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Crosstalk between peripheral and local immune response in the pathophysiology of stroke and neurodegeneration diseases, volume II

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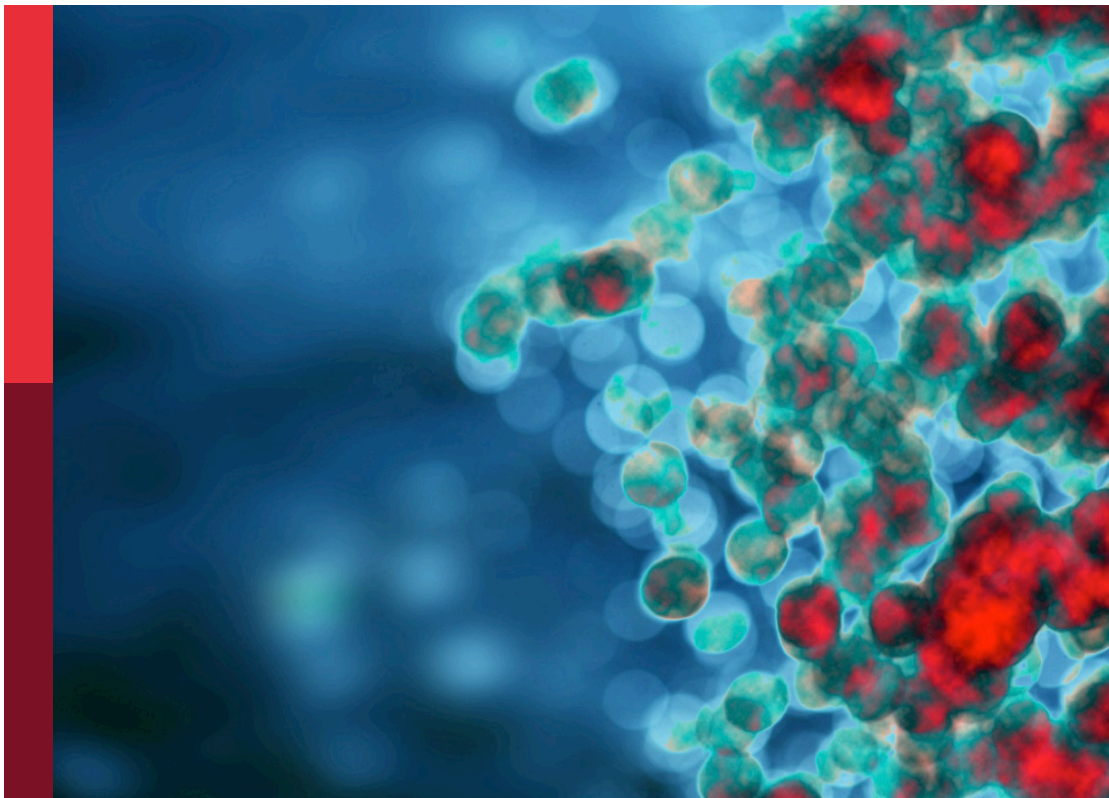

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Crosstalk between peripheral and local immune response in the pathophysiology of stroke and neurodegeneration diseases, volume II

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Editorial: Crosstalk between peripheral and local immune response in the pathophysiology of stroke and neurodegeneration diseases, volume II

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Editorial on the Research Topic
Crosstalk between peripheral and local immune response in the pathophysiology of stroke and neurodegeneration diseases, volume II

The last Research Topic "*Crosstalk between peripheral and local immune response in the pathophysiology of stroke and neurodegeneration diseases*" published in Frontiers in Cellular Neuroscience (Fang et al., 2023) featured 29 papers focusing on peripheral and local immune response in the pathophysiology of stroke and neurodegenerative diseases. The Research Topic covered aspects including pathomechanisms, experimental treatments, and clinical management (Fang et al., 2023). In this volume, nine additional manuscripts were published, including six reviews and three original articles. These new publications primarily focused on advances in mechanisms of immune responses in stroke and neurodegenerative diseases, along with targeted therapies for these diseases.

