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The role of inflammasome activation in post-stroke regeneration and recovery

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Zusammenfassung (Deutsch):

Schlaganfall ist die zweithäufigste Todesursache weltweit und verursacht jährlich etwa 6 Millionen Todesfälle sowie eine bedeutende Ursache für Langzeitbehinderungen. Ein Schlaganfall tritt auf, wenn die Blutversorgung des Gehirns unterbrochen wird, entweder durch eine Verstopfung eines Blutgefäßes (ischämisch) oder durch Blutungen aus einem gerissenen Blutgefäß (hämorrhagisch). Die pathophysiologischen Prozesse, die einem Schlaganfall folgen, sind komplex und umfangreich, wobei die Neuroinflammation eine wichtige Rolle für das Ergebnis nach einem Schlaganfall spielt. Nach einem akuten Schlaganfall wird die sekundäre Neuroinflammation durch proinflammatorische Signale ausgelöst, die von sterbenden Zellen im Infarktbereich freigesetzt werden. Diese Neuroinflammation kann weitere Verletzungen verstärken und zum Zelltod führen, spielt jedoch auch eine vorteilhafte Rolle bei der Förderung der Genesung. Der Beginn dieser Entzündungsreaktion wird über den Inflammasomweg vermittelt.

Das Inflammasom ist ein hochmolekularer Multiproteinkomplex, der für die Einleitung der frühen inflammatorischen Reaktion verantwortlich ist. Es setzt sich aus einem Mustererkennungssystem (PRR), einem Adaptorprotein namens Apoptose-assoziiertes Speck-ähnliches Protein (ASC) und dem Effektorsprotease Caspase-1 zusammen. Das Inflammasom wird in Reaktion auf pathogen- oder schädigungsassoziierte Molekülmuster (PAMPs oder DAMPs) aktiviert. Bei Erkennung dieser Signale rekrutieren PRRs ASC durch homotypische Proteininteraktionen, was dann Caspase-1 rekrutiert. Die Aktivierung des Inflammasoms führt zur autokatalytischen Aktivierung von Caspase-1, deren Hauptfunktion die proteolytische Aktivierung der pro-inflammatorischen Zytokine Interleukin (IL)-1α, IL-β und IL-18 sowie die Spaltung von Gasdermin D ist, einem löslichen Cytosolprotein, das eine programmierte Form des Zelltodes namens Pyroptose initiiert.

Es wurde festgestellt, dass die Hemmung der Inflammasomaktivierung während der akuten Phase eines Schlaganfalls die Ergebnisse verbessert, und die Aktivierung des Inflammasoms wurde auch mit kognitiven Beeinträchtigungen nach einem Schlaganfall in der chronischen Phase in Verbindung gebracht. Die Mechanismen der Inflammasomaktivierung nach einem Schlaganfall und deren Rolle bei der funktionalen Wiederherstellung bleiben jedoch unklar. In dieser Studie untersuchte ich die spatiotemporale Aktivierung von Inflammasomen im post-stroke Mausgehirn

und erkundete, wie die Inflammasomaktivierung die Neuroinflammation und die funktionale Genesung nach einem Schlaganfall beeinflusst.

Meine Ergebnisse zeigten, dass die Inflammasomaktivierung in verschiedenen Modellen des ischämischen Schlaganfalls variiert. In einem schweren Transienten-Mittleren-Hirnarterienverschluss (MCAO)-Modell hielt die Inflammasomaktivierung bis in die chronische Phase an. Im Gegensatz dazu war die Inflammasomaktivierung in einem moderaten Photothrombotischen (PT) Modell schnell und stark, aber hauptsächlich auf die akute Phase beschränkt. In der chronischen Phase nach MCAO wurde die Inflammasomaktivierung in der Großhirnrinde, dem Striatum und dem Hippocampus festgestellt, wobei der peri-infarct Bereich der Großhirnrinde eine deutlich aktivierte Zone zeigte, während in der Kernläsion kaum bis keine Aktivierung festgestellt wurde.

Die spezifischen Beiträge verschiedener Zelltypen zur Inflammasomaktivierung wurden ebenfalls untersucht. Die Analyse der Daten ergab, dass mehrere Zelltypen zu unterschiedlichen Zeitpunkten an der Aktivierung beteiligt waren. Mikroglia waren überwiegend in der subakuten bis chronischen Phase (1 bis 2 Wochen) aktiv, während Makrophagen/Monozyten eine bedeutende Rolle in der akuten und subakuten Phase spielten. Neutrophile zeigten eine erhöhte Inflammasomaktivierung von der akuten bis zur chronischen Phase.

Zusätzlich wurde entdeckt, dass die akute Neuroinflammation nach einem Schlaganfall stark von der Inflammasomaktivität abhängt. Der Mangel an Caspase-1 führte zu einem signifikanten Rückgang der einwandernden Immunzellen im Gehirn während der akuten und subakuten Phase. In der chronischen Phase wurde jedoch keine Auswirkung auf die sekundäre chronische Entzündung festgestellt, die durch die Ansammlung von T- und B-Zellen gekennzeichnet war. Anschließend untersuchten wir die Rolle der Aktivierung des Inflammasoms bei der post-schlaganfallbedingten Regeneration und Erholung. Caspase-1 Knockout-Mäuse, denen wichtige Komponenten des Inflammasom-Wegs fehlen, wiesen vier Wochen nach dem Schlaganfall eine verringerte postsynaptische Dichte im peri-infarcten Kortex auf, im Vergleich zu Wildtyp-Mäusen (WT), ohne jedoch eine Auswirkung auf die kolokalisierten Synapsen zu zeigen. Des Weiteren zeigten die Verhaltens- und Neurologietests, dass die funktionale Genesung in den inflamasomdefizienten Mäusen verzögert war, was auf eine entscheidende Rolle des Inflammasomwegs im Genesungsprozess nach einem Schlaganfall hinweist.

Insgesamt liefert diese Studie starke Beweise dafür, dass die Inflammasomaktivierung vorteilhafte Effekte in der post-stroke Pathologie hat. Meine Ergebnisse deuten darauf hin, dass das Timing der Inflammasomaktivierung ein kritischer Faktor für das Ergebnis eines Schlaganfalls sein könnte, was die Notwendigkeit unterstreicht, den geeigneten Zeitrahmen für die gezielte Inflammasomaktivierung zu berücksichtigen, um die bestmöglichen therapeutischen Ergebnisse zu erzielen.

Abstract (English):

The WHO identifies stroke as the second most significant cause of global mortality, resulting in roughly 6 million deaths per year and significantly contributing to long-term impairment. A stroke occurs when cerebral blood flow is disrupted, either by the blockage of a cerebral artery or by hemorrhage from a ruptured blood vessel. The following pathophysiological processes are activated, with neuroinflammation significantly influencing the outcomes after a stroke. Following an acute stroke, secondary neuroinflammation is initiated by pro-inflammatory signals released from dying cells in the infarct core area. While this inflammation can exacerbate further injury and lead to cell death, it also plays a beneficial role in promoting tissue repair and recovery. In these processes, the inflammatory response is fundamentally triggered through the crucial inflammasome pathway.

The inflammasome is a high-molecular-weight multi-protein complex responsible for initiating the inflammatory response. It consists of a pattern recognition receptor, an adaptor protein and an effector protease caspase-1. The inflammasome becomes activated when the sensor detects pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Upon detecting these signals, pattern recognition receptors (PRRs) bind to the adaptor protein via homotypic protein interactions, leading to the recruitment of pro-caspase-1. This process results in the autocatalytic activation of pro-caspase-1, which primarily functions to proteolytically activate pro-inflammatory cytokines such as interleukin (IL)-1α, IL-β, and IL-18, and to cleave Gasdermin D (GSDMD). This cytosolic protein then triggers a type of programmed cell death known as pyroptosis.

Inhibiting inflammasome activation during the acute phase of a stroke has been shown to improve outcomes. At the same time, its activation has also been linked to post-stroke cognitive impairment in the chronic phase. However, the mechanisms underlying post-stroke inflammasome activation and its role in functional recovery remain unclear. This study investigates the spatiotemporal activation of inflammasomes in the post-stroke mouse brain and explores their impact on neuroinflammation and functional recovery.

My findings indicate that inflammasome activation differs among various stroke models. In a severe transient middle cerebral artery occlusion model, the activation of the inflammasome persisted into the chronic phase. In contrast, in a moderate photothrombotic (PT) occlusion model, inflammasome activation occurred rapidly but was primarily confined to the acute phase. In the chronic phase following middle cerebral artery occlusion, inflammasome activation was observed in the cortex, striatum, and hippocampus. Notably, the peri-infarct cortex shows prominent activation but minimal activation in the core lesion.

Cell-specific contributions to inflammasome activation were also examined. Microglia were primarily involved in inflammasome activation during the subacute to chronic phase (1 to 2 weeks), whereas macrophages and monocytes exhibited greater activity during the acute and subacute phases. Neutrophils exhibited sustained activation from the acute to the chronic phase.

Additionally, our findings indicate that acute neuroinflammation following stroke is mainly dependent on inflammasome activity. Caspase-1 deficiency significantly reduced the immune cell infiltration into the brain during both the acute and subacute phases. However, it did not impact the secondary chronic inflammation, which is marked by the aggregation of T and B cells. Furthermore, the role of inflammasome activation in the process of post-stroke regeneration and functional recovery was thoroughly examined. Compared to wild-type (WT) mice, Caspase-1 knockout mice, which lack critical components of the inflammasome pathway, exhibited a reduced post-synaptic density within the peri-infarct cortical region four weeks after stroke, while showing no effect on the colocalized synapses. Additionally, behavioral tests demonstrated delayed functional recovery in inflammasome-deficient mice, underscoring the critical role of the inflammasome in post-stroke rehabilitation processes (Fig. **1**).

In conclusion, this study presents compelling evidence indicating that inflammasome activation positively influences post-stroke pathology. Our findings suggest that the timing of inflammasome

activation may be a critical factor in determining stroke outcomes, highlighting the importance of targeting inflammasome activation within an optimal time window to achieve the best therapeutic results.

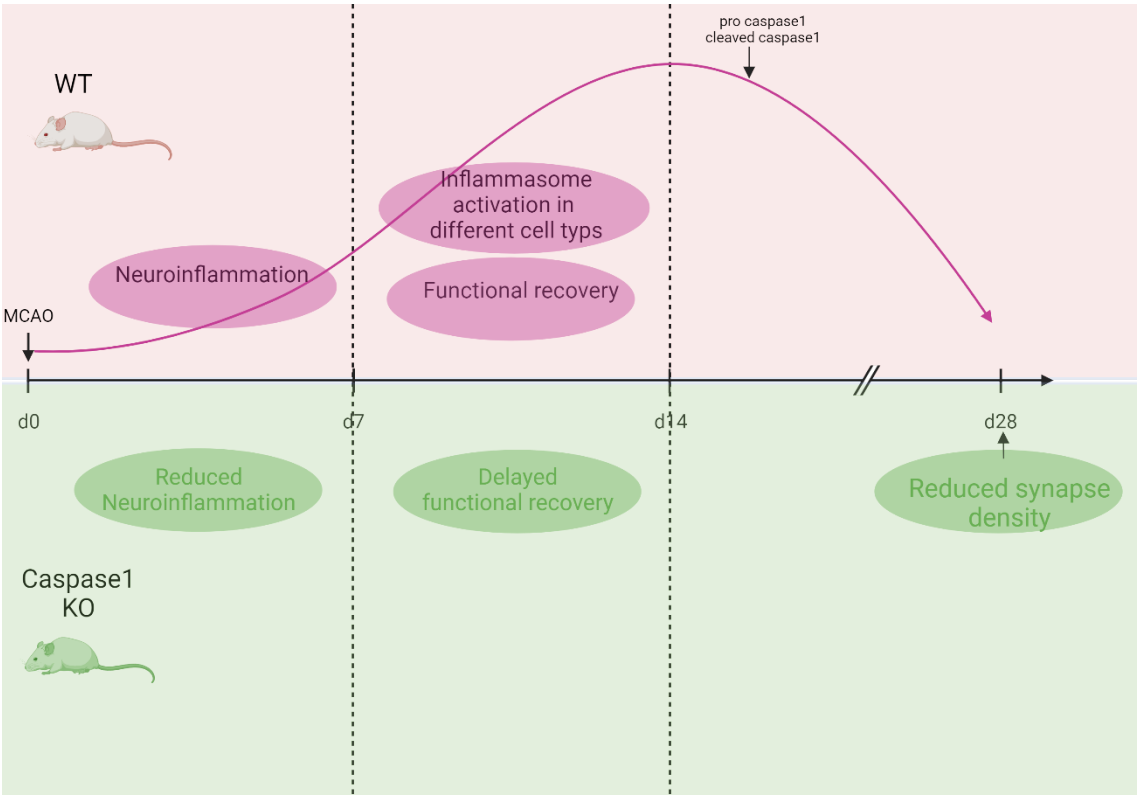

Figure 1 Graphical abstract.

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List of abbreviations

ASC	apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
AIM2	absent in melanoma 2
ATP	adenosine triphosphate
BBB	blood-brain barrier
cGAS	cyclic GMP-AMP synthase
cGAMP	cyclic GMP-AMP
CSF	cerebrospinal fluid
CNS	central nervous system
CPEpiC	Choroid plexus endothelial cells
DALYs	disability-adjusted life-years
DAMPs	damage-associated molecular patterns
FACS	fluorescence-activated cell sorting
FXII	factor XII
GSDMD	Gasdermin D
GFAP	glial fibrillary acidic protein
HMGB1	high mobility group box 1
IFI16	interferon-inducible protein 16
IPAF	ice protease-activating factor
KO	knockout
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta

IL-10	interleukin-10
IL-18	interleukin-18
LPS	lipopolysaccharides
LTD	long-term depression
LTP	long-term potentiation
LRR	leucine-rich repeat
MCAO	middle cerebral artery occlusion
MMPs	matrix metalloproteinases
NLR	Nod-like receptor
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NLRP1	NOD-like-receptor containing a Pyrin domain 1
NO	nitric oxide
PRR	pattern recognition receptor
PYHIN	pyrin and HIN domain-containing
PYD	pyrin domain
PT	photothrombotic
ROS	reactive oxygen species
STING	stimulator of interferon genes
TGF-β	transforming growth factor beta
TLRs	toll-like receptors
VEGF	vascular endothelial growth factor
WT	wild type

1. Introduction

1.1 Stroke

A stroke, which may arise from either the rupture of a cerebral blood vessel (hemorrhagic stroke) or the occlusion of an artery (ischemic stroke), is marked by an interruption in cerebral blood flow (see Fig. 2). While approximately 87% of strokes are categorized as ischemic, around 13% are classified as hemorrhagic in nature[1, 2]. The disruption of oxygen and nutrient delivery in ischemic strokes leads to significant damage within the cerebral tissue. The abrupt manifestation of weakness or numbness in the face, arm, or leg, predominantly impacting one side of the body, is a common sign of a stroke. Further symptoms may include confusion, impaired speech or comprehension abilities, vision problems in one or both eyes, gait abnormalities, dizziness, loss of balance or coordination, a severe unexplained headache (in the case of hemorrhagic stroke), fainting, or loss of consciousness. The results of a stroke differ based on the affected brain region and the extent of damage incurred.

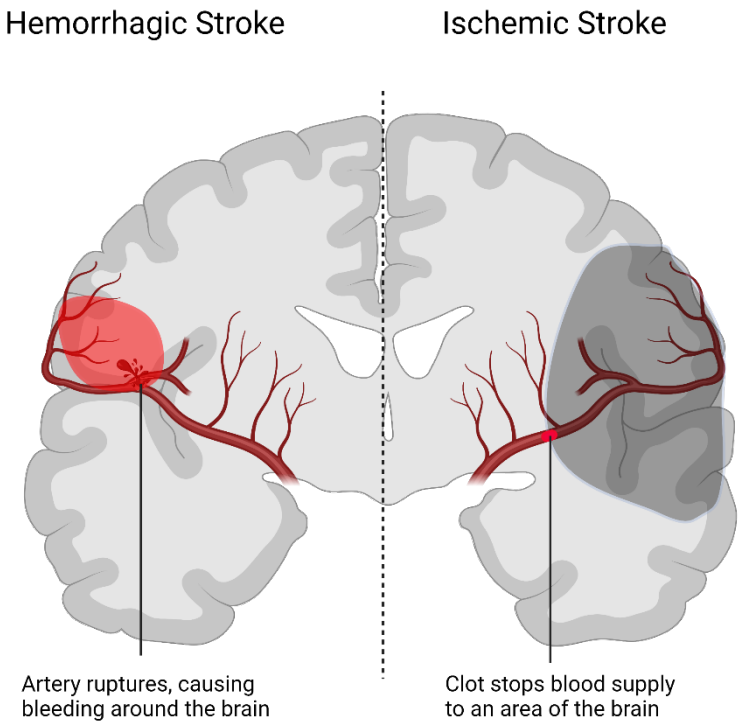


Figure 2 Hemorrhagic and ischemic stroke. A hemorrhagic stroke results from bleeding inside the brain and cranial cavities. An ischemic stroke happens when a blood clot or other material blocks a cerebral vessel.

Despite advancements in medical care, the increasing global burden of stroke-related disability and mortality indicates that stroke continues to pose a substantial challenge to public health systems worldwide. Despite a marked decline in stroke-related mortality rates and death-to-incidence ratios over the past two decades, the worldwide burden of stroke continues to be substantial and is progressively increasing, characterized by a striking annual incidence of new cases, stroke survivors, and associated fatalities, particularly in low- and middle-income nations. In 2019, the global burden of stroke was substantial, with 12.2 million new cases reported and 101 million individuals living with the condition, consequently resulting in 143 million disability-adjusted life-years (DALYs) and approximately 6.55 million stroke-related fatalities[3]. Between 1990 and 2019, the global incidence of strokes demonstrated a significant escalation of 70%, accompanied by an 85% increase in prevalence, a 43% rise in stroke-related mortality, and a 32% growth in DALYs attributable to stroke[3]. According to the World Bank, low-income countries exhibited an age-standardized stroke mortality rate that was 3.6 times higher and an age-standardized stroke-related DALYs rate that was 3.7 times higher compared to high-income countries[1]. In 2019, 86% of stroke-related deaths and 89% of stroke-related DALYs occurred in low-income, lower-middle-income, and upper-middle-income nations[3]. These figures underscore the urgent need for a deeper understanding of the mechanisms underlying stroke to enhance patient outcomes.

1.1.1 Etiology and Epidemiology

Approximately 87% of strokes are ischemic, while 13% are hemorrhagic[1, 2, 4]. A hemorrhagic stroke transpires when a blood artery within or on the brain's surface leaks or ruptures, resulting in bleeding that induces swelling and pressure in or around the brain. Hemorrhagic stroke is classified based on the bleeding location into intracranial (about 10%) and subarachnoid (3%) hemorrhagic stroke[4]. An ischemic stroke may occur due to an embolus or a local thrombus, leading to the constriction or complete obstruction of a cerebral artery. Based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) guidelines, ischemic stroke can be categorized into five distinct categories. 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel blockage, 4) other specified etiology, and 5) unknown etiology[5]. In large-artery atherosclerosis, a principal cerebral artery is obstructed by atherosclerotic plaques or significantly constricted by stenosis (>50%). Cardioembolism transpires when a thrombus originating from the heart,

frequently associated with supraventricular arrhythmias such as atrial fibrillation, migrates and obstructs a cerebral artery. Small-vessel occlusion occurs when minor blood vessels in the brain become obstructed, often due to hypertension.

Findings from extensive epidemiological studies indicate that the risk factors for stroke and their correlations with stroke are consistent throughout various global regions[6]. Hypertension, active smoking, diabetes, abdominal obesity, suboptimal diet, and physical inactivity contribute to over 80% of the worldwide risk for all stroke types (ischemic and hemorrhagic). Additional risk factors encompass excessive alcohol intake, dyslipidemia, cardiac conditions (such as atrial arrhythmia, prior heart attack, rheumatic valvular disorder, and mechanical heart valves), as well as psychological stress and depression[6]. The evidence indicates that the severity of strokes can be greatly reduced through targeted preventive strategies, such as effective blood pressure management, quitting smoking, enhancing physical activity and adopting a nutritious diet.

Stroke registries based on population data provide crucial insights into stroke incidence. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2016) indicates that stroke ranks as the second leading cause of death globally, just after ischemic heart disease[7]. Each year, stroke, including both ischemic and hemorrhagic types, impacts 13.7 million individuals and causes 5.5 million deaths worldwide, with 2.7 million attributable to ischemic stroke and 2.8 million to hemorrhagic stroke[7]. In 2016, stroke also represented the second most common contributor to global disability-adjusted life years (DALYs), reflecting an increase since 1990[7]. Among global regions, East Asia—particularly China—shows the highest age-standardized stroke incidence, followed by Eastern Europe[7]. While individuals under the age of 55 exhibit similar stroke incidence across sexes, men aged 55 to 75 demonstrate a notably higher incidence than women in the same age group[7].

