse TMEM119 (Abcam, ab209064; 1:100); anti-mouse FPR1 (Antibody Online, AA160-210; 1:100); anti-mouse ly6G (1A8) (Bioxcell, BP0075-1; 1:100); anti-goat AF488 or AF546 (Invitrogen, A-11055 or A-11056, respectively; 1:1000); anti-rabbit AF488, AF546, or AF647 (Invitrogen, A-21206, A-10040, or A-27040, respectively; 1:1000); anti-mouse AF488 or AF594 (Invitrogen, A-21202 or A-21203, respectively; 1:1000); anti-rat AF555 (Invitrogen, A-21434; 1:1000); and isotype control IgG (Abcam, ab37415, ab37361, ab37355, and ab37373). In addition, hematoxylin and eosin staining was performed. Images were taken with a fluorescence microscope (model BX-61, Olympus).

**Isolation of mouse microglia and stimulation with fMLF in vitro**
Before removal of brain tissue, perfusion was performed with 50 ml of ice-cold PBS to exclude contamination of peripherally blood cells. Brain tissue from wild-type or Fpr1−/− adult male mice were washed in ice-cold Hanks’ balanced salt solution and enzymatically dissociated into single-cell suspensions with a papain-based neural tissue dissociation kit. The myelin was removed with 30% Percoll by centrifugation at 700g for 3 min. Microglia were sorted with flow cytometry after staining with anti-CD11b and CD45 antibodies. The purity of microglia (CD11b+ CD45 int) is about 99% assessed by cytometry after staining with anti-CD11b and CD45 antibodies. Microglia were sorted with flow cytometry after staining with anti-CD11b and anti-Ly6G (1A8) antibodies. Neutrophils were purified with a neutrophil isolation kit (Miltenyi Biotec). The purity of neutrophils was confirmed by FACS after staining with anti-CD11b and anti-Ly6G (1A8) antibodies. Neutrophils were cultured in 1640 medium containing 10% FBS, 1% penicillin/streptomycin, and mouse G-CSF (20 ng/ml). For transwell migration assay, we cocultured neutrophils with microglia with a 3-μm polycarbonate filter microchamber filtration system. Neutrophils (2 × 106) were added to the upper well of the upper compartment. Lower chambers were seeded with purified 1 × 104 microglial cells stimulated for 6 hours with vehicle, 1 μM fMLF, 10 μM T-0080, or anti-mouse IL-1α mAb (1 μg/ml). After coculture for 6 hours, the migrated neutrophils were harvested from the lower compartment and counted by flow cytometry.

**Screen and design of FPR1 antagonist T-0080**
The search for antagonists of human and mouse FPR1 was carried out based on the computer-aided drug design as previously reported (25, 26, 52). Briefly, homology modeling of human and mouse FPR1 was performed using the I-TASSER online server (53). The antagonist-binding pocket of FPR1 was decided using receptor-antagonist binding mode. Virtual screening was performed using Glide (54) with high-throughput virtual screening, standard precision, and extra precision modes to screen about 2 million compounds of the ChemDiv, InterBioScreen, Enamine, and National Cancer Institute libraries. After virtual screening and obtaining extra precision scores for protein-antagonist binding affinities, the compounds were visually checked and inspected for substantial interactions with the FPR1 active site, including residues Arg84, Phe102, Arg201, Arg203, and Tyr237. Eighteen compounds were selected on the basis of their best pose and subjected to biological evaluation. A compound named D126-0080 showed the ability to inhibit human FPR1 at micromolar concentrations, purchased from the ChemDiv library. We further modified the substituents of D126-0080 and promoted its binding affinities to the binding pocket of human and mouse FPR1. We finally synthesized and verified a new FPR1 antagonist named T-0080.