Systemic and local immune responses play a key role in the development of stroke (Fang et al., 2020; Kelly et al., 2021). Gong et al. comprehensively summarized the role and the interaction between immune system, tissue inflammation, and cell death in ischemic stroke, encompassing the underlying mechanisms and signal pathways. The functions and signal pathways of immune cells, including microglia, astrocytes, neutrophils, T lymphocytes, and monocytes/macrophages, in the post-ischemic brain inflammatory response were discussed. The signal pathways that mediate programmed cell death including pyroptosis, apoptosis, necroptosis, ferroptosis, and PANoptosis were discussed. Mechanisms of action of natural compounds, including salidroside, baicalin, astragaloside IV, and curcumin, in the treatment of ischemic stroke were reviewed, providing potential future directions for ischemic stroke treatment. Deng et al. summarized the latest works on programmed cell death and ferroptosis in subarachnoid hemorrhage (SAH), focussing on iron

metabolism, lipid metabolism, antioxidant systems belonging to the GSH/GPX4 system, newly discovered GSH/GPX4-independent antioxidant systems, and their related upstream regulators and downstream targets in the context of early brain injury after SAH.

Peripheral T cells lymphocytes are widely reported to be involved in brain homeostasis as well as neurological diseases (Evans et al., 2019). $CD8^+$ T cells are an important population of T cell lymphocytes. Accumulating evidence revealed the roles of CD8+ T cells in acute brain injury in slowly progressive and neurodegenerative diseases. Zhang et al. reviewed the involvement of CD8+ T cells in the regulation of brain injury including stroke, traumatic brain injury, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Understanding these processes will promote the investigation of T cell immunity in brain disorders, and provide new intervention strategies for the treatment of brain injuries.

Atherosclerosis, a common cardiovascular disease, is characterized by the dysregulated expression of multiple factors and genes influenced by both environmental and genetic factors. Atherosclerosis is highly correlated with the incidence of ischemic stroke (Tuttolomondo et al., 2020). It has been widely recognized that immune cell infiltration and the interaction of cytokines and chemokines released by these cells contribute to atherosclerotic plaque formation, progression, and regression. Zhao et al. presented a comprehensive overview of the metabolic alterations associated with atherosclerosis, elucidated the impact of inflammatory responses on atherosclerotic plaques, and explored the underlying mechanisms by which statins contribute to plaque stabilization. Furthermore, they investigated the synergistic effects of statins in combination with other pharmacological agents for managing atherosclerosis.

In addition to the reviews concerning the cellular and molecular mechanisms underlying the pathophysiological processes of immune responses in brain injury and neurodegenerative diseases, Sheng et al. and Dong et al. provided a comprehensive summary of the clinical applications of neuroinflammatory molecular imaging and repetitive transcranial magnetic stimulation (rTMS) in brain malignancies and stroke rehabilitation. rTMS, as a novel treatment modulating neural excitability in specific brain regions, has shown promising results in improving post-stroke neurofunction (Starosta et al., 2022). Sheng et al. reviewed the clinical benefits of rTMS for stroke rehabilitation, including enhancements in motor impairment, dysphagia, depression, cognitive function, and central post-stroke pain. They also discussed the underlying molecular and cellular mechanisms involved in rTMS-mediated stroke rehabilitation, particularly focusing on immune regulatory mechanisms such as modulation of immune cells and inflammatory cytokines. Furthermore, they highlighted the current challenges faced by rTMS-mediated stroke rehabilitation along with its future prospects to promote widespread clinical implementation.

Sheng et al. emphasized that incorporating neuroimaging techniques into rTMS-mediated stroke rehabilitation protocols could provide valuable insights into the underlying mechanisms responsible for its effects. Similarly, Dong et al. pointed out that positron emission tomography and magnetic resonance imaging play a crucial role in diagnosing and evaluating brain tumors and associated immune responses. Differentiating between brain tumors and necrotic lesions or inflamed tissues remains a significant challenge in the clinical diagnosis and immunotherapy of brain tumors, which emphasizes the importance of clinically applicable imaging measures monitoring neuroinflammation. They also summarized recent advances in neuroimaging methods aimed at enhancing the specificity of brain tumor diagnosis and evaluating inflamed lesions, which may facilitate the development of non-invasive prognostic and predictive imaging strategies in clinical practice.