A study conducted by Valery L. Feigin and colleagues revealed substantial disparities in stroke incidence and mortality between high-income nations and developing countries[8]. Over the past four decades, a significant 42% reduction in age-adjusted stroke incidence rates has been observed in high-income countries, with the incidence declining from 163 to 94 cases per 100,000 person-years between 1970–79 and 2000–08 ($p = 0.0004$). In contrast, within low- to middle-income nations, the incidence of stroke exhibited a dramatic increase, more than doubling from 52 to 117 cases per 100,000 person-years ($p < 0.0001$), thereby exceeding the rates documented

in high-income regions over the past decade[8]. This divergence marks the first time stroke incidence in low- to middle-income countries has exceeded that in wealthier nations. Regarding case fatality, high-income countries saw a decline from 35.9% in 1970–79 to 19.8% in 2000–08, while low- to middle-income countries experienced fluctuating rates, with 35.2% in 1980–89, 23.0% in 1990–99, and 26.6% in 2000–08[8].

Although age-standardized DALY rates due to stroke decreased from 1990 to 2006, the absolute number of DALYs rose, primarily due to population aging and growth. Despite improvements in incidence and survival, the overall burden of stroke remains substantial. In 2016, over 80 million individuals were living with the consequences of stroke, imposing considerable economic strain. The projected increase in direct costs related to stroke care is estimated to rise from $71.6 billion in 2012 to $184.1 billion by the year 2030[4].

1.1.2 Post-stroke neuroinflammation and regeneration

Post-stroke neuroinflammation is characterized by the liberation of damage-associated molecular patterns (DAMPs), activation of glial cells, compromise of the blood-brain barrier (BBB), and infiltration of peripheral immune cells from the periphery[9-12]. The initial pathological event following cerebral ischemia is reduced cerebral perfusion, which disrupts the delivery of oxygen and glucose to neural tissue, resulting in energy depletion and a decline in adenosine triphosphate (ATP) synthesis[13-15]. This metabolic deficiency triggers a cascade of oxidative stress, biochemical alterations, and hormonal imbalances that collectively initiate the neuroinflammatory response. Notably, the inflammatory cascade begins almost immediately after vascular occlusion, affecting both the cerebral vasculature and brain parenchyma in parallel[16-18].

1.1.2.1 Inflammation in the cerebrovascular compartment

The inflammatory cascade begins in the vasculature due to initial hypoxia, changes in shear forces, and the production of reactive oxygen species (ROS). These factors swiftly trigger intravascular inflammation by activating the coagulation system, complement pathways, platelets, and endothelial cells[19-21].

Platelets bind to exposed or released von Willebrand factor (vWF), particularly ultralarge vWF (UL-vWF), via the glycoprotein Ibα receptor, which promotes platelet activation and subsequent

secretion of polyphosphates[17]. These polyphosphates, along with negatively charged elements such as cell-free DNA, stimulate coagulation factor XII, the protease responsible for initiating the intrinsic coagulation cascade[17]. Factor XII also activates the contact-kinin system by converting plasma prekallikrein into kallikrein, a serine protease[17].

Coagulation processes, in turn, amplify complement system activation; thrombin plays a central role by directly cleaving C3 and C5 complement components, enhancing ROS production and prompting inflammatory cytokine secretion and myeloid cell degranulation[18]. Fibrin generated within vessels entraps platelets and leukocytes, promoting microvascular occlusion and restricting blood flow, which further exacerbates oxygen and glucose deprivation in ischemic regions[22, 23]. Persistent hypoxia imposes shear stress on both endothelial cells and platelets, stimulating the translocation of adhesion molecules such as P-selectin to the cell surface within minutes of activation[18, 24]. P-selectin, along with E- and L-selectins, facilitates leukocyte rolling and recruitment to the endothelium, with L-selectin guiding unstimulated leukocytes[9, 17, 25].

Elevated expression of E- and P-selectins on endothelial cells enables low-affinity interactions with leukocyte glycoproteins. Firm adhesion is mediated by the interplay of leukocyte β2 integrins with endothelial immunoglobulin superfamily members including ICAM-1, ICAM-2, and VCAM-1, as well as integrins like CD11a–c[26, 27]. These adhesion mechanisms collectively coordinate leukocyte recruitment, firm attachment, and transmigration across the endothelium. Concurrently, ROS accumulation within endothelial cells reduces nitric oxide (NO) levels, a crucial vasodilator and blocker of platelet aggregation and leukocyte activation. Diminished NO further promotes vascular occlusion and aggravates ischemic injury. Oxidative stress also activates pericytes and other contractile cells, contributing to additional microvascular blockages.

Inflammatory mediators, which encompass cytokines, chemokines, NO, ROS, and matrix metalloproteinases (MMPs), induce endothelial dysfunction and the detachment of pericytes, ultimately leading to the disruption of the blood-brain barrier (BBB)[28-32]. A compromised BBB allows the free entry of circulating molecules into the brain tissue and promotes leukocyte extravasation. In the perivascular microenvironment, complement fragment C5a binds to CD88 receptors on mast cells, initiating the release of histamine and proteases that increase BBB permeability. Additionally, cytokines like TNF-α and IL-1β, generated by mast cells and perivascular macrophages, further guide leukocyte migration through the vessel wall[16]. This

sequence of events sets the stage for BBB breakdown and leukocyte invasion into the ischemic tissue.

1.1.2.2 Inflammation in the brain parenchyma

The abrupt cessation of cerebral blood flow followed by reperfusion initiates a cascade of cellular and molecular responses. In areas where perfusion is critically reduced, irreversible damage occurs rapidly, forming the ischemic core[33-36]. Neurons within this region experience profound bioenergetic collapse, characterized by ATP depletion, disrupted ion gradients, cytoskeletal disassembly, and activation of degradative processes such as lipolysis and proteolysis. These neurons, exposed to hypoxic-ischemic conditions, rapidly lose function and viability[37].

Adjacent to the ischemic core region is the penumbra, an area in which cellular structures are preserved; however, their functionality is diminished owing to partial reductions in perfusion[38-41]. In this area, metabolic activity is inadequate but not entirely suspended, resulting in a susceptible environment for progressive damage. This region serves as the focal point of the ischemic cascade, where diverse injurious pathways are triggered, resulting in ongoing neuronal deterioration. The penumbra's relative preservation is maintained by residual perfusion from collateral circulation, which temporarily supports tissue survival beyond thresholds for immediate necrosis[42]. However, cells in this area remain highly sensitive to metabolic imbalance and secondary insults.

Glutamate, released in excessive amounts from depolarized or dying neurons, accumulates in the extracellular space, exacerbating excitotoxic injury. The excessive activation of N-methyl-D-aspartate (NMDA) receptors promotes the influx of intracellular Ca^{2+}, triggering the downstream activation of calcium-dependent enzymes, including but not limited to calpains, caspases, nitric oxide synthases, as well as those responsible for generating reactive oxygen species and arachidonic acid metabolites[43]. The combined effects of these molecular events culminate in necrotic or apoptotic cell death, depending on damage severity and neuronal metabolic status.

Following neuronal damage, injured and dying cells emit a variety of damage associated molecular patterns (DAMPs) that serve as alarm signals to trigger the immune response. These DAMPs encompass nucleotides like ATP and UTP, high mobility group box 1 (HMGB1), heat shock proteins, peroxiredoxins, as well as N-formyl peptides derived from mitochondria[44-47].

Pattern recognition receptors (PRRs), broadly expressed on microglia, astrocytes, perivascular macrophages, and endothelial cells in the brain, including Toll-like receptors (TLRs) and scavenger receptors, play a crucial role in detecting these signals[48-50].

Among the key glial components in the central nervous system (CNS), microglia, astrocytes, and oligodendrocytes play pivotal roles in detecting damage, releasing inflammatory mediators, and interacting with other immune cells to orchestrate the neuroimmune response[51]. Microglia are the central nervous system's resident immune cells and are the first to respond to ischemic events. In experimental models of ischemic stroke, microglial activation in peri-infarct regions can be observed within 30 to 60 minutes after arterial occlusion and may persist for weeks[52-56]. Upon activation, microglia rapidly undergo morphological transformation, with retraction of their processes or migration of their cell bodies, driven by decreased perfusion and energy depletion[51, 57, 58].

Microglia primarily respond to DAMPs through a network of receptors, including TLR4, HMGB1-binding receptors, purinergic receptors (e.g., P2X7, P2Y12), glutamate receptors, cytokine/chemokine receptors, and the triggering receptor expressed on myeloid cells 2 (TREM2). These receptors transmit signals through pathways including mitogen-activated protein kinases (MAPKs), nuclear factor-κB (NF-κB), and peroxisome proliferator-activated receptors receptors[51, 59]. Although the complete functions of these receptors are still being explored, it is well established that activated microglia generate proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which exacerbate the inflammatory environment during the acute phase[60].

In addition to cytokine secretion, microglia play a role in disrupting the BBB. Ischemic insults lead to the phosphorylation, degradation, or mislocalization of junctional proteins, thereby enhancing BBB permeability[61]. Microglia release MMPs, notably MMP-9, which further degrade tight junction proteins[62]. The chemokines and cytokines produced by microglia can upregulate endothelial adhesion molecules, promoting leukocyte adhesion and transmigration into brain tissue[63].

Astrocytes, the predominant type of glial cell in the CNS, play a crucial role in various physiological processes, such as maintaining ionic and neurotransmitter balance, regulating cerebral blood flow, modulating synapses, supporting neuronal survival, and preserving the integrity of the BBB[64].

Following an ischemic event injury, astrocytes become reactive, undergoing morphological and molecular changes in response to DAMPs and pro-inflammatory mediators[65-68]. Upon activation, astrocytes undergo enlargement, increased proliferation, and upregulation of structural proteins such as glial fibrillary acidic protein (GFAP), S100 beta, and vimentin, which are characteristic markers of intermediate filaments[69-72].

The role of reactive astrocytes is multifaceted and context dependent. They act both as initiators and regulators of the inflammatory response. In some contexts, reactive astrocytes exacerbate tissue damage through glutamate-mediated excitotoxicity and amplification of inflammation. In others, they contribute to neuroprotection and tissue repair by producing antioxidants like glutathione and secreting neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor[51]. Furthermore, astrocytes help reduce cerebral edema by modulating aquaporin channels, ion transporters, and osmolyte release, and by serving as a physical barrier.

Astrocytes also critically influence BBB integrity after stroke. They can enhance BBB permeability by secreting vascular endothelial growth factor (VEGF), particularly under the influence of IL-1β[70, 73, 74]. In addition, astrocyte-derived matrix metalloproteinases degrade tight junction proteins and extracellular matrix components, further compromising BBB function[75, 76]. Nevertheless, astrocytes also secrete protective factors such as Angiopoietin-1 and Sonic hedgehog, which support BBB stability and endothelial function[77-79]. This dual role underscores the complex and sometimes opposing contributions of astrocytes during stroke pathology and recovery.

Oligodendrocytes, responsible for myelin production in the CNS, are particularly vulnerable during the acute phase of cerebral ischemia. Many undergo apoptosis within hours of injury due to excitotoxicity and ATP depletion[80, 81]. However, in later stages, oligodendrogenesis becomes critical for white matter repair and functional recovery[82]. New oligodendrocytes originate from oligodendrocyte progenitor cells (OPCs) located in regions such as the corpus callosum, striatum, and subventricular zone[83]. Ischemic events trigger the proliferation and migration of OPCs into the penumbra, where they mature into oligodendrocytes, thereby facilitating axonal remyelination and aiding neural repair[84, 85].

The initial vascular response to ischemic stroke results in the recruitment of circulating leukocytes. Through the action of adhesion molecules, these activated leukocytes migrate into the brain parenchyma across the disrupted BBB during the acute phase of the stroke, contributing significantly to neuroinflammation. Neutrophil recruitment to ischemic brain tissue begins swiftly, with cells appearing in microvessels early on and peaking in number between 24 and 72 hours after stroke onset[86-88]. Although the precise role of neutrophils in ischemic stroke remains under investigation, their involvement is generally considered detrimental. Neutrophils contribute to BBB breakdown, the development of the “no-reflow” phenomenon, and the obstruction of blood flow in small vessels, including precapillary arterioles, postcapillary venules, and capillary beds. Additionally, neutrophils exacerbate ischemic injury by releasing proteolytic enzymes, ROS, and pro-inflammatory mediators[89]. Neutrophil extracellular traps (NETs) also contribute to clot formation, thereby further exacerbating ischemic damage[90].

In contrast to neutrophils, monocytes and macrophages infiltrate the ischemic brain at different stages. While neutrophils diminish in number after day 3, macrophages persist for much longer [91]. Macrophages and microglia share similar characteristics in the ischemic brain, which can complicate the differentiation of these two cell types. The infiltration of monocytes/macrophages is typically associated with inflammation and tissue damage.

Adaptive immune cells, including T and B lymphocytes, are also involved in post-stroke pathology, although their exact roles are less well defined compared to those of neutrophils and monocytes. Studies have shown that lymphocyte numbers increase in the ischemic brain at a later stage than neutrophils [92, 93]. Among these, T cells are thought to play a more prominent role in amplifying inflammation, while B cells have a lesser impact[94]. Notably, regulatory T cells (Tregs) may exert a protective effect by suppressing post-ischemic inflammation, highlighting the complexity of lymphocyte involvement in stroke outcomes[18].

Unlike the self-limiting inflammation observed in other tissues, post-stroke neuroinflammation often fails to resolve effectively. This sustained inflammation hinders recovery, underscoring the need for processes that actively resolve the immune response. The resolution of neuroinflammation involves clearing dead cells, creating an anti-inflammatory environment, and producing pro-survival factors that support tissue repair[95, 96]. Phagocytic microglia and infiltrating macrophages are crucial to this process, as they remove dead cells and tissue debris.

These phagocytes are attracted to the injury site by "find-me" signals, which direct them to the damaged area, and "eat-me" signals on apoptotic or necrotic cells that trigger phagocytosis[97, 98]. After engulfing apoptotic cells, Phagocytes contribute to inflammation resolution by releasing anti-inflammatory mediators, including transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10)[96].

TGF-β and IL-10 are multifunctional cytokines involved in tissue regeneration, exhibiting both inflammatory and anti-inflammatory properties depending on context. TGF-β exerts neuroprotective effects by modulating immune responses, notably suppressing T helper type 1 and type 2 activity and promoting regulatory T cell development[99]. Similarly, IL-10, produced by various immune cells including regulatory T cells, plays a key role in limiting inflammation and preserving neural integrity[100]. Their expression following cerebral ischemia contributes to tissue healing by attenuating the immune response and supporting the survival of neurons in the affected region.

Following a stroke, growth factors play a vital role in modulating inflammation and supporting tissue regeneration. Neurons, astrocytes, and immune cells contribute to this process by releasing a variety of growth-promoting molecules that aid recovery after ischemic damage[101, 102]. Microglia, for instance, secrete insulin-like growth factor 1 (IGF-1), which promotes axonal regeneration and neuronal outgrowth[103]. In response to ischemic stress, astrocytes become reactive and secrete vascular endothelial growth factor (VEGF), a key mediator of angiogenesis and vascular remodeling in damaged brain regions[104]. These combined actions foster a reparative environment, encouraging neurogenesis, synaptic reorganization, new blood vessel formation, and extracellular matrix restructuring[105, 106].

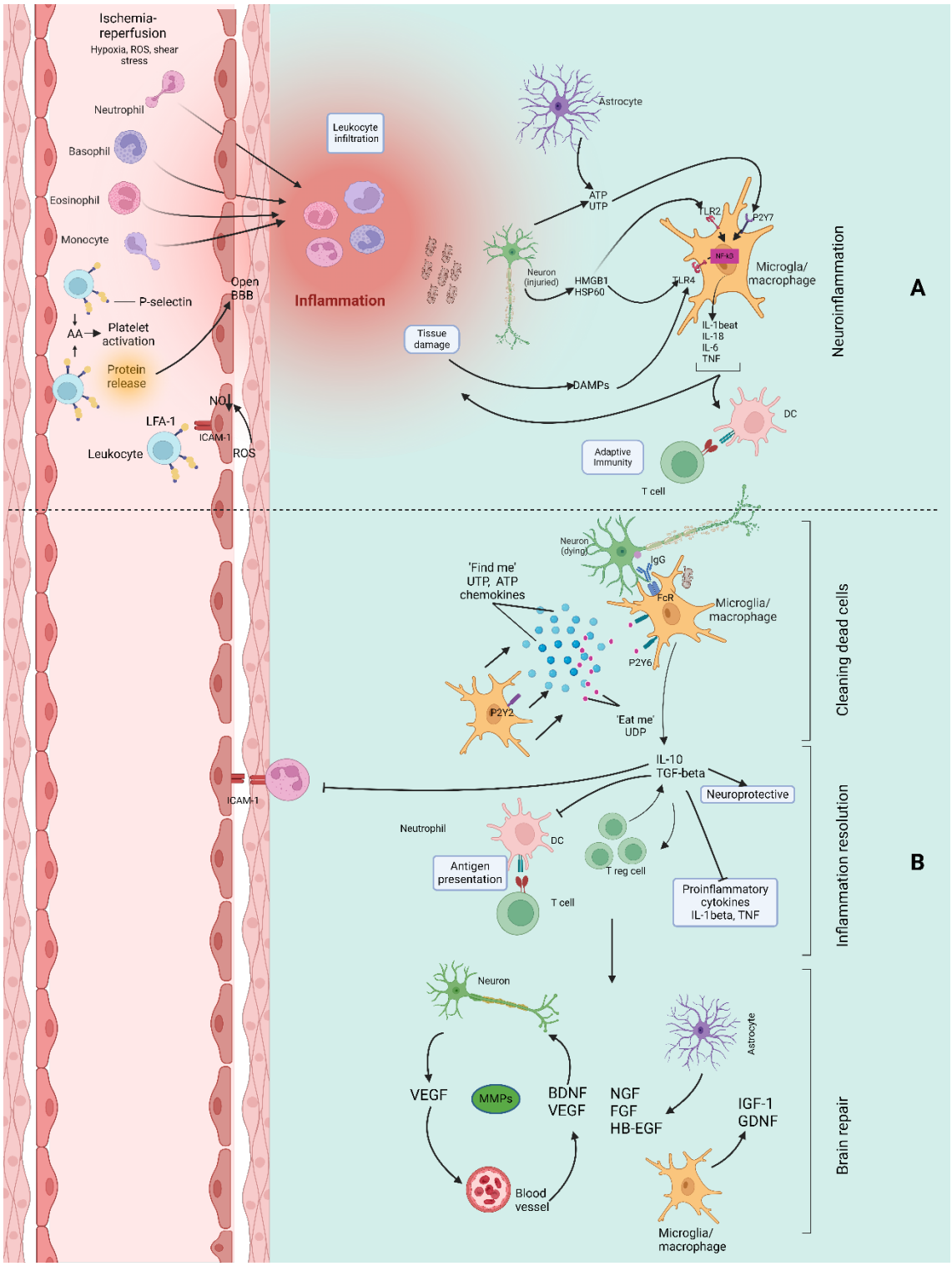


Figure 3 Initiation and resolution of post-stroke neuroinflammation. (A) Ischemic stroke triggers a cascade of events initiated by hypoxia and oxidative stress, which activate coagulation pathways, complement systems, platelets, and endothelial cells. The surface translocation of P-selectin on platelets and endothelial cells facilitates thrombus formation and cytokine secretion. Complement activation and arachidonic acid metabolite release further amplify inflammation. Endothelial expression of E- and P-selectins promotes initial leukocyte tethering via glycoprotein ligands like P-selectin glycoprotein ligand-1, while firm adhesion is stabilized through ICAM-1 interaction with β2 integrins such as LFA-1 and

Mac-1. Reduced nitric oxide availability contributes to vasoconstriction and cellular aggregation. Matrix metalloproteinase (MMP) activity undermines blood-brain barrier integrity and extracellular matrix stability, promoting immune cell infiltration. Damaged neurons release nucleotides that activate purinergic receptors on microglia and macrophages, resulting in pro-inflammatory cytokine production. Cellular necrosis liberates DAMPs—such as HMGB1, heat shock protein 60, and β-amyloid—which engage toll-like receptors (TLRs) (notably TLR2 and TLR4), activating NF-κB signaling. This intensifies cytokine production and leukocyte recruitment, aggravating injury and perpetuating inflammation. Finally, the release of neural antigens activates adaptive immunity via antigen presentation to T cells. **(B)** The resolution of inflammation following cerebral ischemia involves a coordinated response mediated by signaling molecules that suppress immune activation and promote repair[107]. Clearance of necrotic cells is critical for functional recovery, with nucleotides such as UTP and ATP serving as 'find-me' signals to recruit microglia and macrophages via P2Y2 receptors. Concurrently, 'eat-me' signals like UDP and phosphatidylserine engage P2Y6 receptors, facilitating the engulfment of apoptotic cells[108]. Immunoglobulins targeting CNS antigens bind Fc receptors on phagocytes, stimulating the release of anti-inflammatory cytokines like IL-10 and TGF-β[96]. These cytokines contribute to immune suppression by reducing antigen presentation, encouraging Treg differentiation, decreasing adhesion molecule expression on endothelial cells, and dampening pro-inflammatory cytokine production. Both IL-10 and TGF-β also exert neuroprotective effects that support recovery[109, 110]. Specialized pro-resolving lipid mediators, including lipoxins, resolvins, and protectins—derived from arachidonic acid and omega-3 fatty acids—further help to suppress post-ischemic inflammation[93]. Finally, resident CNS cells such as astrocytes, neurons, microglia, and oligodendrocytes secrete growth factors and matrix metalloproteinases, contributing to the structural remodeling required for tissue repair[106]. Diagram modified from [16].