**Functional assay of T-0080**
The efficacy of T-0080 on FPR1 was tested using FPR1-mediated intracellular calcium flux as previously reported (55). Briefly, human or mouse neutrophils were incubated with 1 μM indo-1 AM calcium sensor dye (catalog no. 65-0856-39, ebiosience) and stained with human neutrophil markers (CD45+CD11b+CD16b+) or mouse neutrophil markers (CD45+CD11b+Ly-6G+) for 30 min at 37°C in calcium-free medium. After centrifugation at 1500 rpm for 5 min, cells were resuspended in medium containing 1.3 mM calcium, 1 mM magnesium, and 1% bovine serum albumin and kept at 4°C for T-0080 treatment. T-0080 or vehicle (dimethyl sulfoxide) was given at 10 min before loading to flow cytometry. Cells and chambers for flow cytometry were warmed up to 37°C when adding fMLF (10 nM or 1 μM; Sigma-Aldrich, F3506) and recording the changes of immunofluorescence intensity of indo-1 AM over time. We defined the immunofluorescence intensity of indo-1 AM before adding fMLF as the maximum value and the intensity at the peak reduction after adding fMLF as the minimum value for each sample. The
change of intracellular calcium was calculated as follows: (maximum value – minimum value)/maximum value × 100%.

**Pharmacokinetics analysis in mice**

A single dose of T-0080 (5 mg/kg) was given intraperitoneally to male C57BL/6 (10 to 12 weeks old) mice. Blood and brain samples were acquired at 5 min, 30 min, 1 hour, 3 hours, and 6 hours, after T-0080 administration. The concentration of T-0080 in serum and brain samples was analyzed by liquid chromatography–mass spectrometry (Waters, SCIEX). Each batch of test had a standard for correction. The concentration of T-0080 in blood and brain samples was read out using the standard curve.

**Enzyme-linked immunosorbent assay**

Human serum mitochondrial N-formyl peptides were measured using the human formylmethionine (fMET) ELISA Kit (Babine, KTE62623), according to the manufacturer’s instructions. Serum samples were diluted at 1:5 with sample dilution buffer of the kit when loading to the ELISA plate. The protein concentrations of IL-6, IL-1β, and TNF-α in mouse brain lysates were measured with mouse IL-6, IL-1β, and TNF-α quantikine ELISA kit (catalog numbers M6000B, MLB00C, and MTA00B) according to the manufacturer’s instructions. The ELISA plate was read out by Varioskan Flash 4.00.53 (Thermo Fisher Scientific).

**Western blot**

Cultured microglia were digested with trypsin, washed three times with PBS, and then were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The supernatant was harvested for protein analysis after centrifugation. Proteins were resolved by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat milk solution for 1 hour at room temperature and then incubated with primary antibodies against mouse IL-1α (p18) (1:1000, Abcam) and anti–β-actin (1:1000, Abcam) overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase–conjugated rat anti-mouse and goat anti-rabbit secondary antibodies (1:5000, Invitrogen) for 1 hour at room temperature. The bands were detected and captured using a gel imaging system (Bio-Rad). The intensity of each band was quantified by ImageJ software (National Institutes of Health).

**Proteome profiler mouse cytokine array**

Perihematomal tissue was collected at 24 hours after ICH, and normal mice brain tissues were used as control. Thereafter, brain tissue was lysed with cell lysis buffer (R&D Systems Inc.) supplemented with protease inhibitor mixture (Sigma-Aldrich) at 4°C for 30 min. Total protein was quantified using a protein bicinchoninic acid kit (Thermo Fisher Scientific Inc.). The Mouse XL Cytokine Array Kit (R&D Systems Inc.) was used to measure cytokine and chemokine concentrations. Immunosots were captured using a Gel Doc Imager (Bio-Rad), and spot density was measured using ImageJ software (National Institutes of Health).