Regarding the original articles, Huang et al. investigated the association between the biomarker pan-immune-inflammation value (PIV), which is also called the aggregate index of systemic inflammation (AISI), and all-cause mortality in non-traumatic SAH patients. PIV is calculated by multiplying the counts of neutrophils, monocytes, and platelets, followed by dividing the results by the lymphocyte count. Previous research has demonstrated that PIV serves as a prognostic biomarker for overall survival and progression-free survival in cancer or COVID-19 patients (Yang et al., 2022). The study by Huang et al. included 774 non-traumatic SAH patients, revealing that an elevated PIV upon admission was associated with increased all-cause mortality at various stages (ICU, in-hospital, 30-day, 90-day, and 1-year mortality). These findings emphasize the significance of inflammation-based biomarkers in non-traumatic SAH, and support the predictive value of PIV for predicting outcomes in these patients.

Thougaard et al. isolated peripheral myeloid cells from mice exposed to experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis model, and permanent middle cerebral artery occlusion (pMCAO), a model of ischemic stroke, at different disease time-points, and probed their ability to change the phenotype of primary microglia. They identified peripheral myeloid cell-induced changes in microglia not only dependent on the disease model, but also on the disease phase at which myeloid cells were isolated. Peripheral myeloid cells from acute EAE induced morphological changes in microglia, followed by increases in the expression of genes involved in inflammatory signaling. Conversely, peripheral myeloid cells from the chronic phase of pMCAO induced expression changes in genes involved in inflammatory signaling and phagocytosis, which was not associated with a change in microglia morphology. These finding indicated that neuroprotective and neuroreparative therapies must be tailored to each condition, and no myeloid modulating approach fits all.

Besides, Wang et al. revealed that takinib inhibits microglial M1 polarization and oxidative damage after SAH by targeting the transforming growth factor-β-activated kinase 1 (TAK1)-dependent nod-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome signaling pathway. They demonstrated that takinib administration significantly inhibited phosphorylated TAK1 expression and promoted M2 microglial polarization. Blockade of TAK1 by takinib reduced neuroinflammation, oxidative damage, brain edema, and neuronal apoptosis, as well as improved neurological deficits after SAH. Moreover, TAK1 also mitigated reactive oxygen species (ROS) production and

ROS-mediated NLRP3 inflammasome activation. In contrast, NLRP3 activation by nigericin abated the neuroprotective effects of takinib after SAH. Their findings highlighted that inhibition of TAK1 might be a promising option in the management of SAH.

These studies enriched our understanding of the immune responses in the pathophysiology of stroke and neurodegenerative diseases. We thank all contributing authors, reviewers, and editors who participated in this Research Topic.

Author contributions

YF: Funding acquisition, Writing—original draft, Writing—review & editing. AS: Writing—review & editing. LH: Writing—review & editing. XZ: Writing—review & editing. DH: Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The mechanism of ferroptosis in early brain injury after subarachnoid hemorrhage