The resolution of inflammation creates an environment conducive to regeneration, with angiogenesis and neuronal remodeling becoming key processes driving brain recovery and functional restoration[111-113]. These processes influence each other during post-stroke recovery, with various mediators identified as regulators of angiogenesis and axonal growth within the affected neurovascular units. Key factors include VEGF, TGF-β, angiopoietin-1, platelet-derived growth factor-B, BDNF, and progranulin[114-119].

Angiogenesis, the development of new capillaries from pre-existing vessels, is driven by VEGF signaling in response to oxygen-deprived tissue environments[111, 120]. This process typically begins 4–7 days post-stroke, especially at the border of the ischemic core[121]. Angiogenesis entails the coordinated activity of proteolytic enzymes, angiogenic growth factors, and inhibitors, all of which facilitate the migration and proliferation of endothelial cells[112]. Vascular endothelial growth factor A (VEGF-A) is significantly elevated during this process, stimulating endothelial cell proliferation and activating VEGF receptor 2. This interaction stimulates the release of matrix metalloproteinases (MMPs) and endothelial growth factors, which promote the migration of endothelial progenitor cells and the breakdown of the basal membrane.

Neuronal remodeling after stroke is primarily driven by surviving neurons from the initial injury and new neurons generated through neurogenesis[112]. Neurogenesis is the biological process by which neural stem or progenitor cells generate new, functional neurons through stages of proliferation, migration, and differentiation[111]. In the adult brain, this process predominantly occurs in two regions: the subventricular zone adjacent to the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. Notably, ischemic events have been shown to stimulate increased neurogenic activity in these areas[122]. Axons of undamaged neurons in the penumbra may degenerate if they connect with injured neurons. Axonal sprouting typically begins approximately 14 days after ischemia, even within the ischemic penumbra[121]. Functional recovery is often observed within three weeks post-stroke, with the formation of new cortical circuits[121]. The formation of new blood vessels after brain injury contributes to axonal sprouting by improving perfusion in damaged areas and secreting growth-promoting factors that aid neural regeneration. Cytokines released by microglia and infiltrating immune cells further activate astrocytes, supporting axonal outgrowth and the formation of new neural connections[16, 113, 123, 124].

1.2 Inflammasome in stroke

The immune system comprises two fundamental components: innate immunity and adaptive immunity. Innate immunity offers immediate and rapid protection but lacks specificity, whereas adaptive immunity is highly specific and takes several days to weeks to develop. The effective coordination between these two branches forms a comprehensive defense mechanism. A key function of the immune system is maintaining tissue homeostasis. For example, it must detect, and repair sterile tissue damage caused by injury, while also identifying and eliminating pathogens that invade and damage tissues. To achieve this, the immune system has evolved various receptors that can recognize both foreign structures and self-molecules. These receptors continuously monitor the extracellular space and intracellular compartments for signs of infection, damage, or other stressors. Among the key molecular platforms that mediate this surveillance and response is the inflammasome.

As a central component of the innate immune response, the inflammasome is a multiprotein complex that assembles in reaction to various physiological and pathological signals.

Inflammasome activation is essential for the innate immune response, which is key to eliminating pathogens and damaged cells. Recent research indicates that inflammasomes participate in activities beyond inflammation. For example, studies demonstrate that the AIM2 inflammasome contributes to neurodevelopmental processes, suggesting that inflammasomes may have roles that extend beyond their conventional function as inflammatory mediators[125].

1.2.1 Inflammasome activation

Inflammasomes are multi-protein platforms formed in response to cellular stress or pathogen invasion. These complexes typically comprise a cytosolic pattern recognition receptor (such as an Nod-like receptor (NLR) or pyrin and HIN domain-containing (PYHIN) family member), an adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain), and the zymogen form of caspase-1 (pro-caspase-1)[126]. NLR proteins are characterized by modular domains including a leucine-rich repeat (LRR) domain, a central NACHT domain for oligomerization, and a variable N-terminal domain, often a pyrin domain (PYD). Prominent NLR inflammasomes include NLRP1, NLRP3, and NLRC4, which initiate caspase-1 activation and downstream cytokine maturation[127, 128]. AIM2 and interferon-inducible protein 16 (IFI16), members of the PYHIN family, respond to cytosolic double-stranded DNA[126].

Each sensor is activated by specific stimuli. For instance, NLRP3 is triggered through a two-step process involving transcriptional priming followed by assembly in response to cellular damage or microbial products[129, 130]. Purinergic signaling through receptors like P2X7 can modulate these events[131]. Pathogen-specific signals, such as flagellin or bacterial toxins, engage NAIP/NLRC4 complexes, while dsDNA sensed by AIM2 may also activate cGAS-STING, linking DNA sensing to type I interferon and inflammasome pathways[132-136].

Upon activation, sensor proteins undergo confirmational changes and recruit the adaptor protein ASC, thereby leading to the formation of the inflammasome platform referred to as the ASC speck. This platform then facilitates the cleavage of pro-caspase-1. Although some inflammasomes (e.g., NLRP1 and NLRC4) can directly associate with pro-caspase-1 without ASC, ASC-mediated assembly is crucial for effective cytokine processing[137-140].

Activated caspase-1 cleaves the pro-forms of IL-1β and IL-18, converting them into their mature, bioactive forms that bind to their respective receptors and trigger transcriptional pathways, including those regulated by NF-κB[141]. IL-1β enhances inflammation by promoting immune cell activation and chemokine production, while IL-18 modulates interferon-γ release by T and NK cells. Caspase-1 also cleaves gasdermin D (GSDMD), generating membrane pores that lead to pyroptosis, a lytic and pro-inflammatory form of programmed cell death pyroptosis[142, 143]. During pyroptosis, ASC protein aggregates (specks) are expelled into the extracellular space and can be internalized by nearby cells, thereby amplifying inflammasome signaling in the surrounding environment[144-146].

Apart from this canonical pathway, cytosolic lipopolysaccharide (LPS) is detected through a noncanonical mechanism involving caspase-11 in mice and caspases-4 and -5 in humans[147]. This pathway leads to the activation of caspase-11 in mouse cells and caspase-4 and caspase-5 in human cells[148-150]. These caspases trigger pyroptosis independently of IL-1β and IL-18 maturation, distinguishing their role from caspase-1-mediated cytokine processing[148, 151].

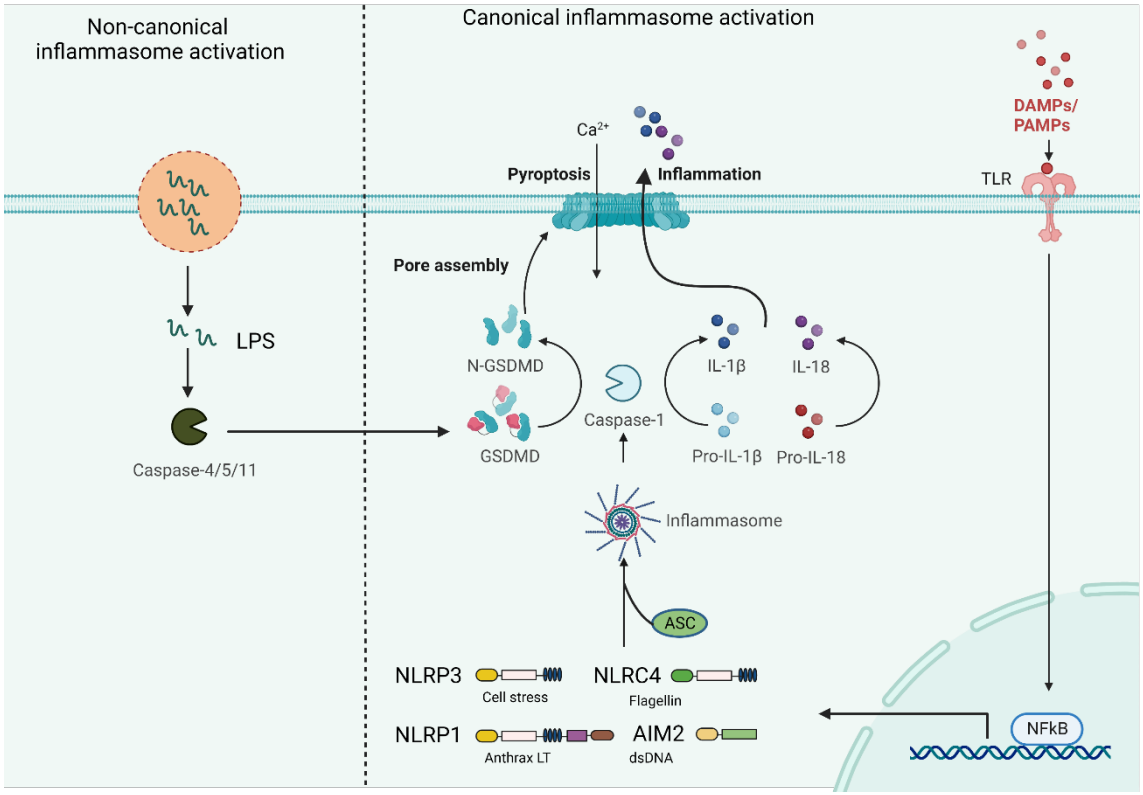


Figure 4 Inflammasome activation. Host detection of pathogens or internal damage signals leads to the activation of several sensor proteins, including NLRP1, NLRP3, NLRP5, NLRC4, and AIM2, which assemble inflammasomes that initiate similar immune responses. The priming step, often mediated through NF-κB activation, enhances the transcription of inflammasome components. Upon sensing cytosolic ligands or biochemical changes, these sensors undergo

oligomerization to facilitate the recruitment and activation of caspase-1. In certain contexts, an adaptor protein called ASC, which contains a CARD domain, is necessary to bridge the sensor and caspase-1. Activated caspase-1 then processes IL-1β and IL-18 into their active forms and induces pyroptotic cell death by cleaving gasdermin D. In parallel, a non-canonical inflammasome pathway exists in which intracellular lipopolysaccharide (LPS) activates caspase-11 in mice and caspase-4/5 in humans. These caspases initiate pyroptosis by cleaving gasdermin D and indirectly stimulate NLRP3 inflammasome activation through the induction of potassium ion efflux. Diagram adapted from [152].

1.2.2 The role of the inflammasome in stroke

Inflammasome components are mainly found in myeloid cells and in tissues abundant with innate immune cells[153-155]. Tissue expression profiles reveal that the CNS is equipped with NLRP1 and NLRP3 inflammasome components[154]. Microglia, a key CNS cell type, expresses both NLRP3 and NLRC4 inflammasomes[156-159]. Recent research has shown that inflammasomes are present not only in myeloid cells but also in non-myeloid cells within the CNS. Activation of NLRP1 and NLRP3 inflammasomes has been found to trigger pyroptosis in neurons[160-162]. Additionally, astrocytes express NLRP3, NLRC4, NLRP2, and NLRP6 inflammasomes[128, 163-165], and endothelial cells have been identified to express NLRP3 inflammasomes[166, 167].

Inflammasome activation has been extensively studied in neurological disorders, including ischemic stroke, where it triggers the neuroinflammatory response during the acute phase following ischemic injury. During ischemic stroke, necrotic cells in the infarcted core release DAMPs that activate inflammasome complexes, leading to pyroptotic cell death and the secretion of IL-1β and IL-18. These cytokines drive a sterile inflammatory response within the brain, amplifying tissue injury. Growing research over recent decades has established a pivotal role for inflammasome activation in the progression of stroke-related pathology.

Research examining mRNA and protein levels indicates a notable rise in NLRP3 expression within just 12 hours after transient middle cerebral artery occlusion (MCAO) in mice, reaching its peak at 24 hours and persisting past 48 hours[168]. A different study revealed that levels of NLRP1, NLRC4, AIM2, ASC, as well as NLRP3 mRNA, increased over time in rats after a stroke in the brain[169]. These inflammasomes have been detected in multiple cell types; for instance, NLRP1 was identified in microglia, astrocytes, and neurons in murine ischemic brain tissue, whereas NLRP3 was found in microglia and endothelial cells in both ischemic and hemorrhagic conditions[168, 170, 171]. Caspase-1 activity studies have further elucidated the function of the

inflammasome in stroke. Expression levels of caspase-1 and its cleaved form, p20, were elevated after both permanent[172, 173] and transient MCAO[174]. Increased caspase-1 expression was observed in neurons and astrocytes following thromboembolic stroke, with microglia showing increased caspase-1 activity 24 hours post-stroke[170].

These findings underline the significant role of inflammasome activation, particularly involving NLRP3, NLRP1, NLRC4, AIM2, and ASC, in ischemic stroke pathogenesis. The activation of these inflammasomes in various cell types within the ischemic brain highlights their significant impact on neuroinflammation. Researchers have investigated the effect of inflammasome activation on post-stroke outcomes to develop novel therapeutic strategies targeting these pathways. Numerous studies have demonstrated that inhibiting inflammasome activation post-stroke offers protective effects. Both pharmacological inhibition and genetic knockout (KO) of NLRP3 have been shown to reduce ischemic damage, leading to improved functional outcomes and less inflammatory pathology[168, 175-179]. However, NLRP3 is not the only inflammasome associated with post-stroke neuroinflammation. Denes et al. demonstrated that the AIM2 and NLRC4 inflammasomes, together with ASC, contribute to acute brain injury independently of NLRP3[180]. Furthermore, the involvement of caspase-1 in stroke pathogenesis has been confirmed through studies using transgenic caspase-1 KO mice and animals with dominant-negative caspase-1. These mice exhibited less cerebral damage compared to wild-type controls following stroke[181, 182]. Intra-cerebroventricular delivery of caspase-1 inhibitors like Ac-YVAD-cmk and VRT-018858 has demonstrated protective effects in stroke models[183-185].

Beyond the canonical inflammasome pathway, initial studies suggest that non-canonical inflammasome activation could also be significant following a stroke. Caspase-11 KO mice showed a notable decrease in apoptosis following permanent MCAO[186].

While most studies focus on the acute phase after stroke, some have also explored inflammasome activity in the chronic phase. A recent study found that the AIM2 inflammasome may influence post-stroke cognitive impairment during the chronic phase[187].

As mentioned, the cGAS-STING pathway is linked to inflammasome activation through its role in sensing dsDNA. DNA fragments released from injured neuronal and apoptotic immune cells accumulate in the ischemic region due to insufficient deoxyribonuclease[188-190]. Microglia engulf these DNA fragments, which are then recognized by cGAS, triggering the STING pathway,

a key player in persistent post-injury neuroinflammation[134, 191-193]. Research suggests that STING is predominantly upregulated in microglia, rather than neurons or astrocytes[194]. Inhibiting the cGAS-STING pathway after ischemic stroke helps reduce neuroinflammation[195-197]. Beyond its effects on innate immunity, cGAS-STING also facilitates T lymphocyte recruitment, maturation, and differentiation, bridging innate and adaptive immunity[198]. However, additional research is required to fully comprehend the molecular mechanisms through which cGAS affects neuroinflammation following ischemic stroke.

In summary, the role of inflammasome activation in the pathogenesis of stroke is increasingly recognized, offering promising avenues for therapeutic intervention aimed at mitigating inflammation-driven injury in ischemic stroke. Several clinical trials have tested interventions aimed at improving post-stroke outcomes. For instance, the IL-1 receptor antagonist (IL-1Ra), which competitively inhibits IL-1α and IL-1β by binding to the IL-1 type I receptor without triggering an intracellular signal, has shown promise[199]. A phase 2 randomized controlled trial, which involved patients presenting within 6 hours of acute stroke onset, showed that IL-1Ra enhanced clinical outcomes, particularly for patients with cortical involvement infarcts[200]. Similarly, in another randomized phase 2 trial, IL-1Ra reduced inflammatory markers associated with worse outcomes, although further exploration of its mechanisms is required[201].

Given the current evidence, it is increasingly important to close the knowledge gap regarding the clinical application of IL-1 inhibition in stroke management. The optimal therapeutic window for targeting IL-1 signaling remains poorly defined. While IL-1 contributes to inflammation, it also supports recovery processes such as angiogenesis, cellular proliferation, and growth factor release[202]. As such, careful consideration of the timing of inflammasome inhibition is necessary to maximize therapeutic benefit while minimizing potential risks associated with premature intervention.

1.3 Open Questions and aims of the thesis

The existing literature not only highlights the crucial involvement of inflammasome activation in the development of stroke but also presents promising avenues for targeted interventions to address the impact of inflammasomes in ischemic stroke. However, significant knowledge gaps persist, particularly in understanding the mechanisms of post-stroke inflammasome activation and

the cell type-specific contributions. Critical information regarding the therapeutic window and potential side effects of inflammasome inhibition remains elusive. As a result, further pre-clinical research is warranted to enhance our understanding in this domain and pave the way for advancements in stroke management.

Therefore, this thesis aims to assess the contribution of inflammasomes to recovery, regeneration, and neuroinflammatory burden during the chronic phase after experimental stroke. To achieve this goal, we will focus on the following specific aims:

1: Characterize the time course of inflammasome activation after stroke.

2: Analyze the cell-type-specific contribution to inflammasome activation.

3: Examine the role of inflammasomes in post-stroke neuroinflammation.

4: Investigate the effect of inflammasome activation on post-stroke regeneration and recovery.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and instrument

Centrifuge 5417 R	Eppendorf (Heidelberg, Germany)
Centrifuge 5810R	Eppendorf (Heidelberg, Germany)
Confocal	Zens LSM980
Cytek NL-3000	Cytek Biosciences
Fusion Fx7	Vilber
Gentle MACS Octo Dissociator	Miltenyi Biotec (Bergisch Galdbach, Germany)
Homogenizer Dounce, 7 ml	Carl Roth (Karlsruhe, Germany)
LightCycler 480	Roche Diagnostics
MACS multistand magnet	Miltenyi Biotec (Galdbach, Germany)
iMark Microplate Absorbance Reader	BioRad
Xcell SureLock Mini-cell Blot Modul	Invitrogen
Mini-Protein-Gele 4 bis 12% Bis-Tris	Invitrogen
MJ Research PTC-200 Thermal Cycler	Marschall Scientific
Nanodrop 2000 spectrophotometers	Thermo Fisher Scientific
PF5010 Laser Doppler Perfusion monitor	YUMPU
TSE system rotarod	TSE systems (Berlin, Germany)
TC 20 Automated Cell Counter	Bio-Rad (Hercules, CA, USA)
Leica VT 1200S Vibratome	Leica Microsystem (Wetzlar, Germany)

2.1.2 Reagents and consumables

1X HBSS no calcium, no magnesium	Thermo Fisher Scientific
7-0 MCAO suture Re L12 PK 5	Doccol (Sharon, MA, USA)
10X HBSS, no calcium, no magnesium	Thermo Fisher Scientific
10X Tris-Glycine-SDS Running Buffer	Bioland
10ml Syringe	BD Biosciences
18G Syringe needle	BD Biosciences
21G Syringe needle	BD Biosciences
4% PFA solution	Sigma-Aldrich (St. Louis, MO, USA)
5ml syringe	BD Biosciences
Adult mice brain dissociation kit	Miltenyi Biotec
Albumin fraction V/ BSA	Sigma-Aldrich
Brilliant Stain Buffer	BD Biosciences
Pierce BCA Protein Assay kit	Thermo Fisher Scientific
Wound Disinfection spray	HPG Health Pharma GmbH
Bepanthen eye and nose ointment	Bayer Vital GmbH (Leverkusen, Germany)
Falcon Round-bottom FACS tube	Fisher Scientific
Flow Cytometry staining	Thermo Fisher Scientific
Falcon tube 15ml	Thermo Fisher Scientific
Falcon tube 50ml	Thermo Fisher Scientific
HEPES	Sigma-Aldrich
Immobilon Western HRP substrate	Merck (Darmstadt, Germany)
Isoflurane	CP-Pharma (Burgdorf, Germany)
Laminin Sample Buffer	Bio-Rad

LS column	Miltenyi Biotec
MACS tubes	Miltenyi Biotec
Myelin removal beads	Miltenyi Biotec
Microglia selection beads	Miltenyi Biotec
Mounting medium	Thermo Fisher Scientific
RIPA buffer	Thermo Fisher Scientific
Percoll Plus	Cytiva (Opfikon, Schweiz)
Phosphatase Inhibitor	Thermo Fisher Scientific
Precision Plus Protein All Blue Standards	Bio-Rad
ProFlow Cell Filters 30um filter	Bio-Rad
ROTI-free stripping buffer	Carl Roth
RPMI Medium 1640	Thermo Fisher Scientific
RNeasy Micro Kit	QIAGEN
Tamoxifen	Sigma Aldrich

2.1.3 Buffers and Solutions

Tissue lysis buffer 50ml

RIPA buffer	50ml
Phosphatase Inhibitor	1 tablet

Western Blot Transfer buffer

NuPAGE Transfer Buffer (20X)	1X	50ml
Methanol		100ml
MilliQ-H2O		Top up to 1L

4% BSA

Albumin Fraction V/ BSA	4%	4g
TBST		100 ml

5% goat serum in PBS

Goat serum	5%	0.5 ml
PBS		10 ml

Tris-buffered saline (TBS)