**Statistical analysis**

Statistical analyses were performed using Prism 8.0.2 software (GraphPad). Data are presented as means ± SEM. Values of P < 0.05 were considered significant. The exact values of sample size (n) are given in figure legends and represent either the number of animals used in vivo or the number of patient samples and cell cultures used in vitro. The experimental design was based on previous publications with similar mechanistic studies done in our laboratory (48, 50, 51). All animals in experimental and control groups were littermates. Experimental groups, data collection, and data analysis were blinded by using different investigators or masking sample labels. All experiments with animals and cell cultures were randomly assigned to experimental groups. Sample exclusion was done as a result of mouse death after surgery. All experiments presented were successfully reproduced for at least three times. For each set of data compared, we evaluated normality using the Kolmogorov-Smirnov test. Two-tailed unpaired Student’s t test was used to determine the significant differences between two groups. One-way analysis of variance (ANOVA) followed by post hoc Tukey test was used for three or more groups. Multiple comparison analyses were assessed with two-way ANOVA accompanied by a post hoc Bonferroni test. Correlation was calculated using Pearson correlation coefficient method. Comparison of survival curves was performed with log-rank (Mantel-Cox) test. DEGs between two samples were defined as genes with FDR ≤ 0.001 and a fold difference of more than two times. Gene ontology terms with a BH-corrected P value <0.05 were considered significant.

**SUPPLEMENTARY MATERIALS**

stm.sciencemag.org/cgi/content/full/13/605/eaeb9890/DC1?figs=s1 to S9

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**


with proinflammatory functions. The last chapter of the book focuses on the future perspectives of these research areas and concludes with a discussion on potential clinical applications.

In summary, this book provides a comprehensive overview of the latest research in the field of inflammation and its role in various diseases. It is an essential resource for researchers, clinicians, and students interested in understanding the complex mechanisms underlying inflammation and its impact on health.

References:


Acknowledgments: We thank J. Lin (High-Throughput Molecular Drug Discovery Center, Tianjin Joint Academy of Biomedicine and Technology, Tianjin 300457, China) for assisting in the creation of the FPR1 antagonist; we also thank S. Wu, N. Yao, and F. Zhang (Department of Neurology, Tianjin Medical University General Hospital, Tianjin 300052, China) for technical support. Controls used for the analyses of perihematoma tissues described in this work were obtained from dbGaP at www.ncbi.nlm.nih.gov/gap through dbGaP accession number phs000424.v6.p2. Funding: This study was supported in part by The National Key Research and Development Program of China (2018YFC1312200 to F.-D.S.); the Advanced Innovation Center for Human Brain Protection, Capital Medical University, Beijing, China; and the National Science Foundation of China (91642205, 81630038, 91949208, and 82071327 to F.-D.S.). Author contributions: F.-D.S. and Y.W. formulated the concept and designed the studies. Z.L., Y.L., J.H., Z.Z., and M.L. performed the experiments. Z.L., Y.L., J.H., Z.Z., and M.L. analyzed and interpreted the results. F.-D.S., Y.W., Z.L., and Q.L. wrote and edited the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: The accession GEO number for sequencing data reported in this paper is GSE167758. Structural data of small-molecule data are available at CCDC (deposition number: 1994208) or PubChem (substance upload ID: 104344). All the remaining data are present in the paper or the Supplementary Materials.

Submitted 26 September 2020
Accepted 2 April 2021
Published 4 August 2021
10.1126/scitranslmed.abe9890

Citation: Z. Li, Y. Li, J. Han, Z. Zhu, M. Li, Q. Liu, Y. Wang, F.-D. Shi, Formyl peptide receptor 1 signaling potentiates inflammatory brain injury. Sci. Transl. Med. 13, eabe9890 (2021).
Formyl peptide receptor 1 signaling potentiates inflammatory brain injury
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Sci Transl Med 13, eabe9890.
DOI: 10.1126/scitranslmed.abe9890

Curbing inflammation in ICH
Intracerebral hemorrhage (ICH) is associated with increased brain inflammation that contributes to exacerbate cell death and neurological deficits. Reducing brain inflammation after ICH could improve clinical outcome. Now, Li et al. performed genome-wide transcriptome sequencing of perihematoma human brain tissue and showed that formyl peptide receptor 1 (FPR1) expression was increased in microglia. In rodent models, FPR1 activation after ICH promoted brain inflammation and increased immune cell recruitment into the brain. The authors developed a blood-brain barrier-permeable FPR1 inhibitor that had therapeutic effects in ICH mice, suggesting that targeting FPR1 could be effective for reducing inflammation and promoting recovery after ICH.