NaCl	150 mM	8.76 g
Tris	20 mM	2.42 g
MilliQ-H2O		1 L

TBS with 0.1% Tween (TBST)

Tween-20	0.1%	1 mL
TBS		1 L

FACS buffer (0.5 % BSA in PBS)

BSA	0.5%	0.5 g
PBS		100 ml

Primary Antibody Dilution Buffer (staining)

1% BSA	2 g
0.1% cold fish skin gelatin	0.2 g

0.5% Triton X-100	1 ml
0.05% sodium azide	0.1 g
0.01 M PBS	200 ml

Adjust pH 7.2-7.4

Secondary Antibody Dilution Buffer (staining)

0.05 Tween 20	0.1 ml
0.01 M PBS	200 ml

Adjust pH to 7.2

100% SIP (stock isotonic Percoll) 100 ml

Percoll Plus	90 ml
HEPES	2 ml
10X HBSS	8 ml

70% SIP 10 ml

100% SIP buffer	7 ml
1X HBSS	3 ml

20% Percoll 50 ml

Percoll Plus	10 ml
1X PBS	40 ml

1 M HEPES in HBSS

1X HBSS	500 ml

HEPES	5 ml

4% Agarose

Agarose	8 g
MilliQ-H2O	200 ml

Phosphate-buffered saline (PBS)

NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
KH2PO4	1.5 mM	0.2 g
Na2HPO4x2H2o	8.1 mM	1.44 g
MilliQ-H2O		1 L

2.1.4 Antibodies

Rabbit polyclonal anti-Caspase-1 antibody	Cell Signaling Technology	3866S
Mouse anti-β-Actin antibody	Cell Signaling Technology	3700S
Goat HRP-conjugated anti-rabbit secondary antibody	Sigma-Aldrich	12-348
Goat HRP-conjugated anti-mouse secondary antibody	Abcam	ab97023
Anti-NeuN antibody, clone A60	Merck Millipore	MAB377
Rabbit anti-Iba1 antibody	FUJIFILM Wako Chemicals	019-19741
Chicken anti-GFAP antibody	Abcam	ab4674
Guinea pig anti-VGlut1 antibody	Merck Millipore	AB5905

Chicken anti-Homer antibody	Synaptic Systems	160006
Goat anti-guinea Pig secondary antibody, Alexa fluor 488	Abcam	ab150185
Goat anti-chicken secondary antibody Alexa fluor 647	Abcam	ab150171
Goat anti-rabbit secondary antibody Alexa fluor 594	Invitrogen	A-11037
Goat anti-mouse secondary antibody Alexa fluor 405	Invitrogen	A-31553
eF450 anti-mouse CD45	eBioscience	48-0459-42
BV421 anti-mouse CD3	BioLegend	100227
APC-Cy7 anti-mouse CD45	BioLegend	103116
BV510 anti-mouse CD8a	BD Horizon	563068
BV570 anti-mouse CD19	BioLegend	115535
FITC anti-mouse CD11b	BD Horizon	562793
PE-eF610 anti-mouse Ly6G	eBioscience	61-9668-82
PerCp-Cy5.5 anti-mouse Ly6C	BioLegend	45-5932-82
PE-Cy7 anti-mouse F4/80	eBioscience	25-4801-82
APC-Fire810 anti-mouse CD4	BioLegend	123166
PE anti-mouse MHC-II	eBioscience	12-5321-82
PerCp-Cy5.5 anti-mouse CD11b	eBioscience	45-0112-82
BV570 anti-mouse Ly6C	BioLegend	108431
PE-eF610 anti-mouse Ly6G	eBioscience	61-9668-82
BV510 anti-mouse CD3	BioLegend	100233
PE anti-mouse O4	Miltenyi Biotec	130-117-357

PE-Vio615 anti-mouse ASCA2	Miltenyi Biotec	130-116-146
PE-Vio770 anti-mouse CD49a	Miltenyi Biotec	130-125-102
Zombie NIR	BioLegend	423106

2.1.5 Software

Carl Zeiss Zen 2010

FlowJo 10.0 software

GraphPad Prism 6.0 software

ImageJ software

Imaris

R (Version 4.2.3)

2.2 Methods

2.2.1 Animals

All animal procedures were conducted in accordance with ethical standards and were approved by the local governmental authority (Regierungspraesidium Oberbayern, Munich, Germany). The mice used in the study, including wildtype (WT) C57BL6/J mice and Casp1$^{-/-}$ mice (B6N.129S2-Casp1$^{<tm1Flv>}$/J) were bred and maintained in the animal facility of the Centre for Stroke and Dementia Research, Munich, Germany. ASC-Citrine reporter mice (B6. Cg-Gt (ROSA)26Sor$^{tm1.1(CAG-Pycard/mCitrine*, -CD2)Dtg}$/J) were originally obtained from the Jackson Laboratory, located in Bar Harbor, USA. Mice were maintained on a 12-hour light/dark cycle with unrestricted access to food and water.

The analysis excluded data from mice that died during surgery, with the exclusion criteria detailed as follows. Through a randomized process, mice were allocated to treatment groups, and all analyses were conducted by investigators who remained blinded to the specific group

assignments. In strict accordance with the ARRIVE guidelines for animal research reporting，all experimental procedures were meticulously conducted[203].

2.2.2 Transient middle cerebral artery occlusion (MCAO) model

Transient middle cerebral artery occlusion was conducted as outlined in a previous study[204]. Mice were anesthetized using isoflurane in a mixture of 30% oxygen and 70% nitrous oxide. A small incision near the temporal bone provided access for skull-mounted placement of a laser Doppler probe to monitor cerebral blood flow. After exposing the carotid arteries via a midline neck incision, the common and external carotid arteries were ligated. A silicone-coated 2-mm filament was advanced through the internal carotid artery until resistance was felt and cerebral blood flow dropped by more than 80%, confirming occlusion. After 60 minutes, the filament was withdrawn under anesthesia, and animals were allowed to recover with unrestricted access to food and water. Control animals underwent an identical procedure without actual occlusion. Throughout the surgery, a temperature-controlled heating pad maintained core body temperature at 37°C.

Animals were excluded based on the following criteria: 1) insufficient occlusion (less than 20% blood flow reduction), 2) surgical mortality, or 3) absence of infarction as verified by histological analysis. The control group had no fatalities, whereas the occlusion group showed a mortality rate of approximately 20%.

2.2.3 Photothrombotic (PT) stroke model

PT was induced as previously described [205]. Prior to being secured in a stereotactic frame for precise positioning, the mice were anesthetized with isoflurane, ensuring accurate experimental conditions. Throughout the procedure, a heating pad was employed to ensure their body temperature remained consistently stable at 37°C. To prevent ocular dryness, Dexpanthenol ophthalmic ointment was applied to both eyes. Before the administration of anesthesia, the animals received an intraperitoneal injection of 1% Rose Bengal (10 μl/g body weight, Sigma-Aldrich, 198250-5g) in saline, which was delivered 5 minutes prior to the introduction of the anesthetic (5% isoflurane). To ensure precise targeting, the skull was exposed through a surgical incision, and the bregma was subsequently identified. The lesion site, located 1.5 mm lateral and

1.0 mm rostral to the bregma, was identified within the left cerebral hemisphere. To focus the laser on the designated lesion area, a shield was strategically placed over the skull. Following a 10-minute interval after the administration of Rose Bengal, a 561 nm laser (Cobolt Jive 50, 25 mV output) was employed to irradiate the lesion site for a duration of 17 minutes, with the beam being transmitted through a fiber collimation package that ensured a beam diameter of approximately 1.4 mm.

2.2.4 Neuroscore assessment

A comprehensive assessment tool, the multiparametric neuroscore, is designed to evaluate both global and focal neurological impairments in a detailed manner. The assessment of global impairments is achieved through the systematic evaluation of grooming behaviors, auditory and visual functions, postural control, spontaneous motor activity, and the manifestation of epileptic episodes. A series of tests, including gait analysis, grip strength assessment, forelimb asymmetry during tail suspension, circling behavior (either whole-body or specific to one forelimb), body symmetry evaluation, and whisker sensitivity measurement, are utilized to assess focal impairments[206]. The total score, which ranges from 0 to 54 points, includes 26 points indicating overall deficits and 28 points representing specific deficits. A more severe level of impairment is indicated by a higher score. Following the induction of stroke, data were meticulously recorded at the baseline and subsequently on days 1, 3, 5, 7, 9, 11, 13, 15, 21, and 28 post-inductions.

2.2.5 Rotarod test

Prior to the baseline assessment and the surgical procedure (transient MCA occlusion or sham), mice were subjected to daily training over a three-day period. The evaluation of baseline performance was conducted through the following protocol[207]: the rod underwent continuous acceleration from 10 to 40 rpm within a 300-second timeframe. At each timepoint, three consecutive trials were carried out for every mouse. The duration until the mice fell off the rod was meticulously recorded.

2.2.6 Adhesive removal test

This test was used to assess sensory and motor deficits. On the palmar side of the forepaw, a 4 mm-diameter adhesive sticker (Neolab, Germany) was carefully positioned, ensuring consistent pressure application during each placement procedure. Two days before the acquisition of baseline data, the mice were acclimatized to both the test cage environment and the adhesive placement protocol[208]. Three consecutive trials were conducted for each mouse to perform both baseline and postsurgical assessments. The time intervals from the initial contact with the adhesive to the complete removal of the adhesive were meticulously recorded. While sensory performance was reflected in the latency to initially contact the adhesive, motor performance was assessed through the differential between the latency to make contact and the latency to remove the adhesive.

2.2.7 Tissue and organ harvesting from mice

Prior to transcardial perfusion with normal saline, mice were initially anesthetized using a combination of ketamine (120 mg/kg) and xylazine (16 mg/kg). Following removal, the brains were carefully positioned in tubes containing Hank's balanced salt solution (HBSS) to facilitate subsequent Western blotting and FACS analysis. Prior to brain extraction, the mice were perfused with 20 ml of aldehyde fixative solution for immunofluorescence staining, after which the extracted brains were subsequently stored in tubes filled with 4% paraformaldehyde.

2.2.8 Enzymatic Brain Dissociation

Utilizing the adult mouse brain dissociation kit provided by Miltenyi Biotec, the brain tissue was enzymatically dissociated through a systematic separation process. The cerebral hemisphere was meticulously divided into smaller fragments and subsequently transferred into a MACS tube. The tissue was treated with a mixture comprising 1,950 µl of enzyme mix 1 and 30 µl of enzyme mix 2. Affixed to the sleeve of the mild MACS Octo Dissociator equipped with heaters, the inverted MACS tube underwent the execution of the 37C_ABDK_01 program. After dissociation, the resulting cell suspension was carefully filtered through a 70-µm cell strainer into a 50 ml Falcon tube, followed by centrifugation at 300 g for 10 minutes at 4°C to facilitate the washing process. In accordance with the manufacturer's methodology, myelin debris was effectively eliminated

through the utilization of Myelin Removal Beads in conjunction with LS columns. Following the generation of the flow-through with individual cells, centrifugation was performed at 310 g for 5 minutes at a temperature of 4°C. The pellet, which comprised a single-cell suspension containing microglia, astrocytes, and vascular endothelial cells, was successfully obtained. For the isolation of oligodendrocytes, the LS column was first rinsed with 3 ml of HBSS, then carefully detached from the magnet, after which its contents were transferred into a sterile 15-ml conical tube, followed by centrifugation at 310 g for 5 minutes at 4°C. At 4°C, the pellet was resuspended in 5 ml of 20% isotonic Percoll PLUS within 1× PBS and subsequently centrifuged at 310 g for 20 minutes with minimal braking applied. The pellet, which was composed of oligodendrocytes, astrocytes, and microglia, underwent a rinsing process with HBSS to remove any remaining Percoll PLUS before being reincorporated into the initial single-cell suspension.

2.2.9 Mechanical Brain Dissociation

Brains were harvested as previously outlined. After the initial removal of the olfactory bulb and cerebellum, the residual brain was subsequently divided into hemispheric sections. Each hemisphere was carefully positioned in a 7 ml Dounce homogenizer, which contained 3 ml of RPMI 1640 medium supplemented with phenol red. Initially, the hemisphere underwent homogenization through 20 strokes with a loose-fitting pestle (size A), and subsequently, an additional 20 strokes were performed using a tight-fitting pestle (size B). The cell suspension obtained was transferred to a 15 ml Falcon tube, into which 4 ml of RPMI and 3 ml of SIP solution were subsequently added, thereby achieving a final SIP concentration of 30%. Subsequently, the suspension was transferred via a 10 ml syringe equipped with a 21G needle into a distinct 15 ml Falcon tube filled with 70% SIP. At 18°C, the tube was subjected to gradient centrifugation at 500 g for 30 minutes, during which the centrifuge was gently stopped to avoid any disturbance to the interphase. The intermediate layer, which contained the cell suspension, was carefully extracted using a 5 ml syringe equipped with an 18G needle, while the upper layer, including myelin, was meticulously excised. The suspension obtained was transferred to a 15 ml Falcon tube containing 8 ml of 1X HBSS/10 mM HEPES and centrifuged at 500 g for 7 minutes at 4°C. Following the removal of the supernatant, the remaining pellet was resuspended in 3 ml of FACS buffer and then subjected to filtration through a 30 µm non-sterile filter. After an additional wash was performed, the cell suspension was carefully prepared for subsequent microscopic examination.

2.2.10 Neural cell caspase-1 detection via FLICA660 flow cytometry

To detect active caspase-1 forms within brain samples, cell suspensions were treated with the fluorescent inhibitor probe 660-YVAD-FMK (FLICA660 Caspase-1 Assay Kit, BioRad, Germany) for a duration of 30 minutes at 37°C, strictly following the manufacturer's recommended protocol. Prior to extracellular antibody labelling, all samples were treated with Fc blocking (Anti CD16/CD32, Invitrogen, US) subsequent to the washing procedure. To delineate immune and non-immune cell types, two antibody panels were utilized in the experiment. For the purpose of identifying caspase-1-positive immune cells within the central nervous system, the suspension was stained with CD45-eF450 (1:250), CD11b-PerCP-Cy5.5 (1:250), Ly6G-PE-AF610 (1:250), Ly6C-BV570 (1:250), CD3-FITC (1:250), and Propidium iodide-PE (1:250), in accordance with the gating strategy depicted in Fig 4. For the purpose of identifying caspase-1-positive cells within the central nervous system, the suspension was subjected to staining with CD45-eF450 (1:250), CD11b-PerCP-Cy5.5 (1:250), Ly6C-BV570 (1:250), Ly6G-PE-eF610 (1:250), CD3-BV510 (1:250), O4-PE (1:250), ASCA2-PE-Vio615 (1:250), CD49a-PE-Vio770 (1:250), and Zombie-NIR (1:200), while employing the gating strategy illustrated in Fig 5. The Northern Light spectral flow cytometer (Cytek) was employed to acquire flow cytometry data, while the analysis was performed using FlowJo software (version 10.0).

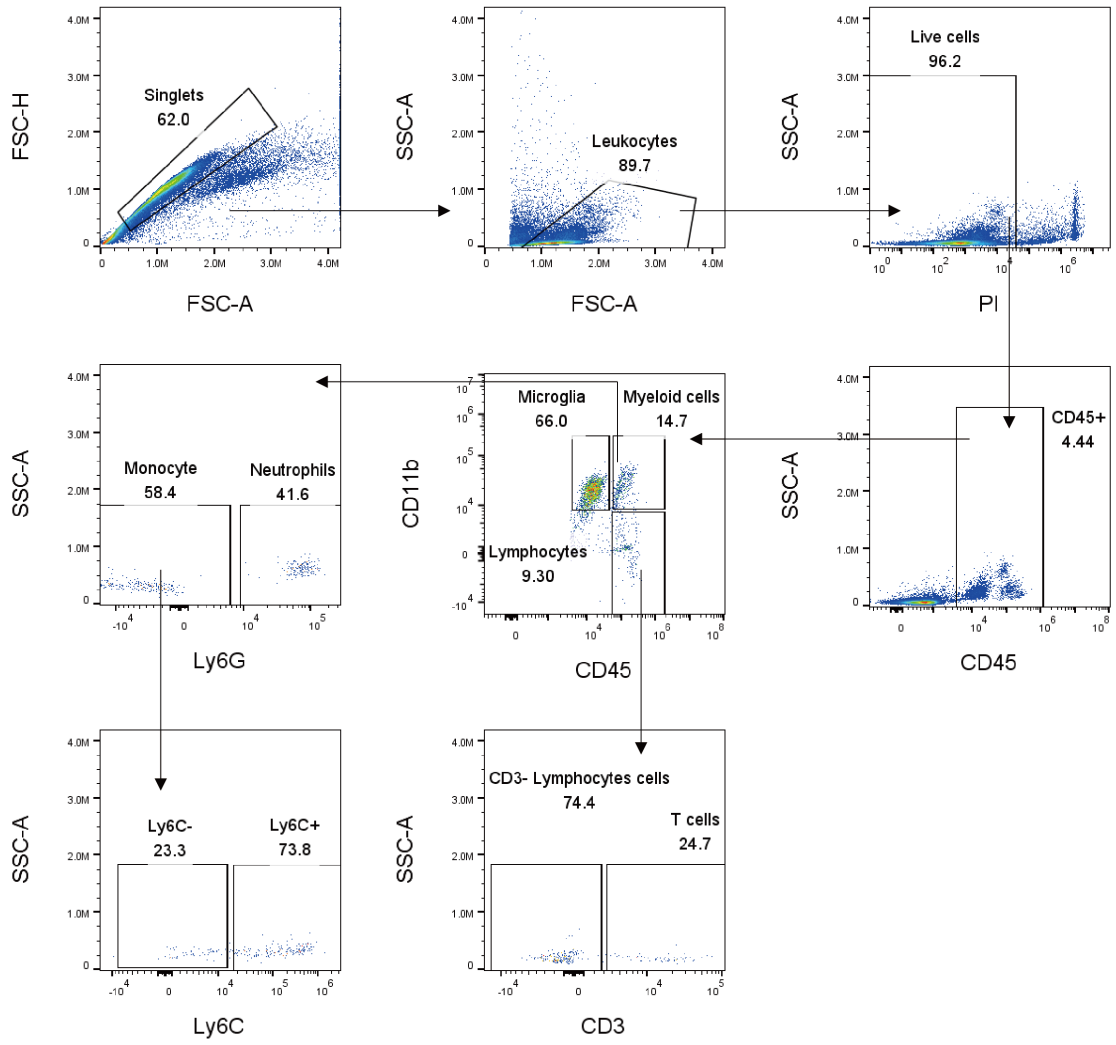


Figure 5 Flow cytometry gating strategy for immune cells in the post-stroke brain. Microglia and infiltrating myeloid cells were identified through a stepwise gating strategy: CD45+ cells (leukocytes) were first gated from singlets. CD45 mid CD11b+ cells were recognized as microglia. CD45 high were recognized as infiltrated leukocytes. CD45 high CD11b - were identified as lymphocytes. CD45 high CD11b+ were recognized as myeloid cells. Neutrophils (CD45 high CD11b+ Ly6G high) and monocytes/ macrophages (CD45 high CD11b+ Ly6G-Ly6C+) were gated from myeloid cells.

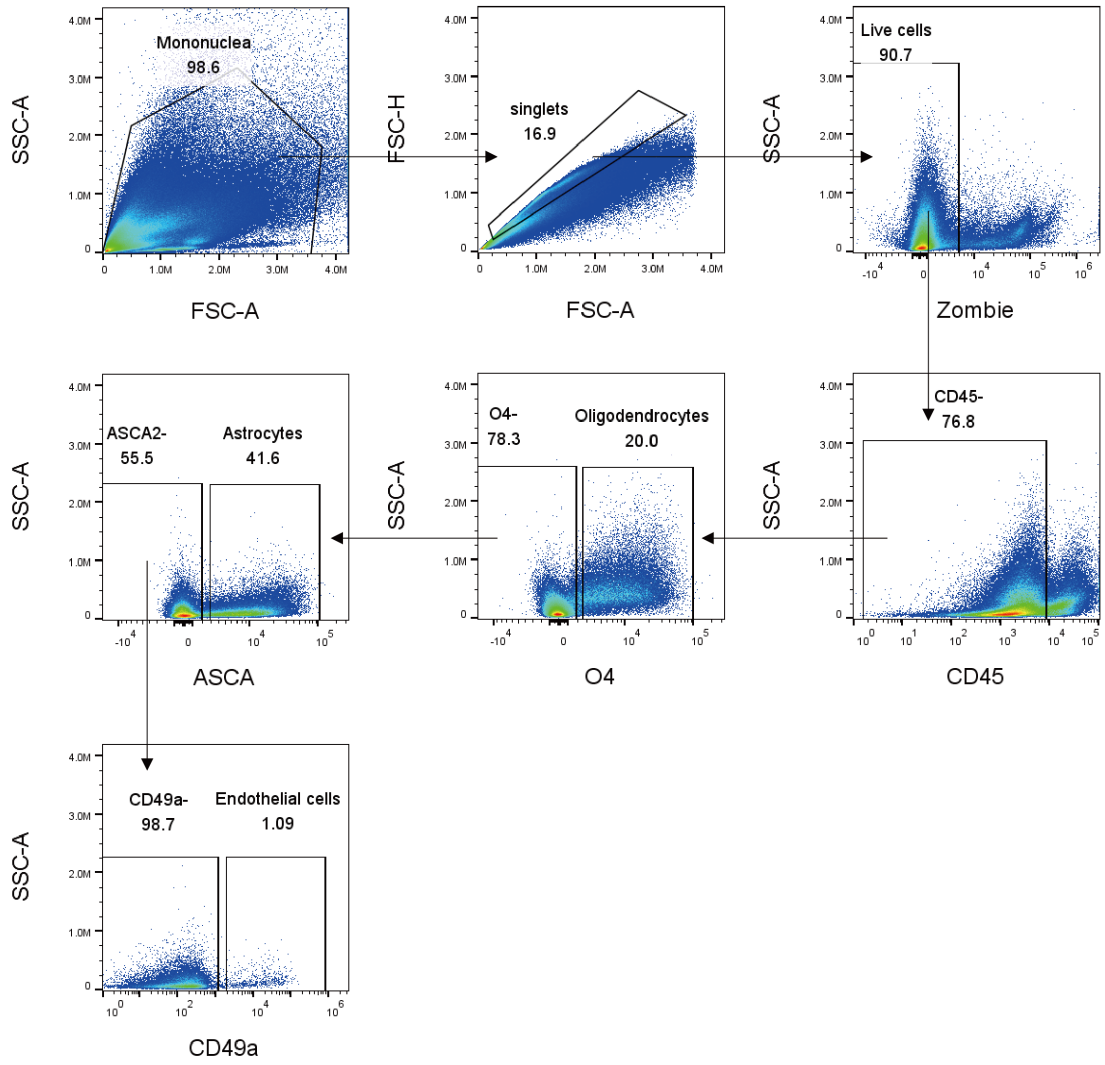


Figure 6 Flow cytometry gating strategy for non-immune cells in the post-stroke brain. CD45- cells were gated first from live single cells. Astrocytes were defined as CD45- ASCA2+, oligodendrocytes as CD45- O4+, and endothelial cells as CD45- CD49a+.

2.2.11 Neuroinflammation FACS for infiltrated immune cells

For the purpose of evaluating neuroinflammation following a stroke, an intravenous injection of 3 µg CD45-APC-Cy7 (103116, clone: 30-F11; BioLegend) was administered to mice three minutes before transcardiac perfusion to effectively reduce blood contamination. Following an initial staining procedure with the Zombie NIR Fixable Viability Kit (1:200; BioLegend), the cell culture subsequently underwent surface marker labeling utilizing Brilliant Stain Buffer (BD Biosciences). The following antibodies were utilized: CD45-eF450 (1:200), CD3-BV421 (1:50), CD8a-BV510 (1:100), CD19-BV570 (1:200), CD11b-FITC (1:200), Ly6G-PE-eF610 (1:200), Ly6C-PerCp-Cy5.5 (1:100), F4/80-PE-Cy7 (1:100), CD4-APC-Fire810 (1:500), and MHC-II-PE (1:500) were utilized

in the study. Figure 7 provides an illustration of the gating technique. Through the utilization of a Northern Light spectral flow cytometer (Cytek), flow cytometry data were acquired, and subsequent analysis was performed using FlowJo software (version 10.0).

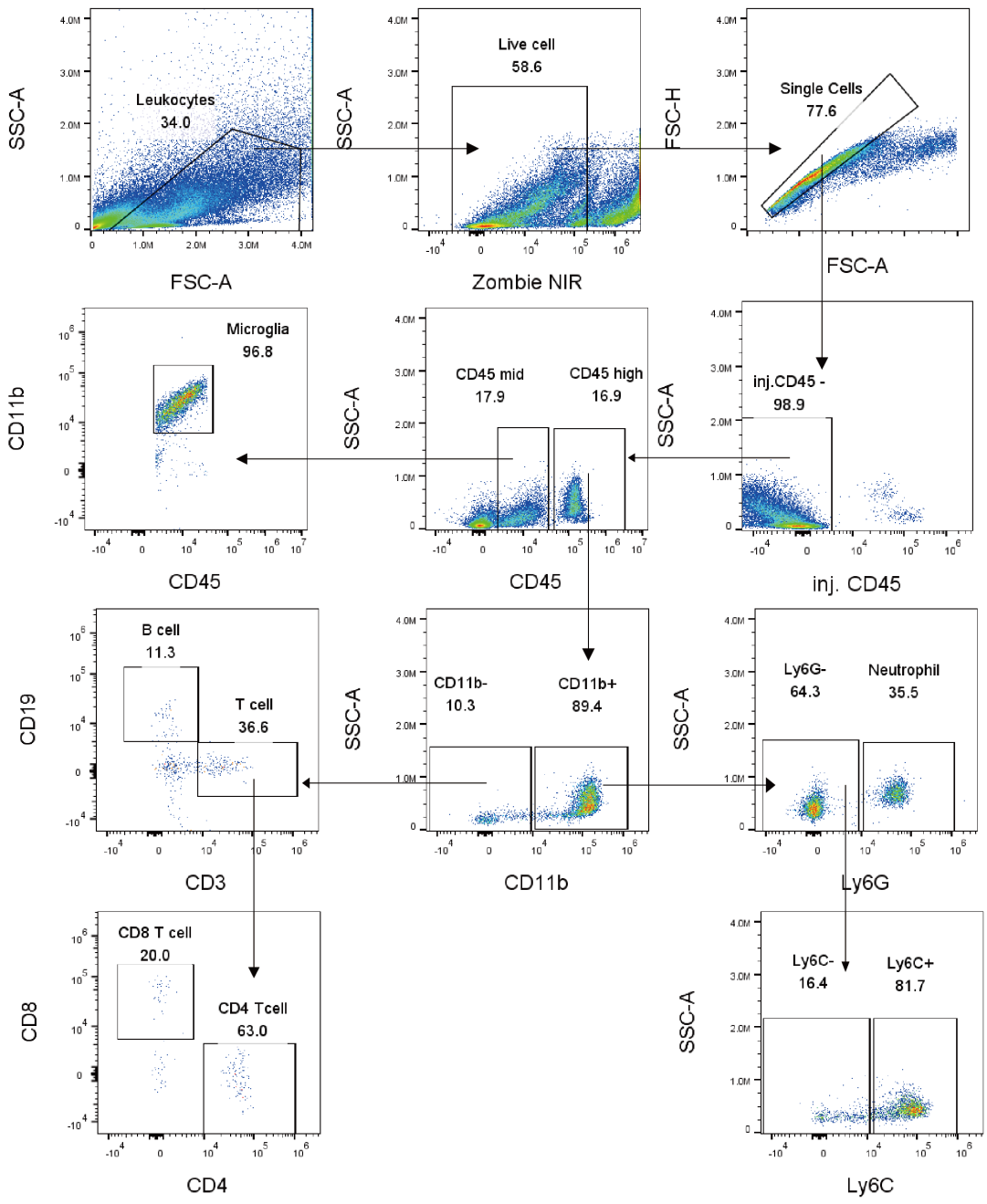


Figure 7 Gating strategy for neuroinflammation. Live single cells were gated for CD45 positivity and subdivided by cell markers. CD45 intermediates were classified as microglia, while CD45 high cells were identified as infiltrating immune cells, which were further separated into myeloid cells and lymphocytes based on CD11b staining. Myeloid cells were assessed for Ly6G and Ly6C: CD11b+/Ly6G+ were considered neutrophils, CD11b+/Ly6C+ as inflammatory monocytes/macrophages, and CD11b+/Ly6C- as tissue-repairing monocytes/macrophages. Lymphocytes were analyzed for CD19, CD3, CD4, and CD8: CD11b-/CD19+ were B cells, CD11b-/CD3+/CD4+ helper T cells, and CD11b /CD3+/CD8+ cytotoxic T cells.

2.2.12 Western Blot

As previously described, brains from deeply anesthetized mice were extracted and hemisected. The tissue was homogenized in RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific, US). Protein concentrations were measured using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, US). Equal amounts of protein were resolved via SDS-PAGE and transferred to PVDF membranes (BioRad, Germany). Membranes were blocked for 1 hour at room temperature in TBS-T (0.1% Tween-20, pH 8.0) containing 4% non-fat dry milk (Sigma, Germany), followed by incubation with primary antibodies against caspase-1 (1:1000; AdipoGen, US) and β-actin (1:1000; Sigma, Germany). After three washes with TBS-T, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000; Dako, Denmark) for 1 hour. Signals were developed using ECL substrate (Millipore, US) and visualized with the Vilber Fusion Fx7 imaging system.

2.2.13 Immunofluorescence staining on free-floating sections

For the purpose of examining the temporal dynamics and distribution patterns of ASC-speck formation among diverse cell types, brain slices obtained from ASC-citrine animals were subjected to immunostaining using the neuronal marker NeuN, the microglial marker Iba1, and the astrocyte-specific marker GFAP. In summary, brain slices were incubated with 5% goat serum at ambient temperature for one hour. Subsequently, the sections were incubated overnight at 4°C with primary antibodies: mouse anti-NeuN, chicken anti-GFAP, and rabbit anti-Iba1, each of which was diluted 1:200 in a primary antibody dilution buffer. After completing the washing procedure, the secondary antibodies (goat anti-chicken Alexa Fluor 647, goat anti-rabbit Alexa Fluor 594, and goat anti-mouse Alexa Fluor 405, all diluted at a ratio of 1:200) were applied and subsequently incubated for a duration of one hour.

To investigate the role of inflammasome activation in post-stroke synaptic plasticity, brain slices obtained after stroke were stained with the pre-synaptic marker VGlut1 (Gp-Millipore, #AB5905, 1:1000) and the post-synaptic marker Homer (Ch-Synaptic Systems, #160006, 1:2000), while the samples were examined under a fluorescence microscope and the resulting images were subsequently analyzed utilizing ImageJ and Imaris software. At room temperature, the sections were incubated with 5% goat serum for a duration of 1 hour. At 4°C, the primary antibodies were

incubated for a duration of three days in a specifically prepared primary antibody dilution buffer. After six thorough washing steps, the secondary antibodies (goat anti-Guinea Pig Alexa Fluor 488 and goat anti-Chicken Alexa Fluor 647, diluted at a ratio of 1:500 in the secondary antibody dilution buffer) were applied and subsequently incubated for a duration of 18 hours. DAPI (4'-diamidino-2-phenylindole)-containing mounting media was utilized for conducting nuclear counterstaining. Under a fluorescence microscope, samples were meticulously examined, and the captured photographs were subsequently analyzed utilizing ImageJ software..

2.2.14 Statistical analysis

Data were analyzed utilizing GraphPad Prism version 6.0. Unless stated otherwise, all summary data are expressed as mean ± standard deviation (s.d.). The Shapiro-Wilk normality test was employed to evaluate the normality of the data sets. Comparisons for normally distributed independent data were conducted using a two-way Student's t-test for two groups or ANOVA for more than two groups. Dependent data conforming to a normal distribution were examined via a two-way ANOVA. Non-parametric data were evaluated using the Mann-Whitney U test for two groups or the Kruskal-Wallis test for many groups. Homogeneity of variance was maintained across all groups exposed to statistical analysis. P-values were modified for multiple comparisons utilizing Bonferroni correction or Dunn's multiple comparison tests. A p-value below 0.05 was deemed statistically significant.

3. Results

3.1 Inflammasome activation in the CNS in the chronic phase after stroke.

3.1.1 Different stroke models lead to different inflammasome activations in CNS.

As noted earlier, recent reports indicate that the inflammasome plays a role in several CNS diseases, such as Alzheimer's disease, ischemic stroke, and traumatic brain injuries[180, 209, 210]. To investigate the role of the inflammasome in the progression of ischemic stroke, the time-dependent expression of the key enzyme caspase-1 was analyzed at the protein level in peri-infarct brain lysates from two different stroke models (MCAO and PT) at 24 hours, 7 days, 14 days, and 28 days post-ischemia induction (Fig. 8 A). Western blot analysis revealed distinct temporal patterns of caspase-1 expression over time after stroke between the two models. In the MCAO model, pro-caspase-1 levels gradually increased over time after stroke, peaking at 14 days. The cleaved/active form (p20) exhibited a similar trend, with levels rising and peaking at 14 days. Both pro-caspase-1 and cleaved caspase-1 exhibited reduced levels 28 days after stroke (Fig. 8 B, C). In contrast, the PT model demonstrated an early surge in pro-caspase-1 expression, peaking at 7 days post-stroke. Moreover, p20 expression in the PT model rapidly attained its maximum within the first 24 hours post-stroke, followed by subsequent attenuation (Fig. 8 D, E).

Overall, these findings indicate that caspase-1 expression and activation occurred earlier in the PT model compared to the MCAO model, with pro-caspase-1 and its cleaved form peaking at different intervals.

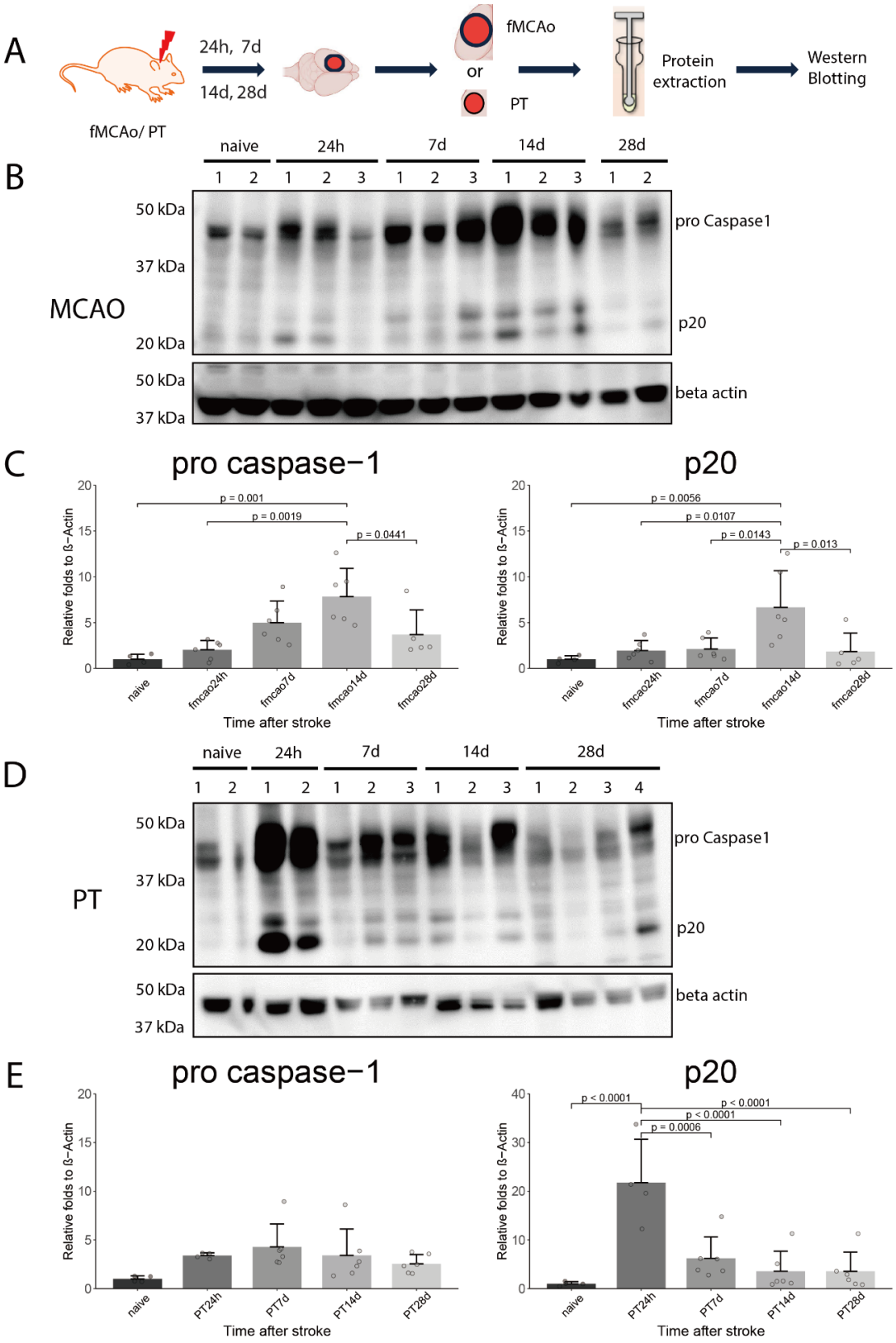
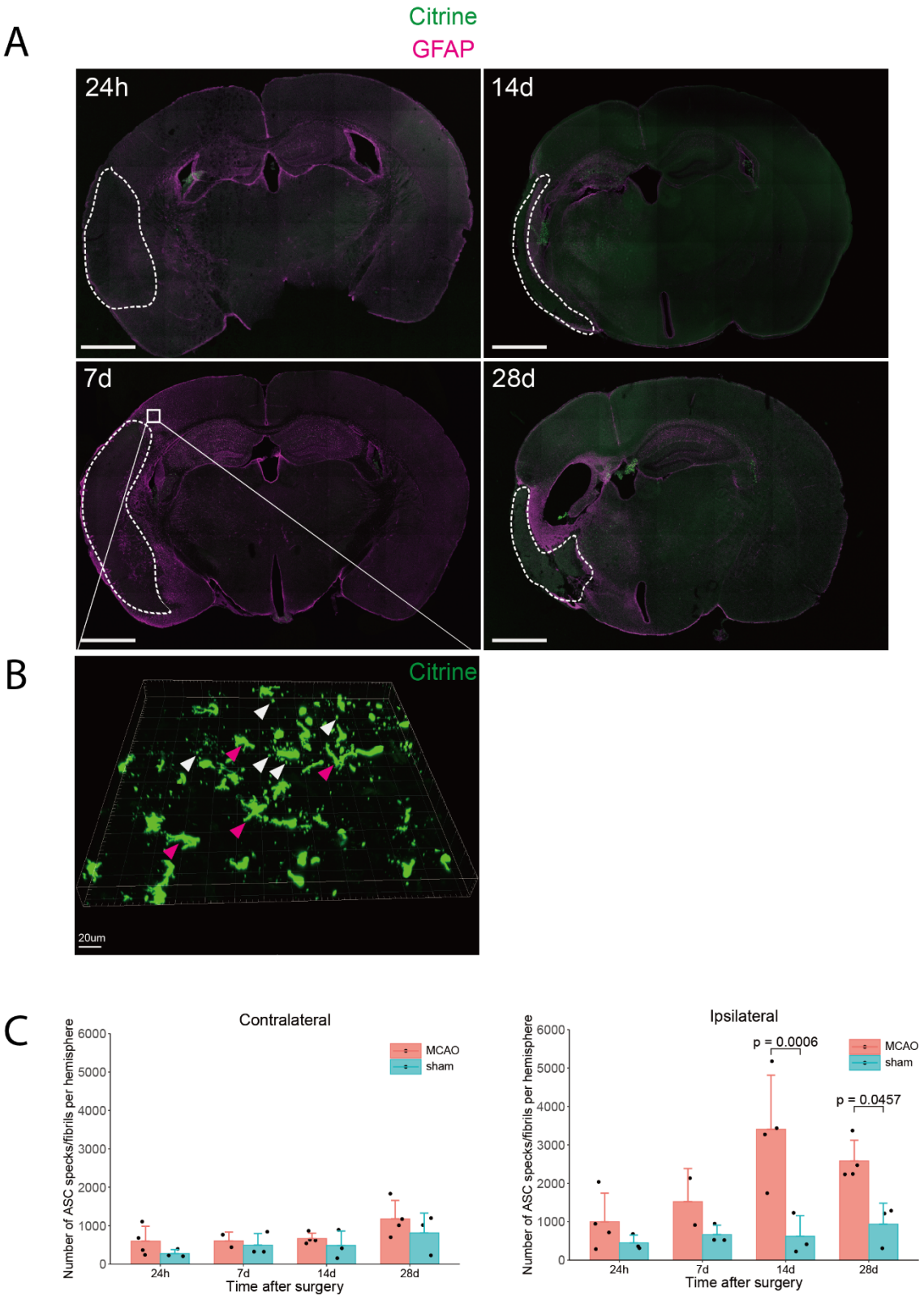


Figure 8 Inflammasome activation in the CNS in the chronic phase after stroke. (A) WT mice underwent ischemic stroke surgery and were sacrificed at various time points. Brain hemispheres were collected for protein lysate extraction, focusing on the infarct core for PT. Caspase-1 levels were measured by Western blotting. (**B, D**) Representative immunoblots of caspase-1 cleavage forms from MCAO (B) and PT (D) mice. (**C, E**) Corresponding quantification of pro-caspase-1 and cleaved p20 caspase-1 intensity normalized to β-actin (n = 3-6 per group; one-way ANOVA).

3.1.2 Inflammasome activation in different cell types in the chronic phase after stroke.

Utilizing inflammasome reporter mice, we employed a comprehensive investigation into the spatiotemporal distribution of inflammasome activation within the CNS after MCAO stroke. The mouse line used for this study (B6. Cg-Gt (ROSA)26Sor$^{tm1.1(CAG\text{-}Pycard/\ mCitrine*,\ -CD2)\ Dtg}$/J) expresses the ASC protein fused to the fluorescent protein citrine[211]. The ASC-Citrine reporter enables the detection of activated inflammasomes in situ through the visualization of ASC specks. Previous research has validated these reporter mice for tissue use by employing immunostaining against ASC[107, 212, 213].

ASC-Citrine mice were subjected to the procedure and sacrificed at various times. Brains were collected and sectioned. Sections were then stained for either neuron (NeuN), microglia (Iba-1), or astrocytes (GFAP) to determine the inflammasome. Tile scan images showed that ASC specks were almost undetectable in the acute phase (24 hours) (Fig. 9 A). They were detected in the chronic phase at days 7, 14, and 28 (Fig. 9 A). Besides focal ASC specks (Fig. 9 B, white arrow), fibrillar ASC-Citrine formations were observed (Fig. 9 B, red arrow). Consistent with Western blot data, the total ASC speck and fibril numbers gradually increased in the chronic phase, also with a peak at day 14 (Fig. 9 C). An interesting finding was that ASC specks and fibrils were mainly distributed in the peri-infarct cortex, striatum, and hippocampus of the ipsilateral hemisphere, but not in the infarct core area (Fig. 9 A). In addition, ASC specks were also found in the lateral ventricles of both sides (Fig. 9 A). The ASC specks and fibrils were found mainly colocalized with GFAP, Iba1 signal. In contrast, only a few were colocalized with NeuN, indicating that astrocytes and microglia activate inflammasome throughout the chronic phase (Fig. 9 D, yellow arrow).


A
Citrine
GFAP
24h
14d
7d
28d
B
Citrine
20um
C
Contralateral
Ipsilateral
MCAO
sham
p = 0.0006
p = 0.0457
Number of ASC specks/fibrils per hemisphere
Time after surgery
24h
7d
14d
28d
0
1000
2000
3000
4000
5000
6000

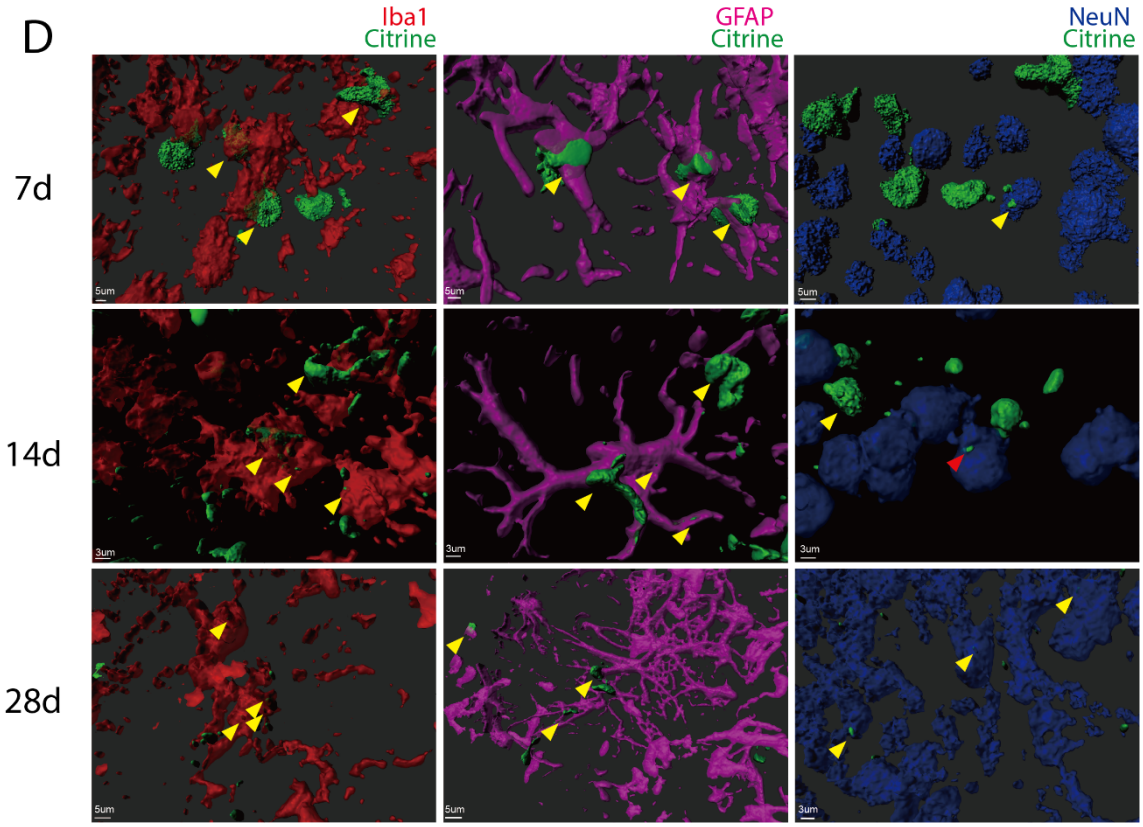


Figure 9 ASC-speck formed in different cell types in the CNS in the chronic phase after stroke. (**A**) Confocal microscopy images of ASC-citrine brain sections co-stained for GFAP, Iba1, and NeuN: scale bar, 1000 μm. Dotted lines indicate the infarcted brain areas. (**B**) Three-dimensional (3D) z-project fluorescence images of ASC specks and fibrils (white arrows indicate ASC specks. Red arrows indicate ASC fibrils. (**C**) Quantification of total ASC speck and fibril numbers in the ipsilateral and contralateral hemispheres (n = 2-4 per group; 2-way ANOVA test). (**D**) 3D reconstructions of representative astrocytes, microglia, and neurons containing ASC specks or fibrils at different time points (7 day, 14 day, and 28 day). Yellow arrows indicate co-localized ASC specks and fibrils.

3.2 Cell type-specific contribution to inflammasome activation.

To further assess the cell-type-specific inflammasome activation across varying time frames after stroke, brain flow cytometry investigating caspase-1 activity was performed. FLICA (Fluorochrome Inhibitor of Caspases) is a unique cell-permeable and non-cytotoxic reagent. Activated caspase-1 enzyme in living cells can be detected by FLICA660 employing the fluorescent inhibitor prob 660-YVAD-FMK.

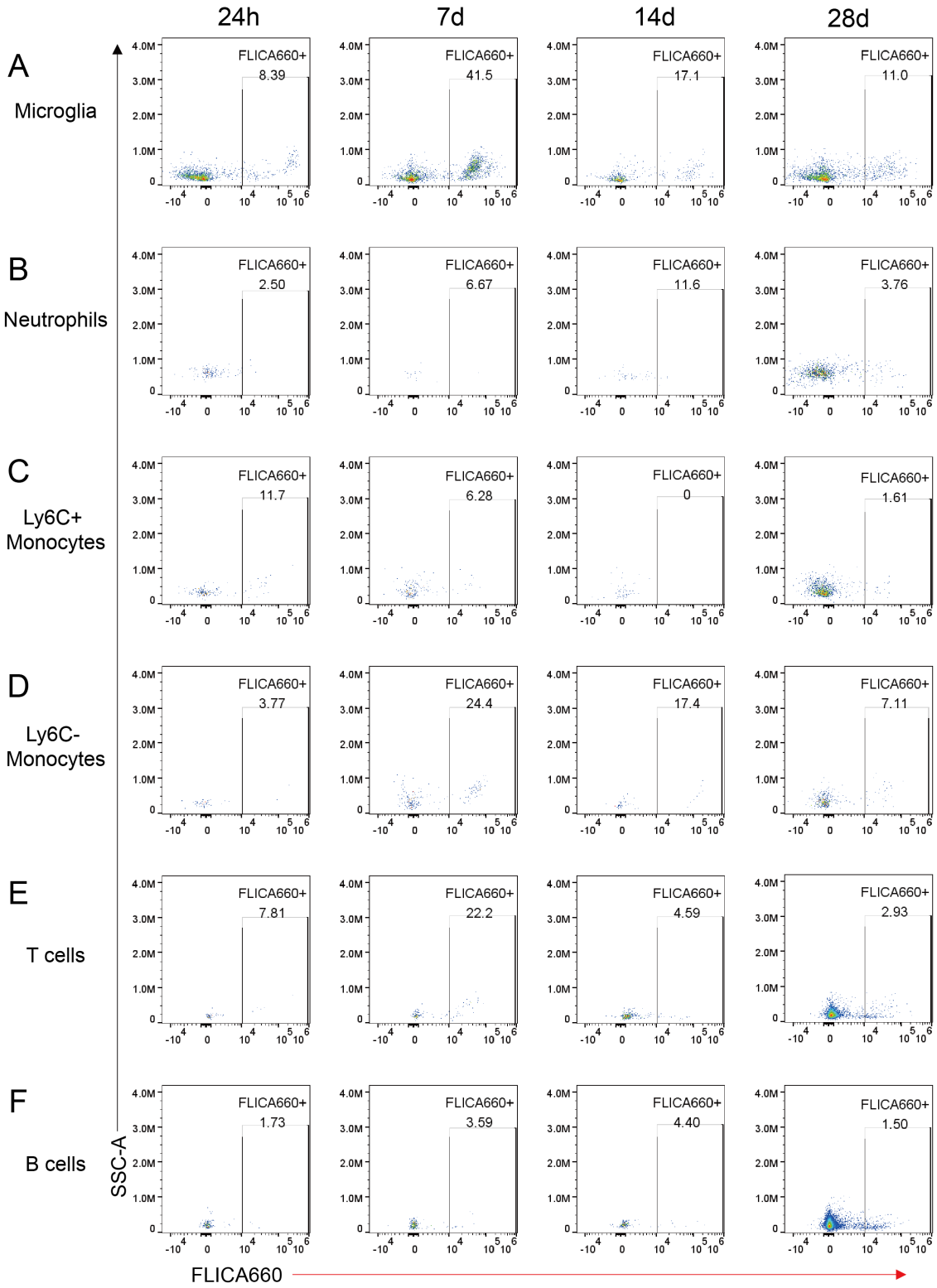
Caspase-1 activity was quantified by measuring the percentage of FLICA-positivity in different cell types (Fig 10 A-I). FLICA660 FACS data demonstrated a slight increase in the FLICA-positive

microglia population 24 hours after stroke, followed by a significant increase in the ipsilateral hemisphere compared to the contralateral hemisphere one week later (Fig. 10 A, J). Then, the FLICA-positive microglia population remained elevated at day 14 and decreased at day 28 (Fig. 10 A, J).

In contrast, neutrophils did not exhibit changes in the FLICA-positive population during the initial week after stroke. However, they showed increased caspase-1 activity at later time points, specifically two weeks after the surgery (Fig. 10 B, N), indicating delayed inflammasome activation in neutrophils. Monocytes did not display differences in the caspase-1 positive population between ipsi- and contra-hemispheres at all time points (Fig. 10 C, D, O). However, the data indicated that approximately 10% of monocytes were caspase-1 positive, suggesting sustained inflammasome activation in this cell type (Fig. 10 C, D, O).

Among non-immune cells, astrocytes exhibited an increase in caspase-1 activity 2 weeks after surgery (Fig. 10 H, K). Oligodendrocytes also displayed a sustained caspase-1 positive population in both ipsilateral and contralateral hemispheres, with no significant difference between the two sides (Fig. 10 G, L). Notably, endothelial cells exhibited a sustained increase in caspase-1 activation from 24 hours to 4 weeks after stroke in both hemispheres (Fig. 10 I, M).

This analysis using brain flow cytometry provided cell-type-specific insights into the activation of caspase-1 at different time points after stroke. The observed patterns of caspase-1 activity in microglia, neutrophils, monocytes, astrocytes, oligodendrocytes, and endothelial cells contribute to our understanding of inflammasome dynamics in stroke.


24h
7d
14d
28d
A
Microglia
FLICA660+ 8.39
FLICA660+ 41.5
FLICA660+ 17.1
FLICA660+ 11.0
B
Neutrophils
FLICA660+ 2.50
FLICA660+ 6.67
FLICA660+ 11.6
FLICA660+ 3.76
C
Ly6C+ Monocytes
FLICA660+ 11.7
FLICA660+ 6.28
FLICA660+ 0
FLICA660+ 1.61
D
Ly6C- Monocytes
FLICA660+ 3.77
FLICA660+ 24.4
FLICA660+ 17.4
FLICA660+ 7.11
E
T cells
FLICA660+ 7.81
FLICA660+ 22.2
FLICA660+ 4.59
FLICA660+ 2.93
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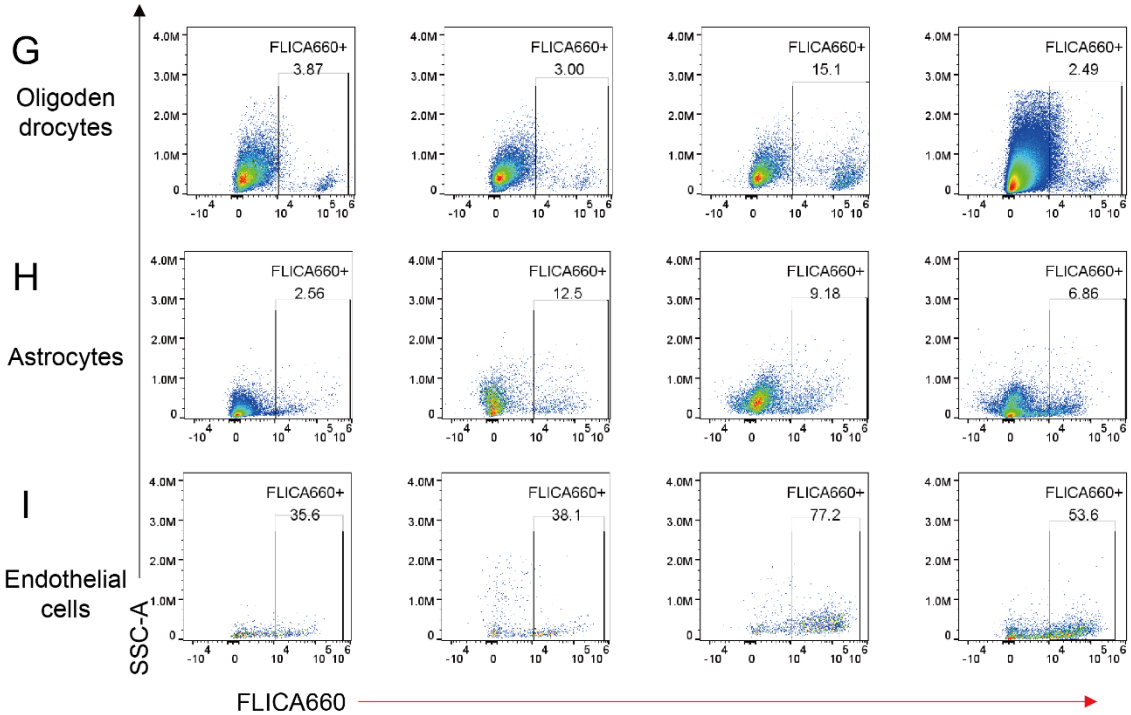
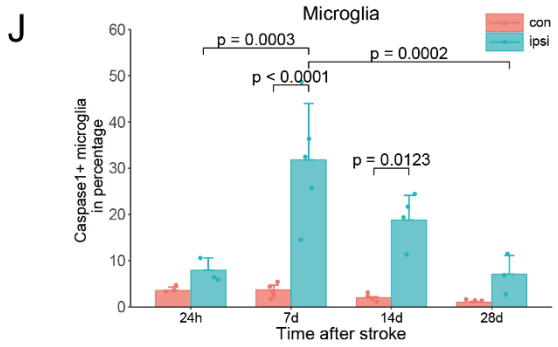
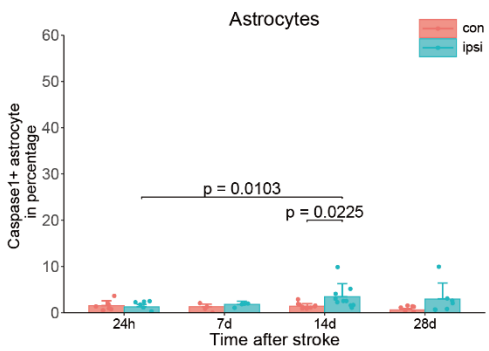
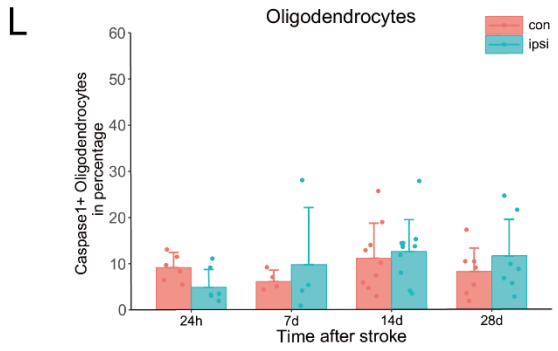
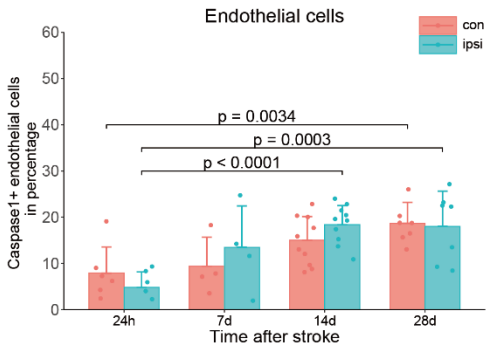
FLICA660+ 4.40
FLICA660+ 1.50
SSC-A
FLICA660


G
Oligoden drocytes
H
Astrocytes
I
Endothelial cells
FLICA660+
3.87
3.00
15.1
2.49
2.56
12.5
9.18
6.86
35.6
38.1
77.2
53.6
SSC-A
FLICA660


J
Microglia
con
ipsi
p = 0.0003
p = 0.0002
p < 0.0001
p = 0.0123
Caspase1+ microglia in percentage
24h
7d
14d
28d
Time after stroke


K
Astrocytes
con
ipsi
p = 0.0103
p = 0.0225
Caspase1+ astrocyte in percentage
24h
7d
14d
28d
Time after stroke


L
Oligodendrocytes
con
ipsi
Caspase1+ Oligodendrocytes in percentage
24h
7d
14d
28d
Time after stroke


M
Endothelial cells
con
ipsi
p = 0.0034
p = 0.0003
p < 0.0001
Caspase1+ endothelial cells in percentage
24h
7d
14d
28d
Time after stroke

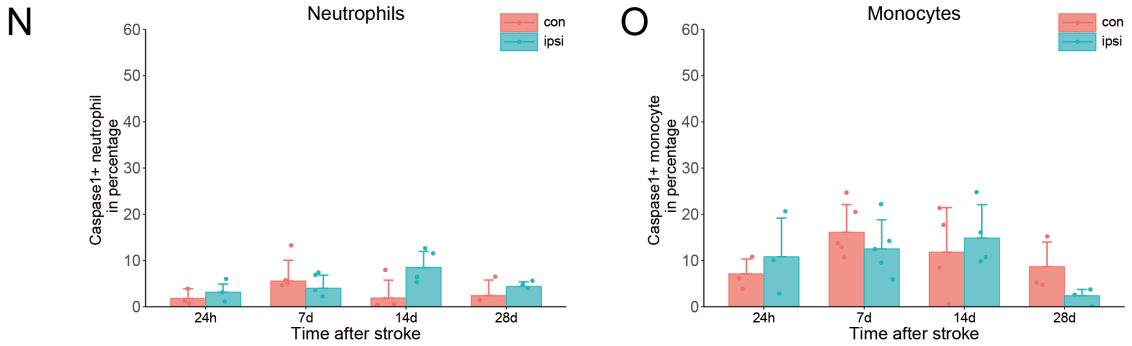


Figure 10 Cell type-specific contribution to inflammasome activation after stroke. (**A-I**) Caspase-1 positive population of microglia(**A**), neutrophils(**B**), Ly6C+ monocytes(**C**), Ly6C- monocytes(**D**), T cells(**E**), B cells(**F**), oligodendrocytes(**G**), astrocytes(**H**), and endothelial cells(**I**) at different time points after stroke. (**J-O**) Flow cytometry analysis of caspase-1 activation in microglia (**J**), astrocytes (**K**), oligodendrocytes (**L**), endothelial cells (**M**), neutrophils (**N**), and monocytes (Ly6C+ and Ly6C-) (**O**) at different time points after stroke (n =7-10 per group; 2-way ANOVA).

3.3 Caspase-1 deficiency reduces neuroinflammation in the acute and subacute phase after stroke.

Considering previous findings that distinct cell types contribute to post-stroke inflammasome activation at varying time points, a subsequent question arises regarding the specific role of inflammasome activation in post-stroke neuroinflammation. To address this, a comparative analysis was conducted using brains from caspase-1 KO mice and WT counterparts at different intervals following MCAO stroke.

The total number of leukocytes ($CD45^{high}$) exhibited a significant increase in the ischemic brains of WT mice from 24 hours up to 4 weeks after surgery, peaking at 24 hours post-stroke. In contrast, caspase-1 KO mice did not exhibit significant changes in total circulating immune cells in the acute and subacute phases. However, interestingly, these mice displayed augmented leukocyte numbers in the chronic phase (2 weeks and 4 weeks after surgery) but not in the acute and subacute phase (24 hours and 1 week). This observation implies a reduced extent of post-stroke neuroinflammation in the acute phase when caspase-1 is lacking (Fig. 11 A).

In WT mice, the influx of specific leukocyte subsets included neutrophils, monocytes/macrophages, B cells, and T cells, each showing distinct temporal patterns. Neutrophils rapidly infiltrated the ischemic brain as early as 24 hours after injury, followed by a decrease at 1 week and then a subsequent surge at 2 weeks (Fig. 11 B). Monocytic cells also

exhibited swift appearance in the ischemic hemisphere and kept expanding until 2 weeks after surgery before its number dropped at 4 weeks (Fig. 11 C). Both Ly6C+ and Ly6C- monocytes/ macrophages displayed a similar time pattern (Fig. 11 D, E). B cells started appearing in the ischemic brains 2 weeks after injury and demonstrated significant accumulation at 4 weeks (Fig. 11 F). T cells appeared in the ischemic brain 1 week after surgery, then gradually increased until 4 weeks (Fig. 11 G). CD4+ and CD8+ T cells contributed equally to this process (Fig. 11 H, I).

Notably, the differences between WT and caspase-1 KO mice were most pronounced in the first week post-stroke. Caspase-1 KO mice did not exhibit early infiltration of neutrophils and monocytes/macrophages but displayed accumulation of these cell types, along with B and T cells, during the chronic phase (Fig. 11 B-I).

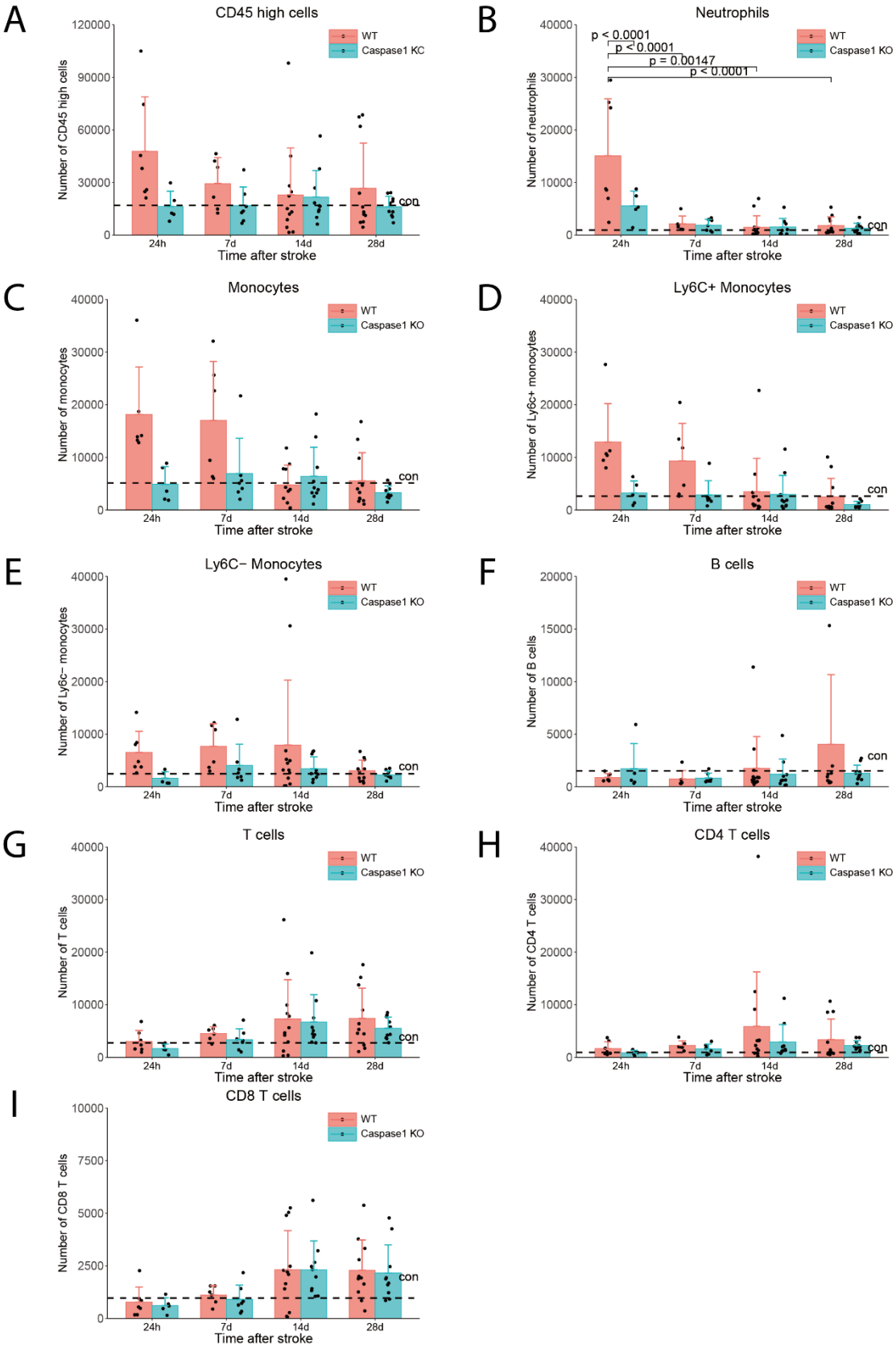


Figure 11 Inflammasome activation affects post-stroke neuroinflammation. (**A-I**) Quantification of brain tissue immune cell numbers obtained after 24 hours (WT, n = 10; caspase-1 KO, n= 5), 7 (WT, n = 6; caspase-1 KO, n= 7), 14 (WT, n= 15; caspase-1 KO, n= 13) and 28(WT, n= 6-15; caspase-1 KO, n= 11) days after MCAO are shown for the ipsilateral and contralateral hemispheres (2-way ANOVA).

3.4 Inflammasome activation does not affect post-stroke synaptic density

Subsequently, an investigation was undertaken to explore the potential impact of inflammasome activation on post-stroke neuronal remodeling. The assessment focused on synapse density in both wild-type (WT) and caspase-1 deficient brains precisely 28 days after experimental MCAO-induced stroke. Measurements included presynaptic (vGluT1), postsynaptic (Homer), and colocalized synaptic densities in the perilesional sensory cortex and remote motor cortex of both the ipsilateral and contralateral hemispheres (Fig. 12 A).

Quantitative analysis revealed that in the perilesional cortex of both WT and caspase-1 deficient brains, presynaptic formations decreased at 28 days post-stroke compared to the contralateral homotypic area. No noticeable differences were observed between the two genotypes in the contralateral hemisphere (Fig. 12 B-D). Notably, caspase-1 deficient brains exhibited reduced postsynaptic staining in the sensory cortex compared to WT brains 28 days after stroke (Fig. 12 C). Furthermore, caspase-1 deficient brains also displayed fewer pre-/post-synaptic colocalizations in the ipsilateral sensory cortex compared to the contralateral hemisphere, but with no significant differences between the hemispheres of WT and caspase-1 deficient brains (Fig. 12 B-D).

These findings collectively indicate that inflammasome activation had a limited influence on post-stroke synaptic density.

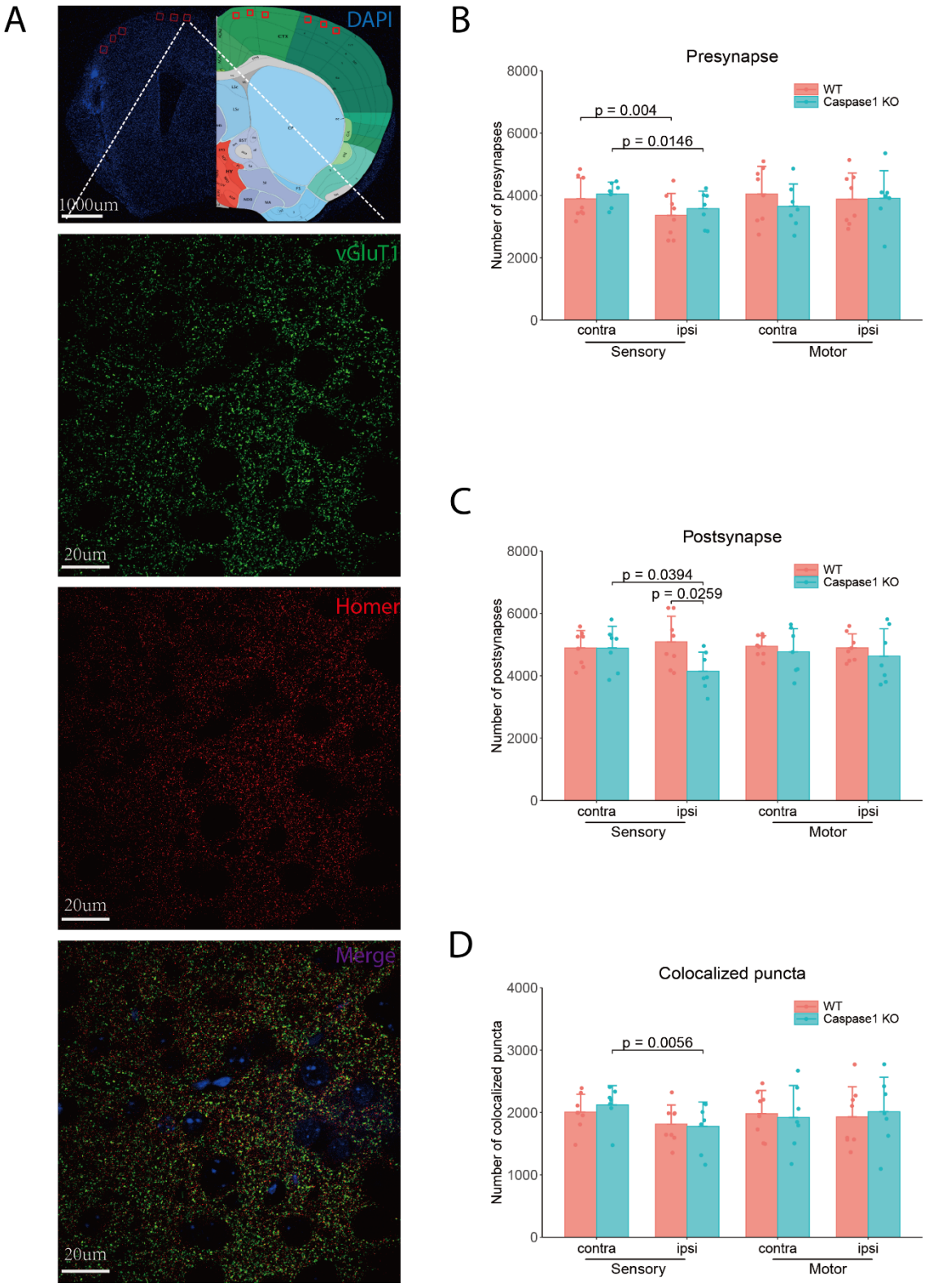


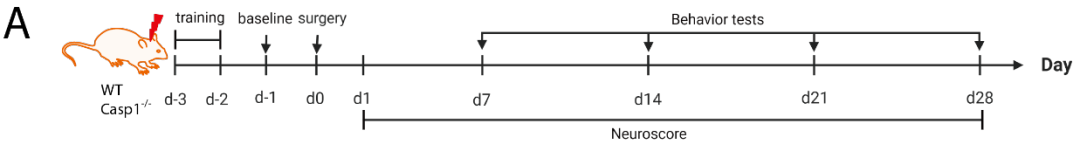
Figure 12 Effect of inflammasome activation on post-stroke synaptic density. (**A**) Confocal microscopy images of brain sections co-stained for vGluT1, Homer, and DAPI. (**B-D**) Quantification of synapse staining. The number of pre-synapse (**B**), post-synapse (**C**), and co-localized synapses (**D**) (n = 7-8 per group; 2-way ANOVA).

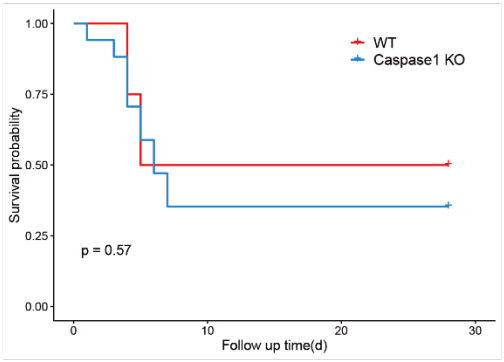
3.5 Inflammasome activation affects post-stroke recovery

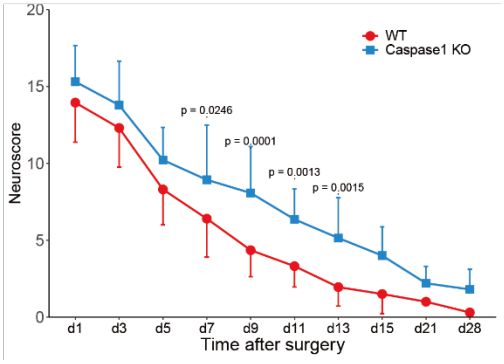
The synapse staining unveiled a notable influence of inflammasome assembly on post-stroke synapse plasticity. This prompted an investigation into whether such an impact affects post-stroke functional recovery. To explore this, behavioral tests, including neuroscore, the adhesive removal test, and rotarod tests, were conducted on WT and caspase-1 KO mice. Sensorimotor function was evaluated using the adhesive removal test, which involved timing how long it took each mouse to detect and remove small adhesive patches affixed to the forepaws. Motor coordination and balance were further assessed through the rotarod performance test, where latency to fall from an accelerating rotating rod was recorded. Baseline measures were taken one day prior to surgery, followed by daily neuroscore evaluations after surgery. Other behavior tests were conducted once a week to monitor post-stroke recovery (Fig. 13 A).

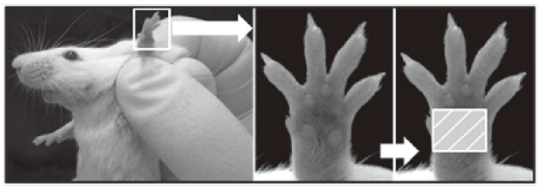
During the adhesive removal test, four parameters were collected for each mouse and trial: the left and right time-to-contact and time-to-remove the adhesive tapes. Time-to-contact reflects the sensory deficit, while time-to-remove reflects the motor deficit (Fig. 13 D). For the rotarod test, the time on the rotarod was recorded (Fig. 13 E).

Initially, a comparison of the mortality rates between WT and caspase-1 KO mice revealed high rates with no significant difference between the two genotypes (Fig. 13 B). Neuroscore assessments indicated both genotypes exhibited comparable neurological deficits for up to 5 days after surgery. However, from 7 to 13 days, caspase-1 KO mice demonstrated worse scores than WT mice, suggesting a slower recovery process during this period (Fig. 13 C). Regarding the adhesive removal test, one week after surgery, both WT and caspase-1 KO mice displayed comparable sensory deficits in the affected right paw (Fig. 13 G). However, this sensory deficit resolved in WT mice by 2 weeks post-stroke, while remaining significant in caspase-1 KO mice. Even 4 weeks after the stroke, caspase-1 KO mice continued to perform worse. They spent more time recognizing the adhesive compared to WT mice (Fig. 13 G). In terms of motor deficits, WT mice did not exhibit such deficits in the right paw after stroke, whereas caspase-1 KO mice displayed significant motor deficits at days 7 after injury (Fig. 13 I). No sensory or motor deficits were detected in the unaffected left hand of either genotype (Fig. 13 F, H). In the rotarod test, WT and caspase-1 KO mice exhibited motor deficits after the stroke, with no significant difference in motor performance between the two genotypes (Fig. 13 J).



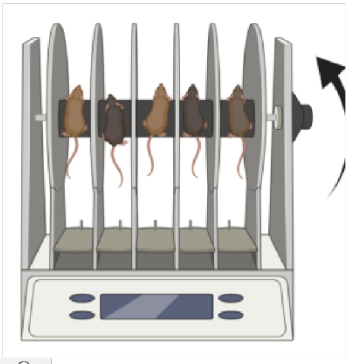




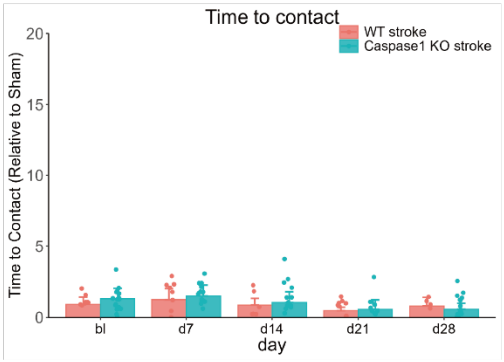


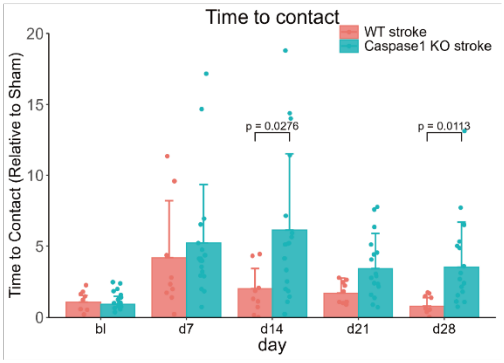
Time to contact (TTC)

Time to remove (TTR)



Latency to fall





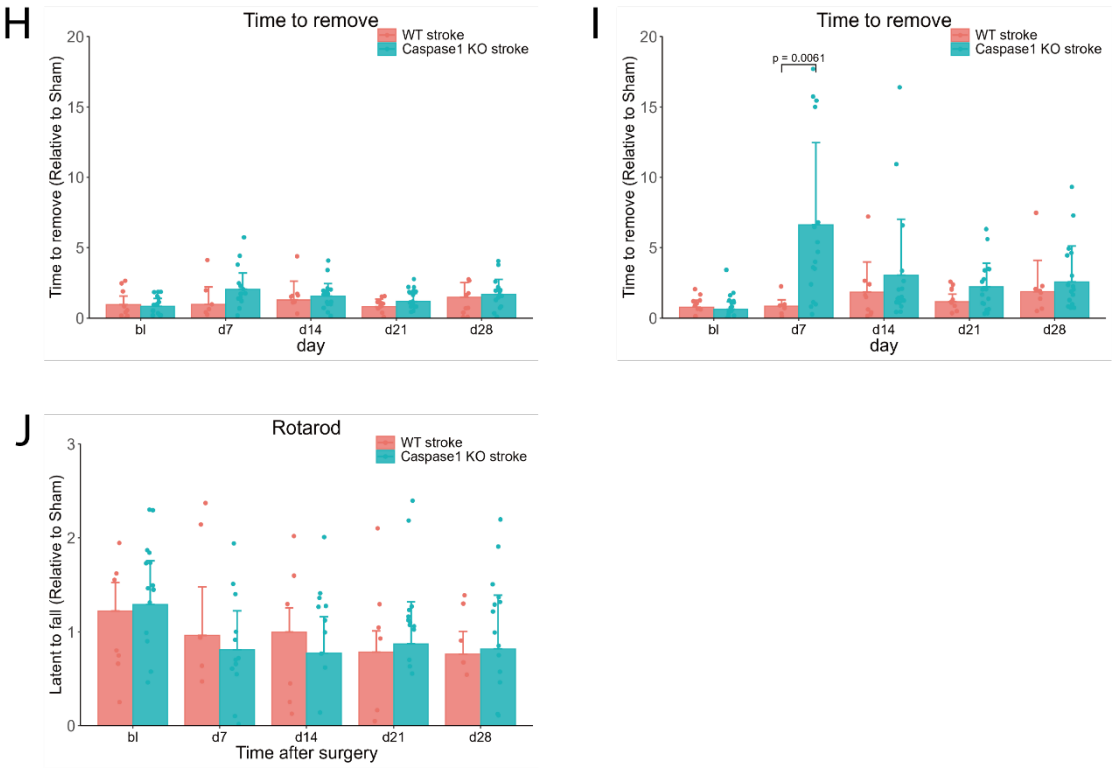


Figure 13 Inflammasome activation affects post-stroke recovery. (**A**) Experimental design: WT and caspase-1 KO mice were trained for rotarod and adhesive removal tests for two days, and a baseline was acquired one day before surgery. Mice have subsequently induced MCAO or sham surgery. The adhesive removal test and rotarod test were performed once a week after surgery. Neuroscore was recorded every other day. 28 days after surgery, mice were sacrificed, and brains were collected. (**B**) Survival curve of WT and caspase-1 KO mice after MCAO strokes (n = 8-17; Kaplan-Meier estimate). (**C**) Neuroscore of WT and caspase-1 KO mice after MCAO stroke (n = 14-20 per group; 2-way ANOVA). (**D**) Schematic of the adhesive removal test requires mice to remove an adhesive tape from their forepaw. (**E**) Schematic of the rotarod test. (**F-I**) Adhesive removal performances in WT and caspase-1 KO mice after stroke, time-to-contact the adhesive tape of left (**F**) and right (**G**) hands, time-to-remove the adhesive tape of left (**H**) and right (**I**) hands (normalized to sham group, n =10-18 per group; 2-way ANOVA). (**J**) Rotarod performance. Relative latency to fall off from the Rotarod (normalized to sham group, n = 7-15 per group; 2-way ANOVA).

4. Discussion

In this study, we first investigated the temporal and spatial dynamics of inflammasome activation following ischemic stroke using MCAO and PT models. Quantification of caspase-1 cleavage revealed significant differences in inflammasome activation between these models. In the MCAO model, inflammasome activation persisted into the chronic phase, whereas in the PT model, it was rapid but primarily confined to the acute phase.

Immunostaining of ASC-citrine reporter brains showed that inflammasomes were predominantly activated in the ipsilateral hemisphere's cortex, striatum, and hippocampus, with notable activity in the lateral ventricles. Specifically, in the cortex, ASC specks are localized to the peri-infarct area.

Next, we explored the cell type-specific inflammasome activation using a caspase-1-specific FLICA dye in flow cytometry. The FLICA flow cytometry data revealed that various cell types, including macrophages/monocytes, microglia, and endothelial cells, exhibited caspase-1 activity after stroke. Notably, microglia activation was predominant in the subacute and chronic phases, while macrophage activity peaked during the acute and subacute phases. Endothelial cells exhibited sustained increases in caspase-1 activity from the acute to the chronic phase.

Further investigations into post-stroke neuroinflammation revealed that inflammasome activation significantly influenced immune cell infiltration in the acute and subacute phases. In caspase-1 KO mice, we observed a marked reduction in immune cell accumulation within the ischemic brain. However, this effect did not persist into the chronic phase, which was characterized by the accumulation of B and T cells. Four weeks post-stroke, caspase-1 KO mice exhibited decreased post synapse density in the peri-infarct cortex compared to WT mice. Additionally, behavior test results indicated a delayed functional recovery in the caspase-1 KO mice, underscoring the critical role of the inflammasome pathway in the post-stroke recovery process.

Below, I discuss the key findings of this thesis and potential approaches for further investigations.

4.1 Sustained inflammasome activation in the ischemic brain after stroke

Post-stroke activation of inflammasomes has been extensively studied at transcriptional, molecular, and cellular levels. While previous research has indicated sustained inflammasome activation from the acute to the chronic phase of stroke, the specific spatial and temporal characteristics of this activation remain poorly understood. In this study, we assessed inflammasome activation through two methods: monitoring caspase-1 cleavage via Western blotting and visualizing ASC oligomerization in ASC-citrine mouse brains.

4.1.1 Differential inflammasome activation in two ischemic stroke models

Our study provides new insights into the temporal characteristics of inflammasome activation in two ischemic stroke models. While numerous studies using the MCAO stroke model have demonstrated the beneficial effects of inhibiting inflammasome on acute stroke outcomes, our data revealed only a limited increase in inflammasome activation within the first 24 hours. In contrast, we observed a gradual rise in inflammasome activation peaking at 2 weeks post-stroke in the MCAO model, followed by a decline (Fig. 8 B, C). This finding was further supported by immunostaining of ASC-citrine brain sections (Fig. 9**)**, suggesting that inflammasomes may play a more critical role in the chronic phase following ischemia.

Conversely, the PT model exhibited a more rapid and robust inflammasome activation within the first 24 hours following injury. Western blot analysis detected a significant increase in cleaved caspase-1 in ischemic brain tissue, which subsequently decreased over the following weeks (Fig. 8 D, E).

The notable differences between the two models can be attributed to two key factors. First, the distinct underlying injury mechanisms lead to different pathological processes. The PT model, first introduced by Watson et al. in 1985, is characterized by rapid ischemic cell death[205]. Dye-sensitized photooxygenation causes endothelial damage, leading to platelet adhesion and aggregation, resulting in thrombi that block cerebral vessels and cause rapid infarction in a small cortical volume. The swift cell death in the core area releases numerous DAMPs, promptly activating the immune system. This activation leads to a marked increase in caspase-1 cleavage

in the first 24 hours (Fig. 8 D, E). Meanwhile, due to intense necrosis in the infarct core, there is little to no ischemic penumbra around the lesion, resulting in a quick resolution of immune responses, as reflected by the subsequent decrease in caspase-1 cleavage (Fig. 8D, E).

In contrast, tissue damage from ischemia-reperfusion in the MCAO model is more progressive and prolonged. This damage can be divided into two phases: ischemia injury and reperfusion injury. After ischemia, metabolic products accumulate, leading to metabolic acidosis[214]. When blood flow is restored, local inflammation and ROS production increase, leading to secondary injury[214]. Prolonged ischemia-reperfusion injury leads to various forms of cell death, including necrosis, necroptosis, apoptosis, and autophagy, in both the infarct core and penumbra[215-217]. This prolonged injury period stimulates the immune system, explaining the sustained increase in p20 protein levels observed over the first 2 weeks post-stroke (Fig. 8 B, C).

Second, as explained in Fig. 8 A, the brain tissues isolated from the ischemic hemisphere for western blotting differed between the two models. In the PT model, only perilesional cortex tissue was isolated, while in the MCAO model, the entire hemisphere was used. Consequently, the PT model showed a much stronger increase in cleaved caspase-1 expression than the MCAO model during the acute phase.

4.1.2 The temporospatial character of inflammasome activation in MCAO

Immunostaining of ASC-citrine brain sections provided critical insights into the temporospatial dynamics of post-stroke inflammasome activation (Fig. 9). It is important to note that the limited detection of ASC specks during the acute phase does not necessarily rule out inflammasome activation, as certain inflammasomes, such as NLRP1 and IPAF, can function independently of ASC speck formation inflammasomes, inducing caspase-1 cleavage without ASC speck formation[218].

Immunofluorescence images revealed that ASC specks in the ipsilateral cortex were primarily localized to the peri-infarct border area, with few specks observed in the ischemic core lesion. Previous studies have shown that 7 days following MCAO stroke, astrocytes accumulated at the infarct border to form a glial scar. At the same time, Iba1+ microglia/macrophages in the ischemic core area are involved in phagocytosing dead neurons[86, 119]. Additionally, IL-1β was detected in GFAP+ astrocytes and Iba1+ microglia/macrophages 7 days after MCAO stroke[86].

Consistent with these findings, we observed colocalization of ASC specks with GFAP+ astrocytes and Iba1+ microglia in the peri-infarct area, indicating that inflammasomes may be involved in the activation of astrocytes and microglia during the formation of glial scar surrounding the ischemic core.

Furthermore, ASC specks were also observed in the lateral ventricle of the hemisphere opposite to the lesion site. The choroid plexus, located within the brain ventricles, is composed of capillary networks surrounded by a single epithelial cell layer that forms the blood–cerebrospinal fluid (CSF) barrier[219]. These choroid plexus epithelial cells (CPEpiCs) play a central role in controlling molecular and ionic transport between the CSF and brain parenchyma via specialized junctional structures[219]. Previous studies have demonstrated that Choroid plexus epithelial cells (CPEpiCs) respond to ischemic and traumatic impacts within the CNS[219]. Notably, an in vitro study reported that when exposed to hemorrhagic CSF, cultured CPEpiCs exhibited a marked upregulation of IL-1β mRNA expression within four hours[220]. These transcriptional data suggest that CPEpiCs respond to injury by activating the inflammasome. Our immunofluorescence data indicate that ASC specks detected in the ventricle during the chronic phase post-stroke may have originated from the CPEpiCs. However, this hypothesis requires further confirmation through specific cell-type staining. The direct implication of these specks in inflammasome activation within this context necessitates further elucidation.

To conclude, the distribution of ASC specks indicates widespread and persistent inflammasome activation in different cell types within the ischemic brain following MCAO stroke.

4.2 Cell-type specific contribution to inflammasome activation

Inflammasome components are primarily expressed in immune cells[221]. Traditionally, inflammasomes are regarded as initiators of inflammatory responses, playing crucial roles in infectious, immune, and inflammatory processes. However, they also exhibit non-canonical inflammation-independent functions. For instance, Tyrkalska et al. demonstrated that inflammasomes can regulate the balance between erythroid and myeloid differentiation[222]. Another study revealed that AIM2 inflammasome surveillance of DNA damage shapes neurodevelopment[125]. In the CNS, inflammasomes can be activated in various cell types, including microglia, astrocytes, neurons, and endothelial cells[126].

In the context of ischemic stroke, the inflammasome has been reported to significantly impact pathology, with different cell types contributing to this process. For example, the NLRC4 inflammasome mediates the inflammatory response in microglia cells[223], while NLRP1 and NLRP3 inflammasomes are activated in primary cortical neurons under ischemic conditions[161]. However, most studies have focused on a single cell type and have primarily examined only the acute phase post-stroke.

This study's immunofluorescence images of ASC-citrine brains demonstrated ASC speck formation in astrocytes and microglia during the chronic phase after stroke. Using brain FLICA-660 flow cytometry to measure caspase-1 activity, we provided the first comprehensive analysis of inflammasome activation across both immune and non-immune cell types, including microglia, monocytes/ macrophages, neutrophils, astrocytes, oligodendrocytes, and endothelial cells (Fig. 10).

Data from ischemic brains revealed that 24 hours after MCAO stroke, caspase-1 activity did not significantly increase in any cell type in the ipsilateral hemispheres compared to the contralateral side (Fig. 10). This aligns with immunoblot data, which indicated only a slight increase in p20 protein expression during the acute phase after MCAO stroke (Fig. 8 B, C). In contrast, the microglia population with activated caspase-1 dramatically increased seven days post-surgery (Fig. 10 A, J) and remained elevated at 14 and 28 days, despite a decreasing trend. Endothelial cells exhibited a consistent percentage of the population with activated caspase-1 throughout the chronic phase (Fig. 10 I, M). Monocytes maintained a stable population with activated caspase-1 across both the acute and chronic phases, with no significant differences between the ipsilateral and contralateral hemispheres (Fig. 10 O).

Current understanding of specific inflammasome subtype activation in distinct cell types following stroke remains limited. Even within microglia, the precise inflammasome subtypes responding at different time points are unclear. Moreover, identifying the specific DAMPs that stimulate these inflammasome sensors remains an open question. Further research is essential to address these gaps and enhance our understanding of inflammasome activation and its role in post-stroke pathogenesis.

4.3 Inhibition of inflammasome activation partly reduced post-stroke neuroinflammation

Following a stroke, neuroinflammation arises as a response to disrupted tissue homeostasis. The inflammatory cascade begins almost immediately within the vascular system upon arterial blockage. Subsequent hypoxia altered shear forces, and increased production of reactive oxygen species activate the coagulation system, initiating a chain of events involving the complement system, platelets, and endothelial cells[19-21, 224]. The principal role of neuroinflammation is to eradicate initial cellular damage, remove necrotic cells affected by cerebral ischemia and the inflammatory response, and commence the restoration of brain tissue. Inflammasomes are crucial in mediating post-stroke neuroinflammation.

This study shows that caspase-1 deficiency significantly dampens early neuroinflammatory responses following ischemic stroke. Flow cytometric analysis revealed extensive infiltration of peripheral immune cells into the brains of wild-type (WT) mice during both acute and subacute phases, a phenomenon markedly reduced in caspase-1 knockout mice (Fig. 11). These findings are consistent with previous research indicating that suppression of inflammasome activity mitigates acute inflammatory responses after stroke onset[162, 170, 225]. The observed effects likely stem from inhibited caspase-1 activity, which prevents the maturation and release of key inflammatory cytokines such as IL-1β and IL-18, and blocks pyroptotic cell death.

IL-1β is a key prototypical proinflammatory cytokine that stimulates both local and systemic immune responses[226]. It facilitates immune cell recruitment by inducing the endothelial adhesion molecule expression and promoting chemokine secretion by stromal cells[226]. The injection of an IL-1β receptor antagonist has demonstrated a reduction in ischemic brain damage and inflammation within 24 hours post-stroke[227]. Caspase-1 also activates GSDMD, forming membrane pores that allow the extracellular release of IL-1β and other DAMPs, amplifying the inflammatory milieu. Knocking out caspase-1 can significantly attenuate this inflammatory response by reducing cytokine maturation and preventing programmed cell death, likely explaining the diminished infiltration of circulating immune cells into the ischemic brain tissue during the acute phase after stroke.

Previous studies have documented the infiltration of B and T cells into the ischemic brain following stroke[206, 228, 229]. Specifically, B, T, and plasma cells progressively infiltrate the infarct region between 2- and 4-weeks following stroke [228, 229]. Consistent with these studies, our study observed a substantial accumulation of B and T cells in the ischemic hemispheres of WT mice. Importantly, this phenomenon appears to be independent of the inflammasome complex, as caspase-1 KO brains exhibited comparable B and T cell counts during the chronic phase, indicating minimal impact of the inflammasome on this progression.

The underlying mechanisms remain unclear, although research has provided some insights. For example, findings from the photothrombotic stroke model suggest that the accumulation of T cells in the ischemic brain is primarily due to local proliferation rather than infiltration from the periphery[206]. Moreover, CD4+ T cells appear to play a key role in facilitating B cell entry into the brain, as antibody-mediated depletion of CD4+ T cells significantly reduces B cell infiltration by day 14 following MCAO[230].

4.4 Inflammasome inhibition delayed post-stroke recovery without affecting post-stroke synaptic density

Given that caspase-1 deficient mice exhibited reduced neuroinflammation in the acute phase, we hypothesized that caspase-1 deficiency might also impact long-term post-stroke regeneration and functional recovery. Post-stroke recovery relies heavily on neuroplasticity, which involves activity-dependent rewiring and synapse strengthening[231]. To assess the potential impact of caspase-1 deficiency on post-stroke neuroplasticity, we measured synapse density in both the motor and sensory cortices 28 days after stroke. We found that stroke led to a decreased density of pre- and post-synaptic puncta (VGluT1 and Homer), with this effect persisting for at least 4 weeks (Fig. 12 B, C). Notably, caspase-1 deficient mice showed reduced density of Homer puncta in the perilesional sensory cortex compared to WT brains (Fig. 12 C), although no difference was found in colocalized synapses between WT and caspase-1 deficient brains.

Synapses serve as asymmetric intercellular junctions, facilitating point-to-point communication between neurons[232]. During development, synapses develop as newborn neurons move to designated locations and grow axons and dendrites[233]. Synapses are particularly vulnerable to

changes in neuronal homeostasis[234]. Synaptic plasticity refers to how activity can alter the strength or effectiveness of existing synaptic connections[235]. These synaptic changes may be transient, occurring within seconds to minutes, or long-lasting, with enduring forms of plasticity being essential for strengthening neural circuits during development and in cognitive processes like learning and memory[235].

The vulnerability of synapses to ischemic stroke is well-documented. Reduced blood flow damages neuronal networks, disrupting established patterns of synaptic activity in the peri-infarct area and even in distant functional structures for days to weeks post-stroke [236-238]. This disruption is likely due to signal loss from adjacent tissue affected by infarction, edema, reduced cerebral blood flow, and metabolic factors[239]. Despite this damage, synaptic plasticity allows future post-stroke recovery. Research from animal studies indicates a limited timeframe for neuroplasticity, during which the greatest recovery gains are observed[231]. Research conducted on animals suggests that enhanced rehabilitation in the initial weeks following a stroke boosts recovery of forelimb reaching and fosters dendritic branching in cortical neurons, whereas delaying rehabilitation significantly hampers recovery[240].

The reduction in synaptic puncta observed in caspase-1 deficient mice post-stroke may be mediated through mechanisms involving IL-1β and caspase-3 signaling. Caspase-1 cleaves pro-IL-1β into its mature, active form, which, during the acute phase of stroke, facilitates leukocyte recruitment by upregulating endothelial adhesion molecules and enhancing chemokine release from stromal cells[226]. Beyond its acute effects, IL-1β also influences synaptic remodeling during the chronic recovery phase. Experimental studies have shown that elevated IL-1β levels disrupt hippocampal synaptic plasticity and impair long-term potentiation (LTP). Specifically, direct application of IL-1β to hippocampal slices diminishes LTP in both the CA1[241] and CA3[242] regions, suggesting a negative impact on learning-related synaptic strengthening. This adverse effect has also been demonstrated in vivo; intracerebroventricular injection of IL-1β suppressed LTP at the perforant path-granule cell synapses in rats under urethane anesthesia[243-246], the IL-1 receptor antagonist (IL-1Ra) reduced the alterations induced by IL-1β[247]. However, despite the negative impact of IL-1β on LTP, several studies have suggested that the changes induced by IL-1 are likely dose-dependent. Inhibiting IL-1R with IL-1Ra has been shown to reduce LTP[247-250], and a temperature-dependent influence of IL-1Ra on LTP has also been noted[251]. These findings imply that physiological concentration of IL-1β is essential for the

induction and maintenance of LTP. This may explain the reduced synapse density observed in caspase-1 deficient brains compared to WT brains 28 days post-stroke.

Caspase-3 may also be involved in the synapse plasticity following a stroke. While most caspase family members are involved in apoptotic signaling pathways [249, 250], caspase-3 has also been associated with LTD induction via mitochondrial pathways[252]. In mice lacking caspase-3, long-term depression (LTD) in CA1 hippocampal neurons, dependent on NMDA receptor signaling, is abolished. Similarly, pharmacological inhibitors of caspase-3 and caspase-9, a key upstream activator in the intrinsic apoptotic pathway, can suppress LTD induction. The caspase-1 and caspase-3 pathways are functionally linked: while the absence of caspase-1 prevents pyroptosis, it may unintentionally promote apoptosis by enabling caspase-3 activation. In macrophages deficient in caspase-1, inflammasome assembly can engage caspase-8 via ASC specks, redirecting the cell death response toward apoptosis. These ASC aggregates support activation of both caspase-1 and caspase-8, but in the absence of caspase-1, the apoptotic pathway driven by caspase-8 predominates[253]. In neurons, such a shift toward apoptosis may contribute to synaptic loss and structural remodeling.

To further investigate the impact of caspase-1 on post-stroke recovery, we conducted behavioral tests, including neuroscore, adhesive removal test, and rotarod test, to monitor the recovery process (Fig. 13 A). Results indicated that caspase-1 deficient mice exhibited delayed recovery in the subacute phase after stroke (Fig. 13 C, G, I). Although reduced synaptic density may partly explain this delay, the attenuation of post-stroke neuroinflammation due to caspase-1 deficiency likely also contributes.

Flow cytometry data revealed that caspase-1 deficiency significantly reduced the number of infiltrated leukocytes (Fig. 11). While post-stroke neuroinflammation can exacerbate secondary brain damage by harming surviving neurons, it also has beneficial functions in post-stroke recovery. Evidence suggests that the subacute inflammatory phase is crucial for endogenous repair, including BBB repair, neurogenesis, and angiogenesis[12]. During this phase, microglia exhibit an anti-inflammatory phenotype that not only suppresses inflammatory responses but also promotes tissue repair through the secretion of anti-inflammatory cytokines such as IL-10, TGF-β, IL-4, and IL-13, as well as scavenger receptors[254]. In the hippocampus, neurogenesis takes place in the subgranular zone of the dentate gyrus, while in the region adjacent to the third

ventricle, it occurs in the subventricular zone[255]. Following experimental strokes, this mechanism is exacerbated and regulated by inflammatory mediators generated during the acute phase[256, 257]. Nonetheless, the function of neurogenesis in human functional recovery remains ambiguous. Angiogenesis is crucial for the oxygenation of post-ischemic brain tissue, as well as for the restoration of the blood-brain barrier, the promotion of neurogenesis, and the enhancement of neuronal synaptic plasticity[12]. IL-1β is necessary to induce the angiogenic response, which can be abrogated by neutralizing IL-1β[258].

In conclusion, our study reveals that although caspase-1 exhibits a relatively modest impact on synapse density following stroke, it exerts a crucial influence on the functional recovery processes post-stroke. Our observations showed that the stroke-induced diminutions in post-synaptic density not only persisted for a minimum duration of 4 weeks but also were associated with exacerbated outcomes in mice lacking caspase-1(Fig. 12, 13). The results indicate that the inflammasome, particularly caspase-1, might facilitate post-stroke recovery through its involvement in promoting a regenerative process.

4.5 Conclusion

This study offers a detailed and comprehensive investigation into the activation of inflammasome within the murine brain following ischemic stroke, detailing its spatial and temporal dynamics, influence on neuroinflammation, and role in recovery processes. It reveals distinct patterns of inflammasome activation across various ischemic stroke models. In the MCAO model, activation was sustained into the chronic phase. In contrast, in PT model, rapid and robust activation was predominantly observed during the acute phase.

We identified specific cell types involved in inflammasome activation, with microglia playing a central role during the subacute to chronic phases and macrophages/monocytes predominating in the acute and subacute phases. The research confirmed that inflammasome activation initiates acute neuroinflammation post-stroke. Inhibiting inflammasome activity significantly reduced the infiltration of immune cells during the acute and subacute phases; however, chronic inflammation, marked by persistent T and B cell accumulation, persisted.

The study further investigated the involvement of inflammasome activation in post-stroke regeneration and functional recovery. Although caspase-1 activity did not significantly influence colocalized synapses, the findings revealed that caspase-1 deficient mice exhibited a delayed recovery of motor and neurological function when compared to WT mice. This delay in functional recovery underscores the critical role of the inflammasome signaling pathway in the restoration of brain function following ischemic injury.

In conclusion, this investigation underscores the significant impact of inflammasome activation on post-stroke pathology, with activation timing being a critical factor in determining outcomes. Targeting inflammasome activation within specific temporal windows requires careful consideration. These findings provide valuable insights into the intricate post-stroke recovery processes and have important implications for developing therapeutic strategies.

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Affidavit

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