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Subarachnoid hemorrhage (SAH) is a cerebrovascular accident with an acute onset, severe disease characteristics, and poor prognosis. Within 72 hours after the occurrence of SAH, a sequence of pathological changes occur in the body including blood-brain barrier breakdown, cerebral edema, and reduced cerebrovascular flow that are defined as early brain injury (EBI), and it has been demonstrated that EBI exhibits an obvious correlation with poor prognosis. Ferroptosis is a novel programmed cell death mode. Ferroptosis is induced by the iron-dependent accumulation of lipid peroxides and reactive oxygen species (ROS). Ferroptosis involves abnormal iron metabolism, glutathione depletion, and lipid peroxidation. Recent study revealed that ferroptosis is involved in EBI and is significantly correlated with poor prognosis. With the gradual realization of the importance of ferroptosis, an increasing number of studies have been conducted to examine this process. This review summarizes the latest work in this field and tracks current research progress. We focused on iron metabolism, lipid metabolism, reduction systems centered on the GSH/GPX4 system, other newly discovered GSH/GPX4-independent antioxidant systems, and their related targets in the context of early brain injury. Additionally, we examined certain ferroptosis regulatory mechanisms that have been studied in other fields but not in SAH. A link between death and oxidative stress has been described. Additionally, we highlight the future research direction of ferroptosis in EBI of SAH, and this provides new ideas for follow-up research.

KEYWORDS

ferroptosis, subarachnoid hemorrhage, early brain injury, oxidative stress, reactive oxygen species

1 Introduction

In 2012, Dixon et al. observed that erastin-induced cell death exhibits a distinct series of morphological, biochemical, and genetic features. This form of death is highly dependent on Fe^{2+}, and the accumulation of reactive oxygen species (ROS) and lipid peroxidation (LPO) products is one of the salient features. This process was termed "ferroptosis" (1). Ferroptosis has received widespread interest due to its involvement in development, immunity, aging, and various pathological conditions. Numerous studies have reported that ferroptosis is widely present in multiple diseases such as renal failure, cardiomyopathy, liver cancer, cerebral hemorrhage, stroke, and neurodegeneration (2). After rupture of intracranial blood vessels, the blood enters the subarachnoid space, and this is referred to as subarachnoid hemorrhage (SAH). Within 72 hours after SAH occurs, a sequence of pathological changes occur in the body such as blood brain barrier (BBB) destruction (3, 4), cerebral edema, and neuronal damage that is defined as early brain injury (EBI), and studies have demonstrated that EBI is closely related to poor prognosis. In recent years, researchers and medical professionals have questioned if ferroptosis is involved in early brain injury after SAH. Cao et al. confirmed that ferroptosis is involved in EBI following SAH (2). After SAH, a large number of erythrocytes enter the subarachnoid space and rupture, and the concentration of iron ions increases rapidly (5). Under the mediation of the Fenton reaction using iron as a catalyst, a large number of free radicals such as ROS are generated, and these are a class of molecules that contain partially reduced oxygen such as O_2^-, H_2O_2, OH^-, O_3, and $^1O2$ (6–8). Additionally, under the action of lipoxygenase (LOX), membrane phospholipids containing polyunsaturated fatty acids are directly oxidized to lipid hydroperoxides, and excessive accumulation of reactive oxygen species and lipid peroxides eventually results in cell ferroptosis (9).

2 Iron metabolism

After SAH, the blood flowing into the subarachnoid space carries large amounts of hemoglobin and iron that provides the basis for the formation of LPO (6). Ferroptosis is a form of iron-dependent death. Iron acts as an indispensable inducer of lipid peroxidation and ferroptosis that can result in ROS production via the Fenton reaction. It is also used as a synthetic raw material for lipoxygenase and cytochrome P450 oxidoreductase to produce lipid peroxides (10) that ultimately lead to ferroptosis.

Iron homeostasis plays a critical role in the normal life activities of the body, and the body maintains the stability of iron content inside and outside of cells through various metabolic pathways (Figure 1). Increasing iron intake or decreasing iron excretion increases cellular susceptibility to ferroptosis. The active iron content in cells is primarily adjusted via the following pathways: 1) ferrotinophagy (11) that is a specific autophagic process that uses ferritin as a substrate; 2) iron uptake mediated by transferrin (12, 13); 3) ferroportin (FPN) that can transfer intracellular iron from cells (14); 4) iron regulatory proteins (IRP) that maintain iron homeostasis by binding to iron response elements in different tissues (15, 16).

Iron is primarily stored and transferred in the form of ferritin complexes that are inert forms of iron that are inactive and cannot promote lipid peroxidation. Ferrotinophagy is an autophagic cell death pathway that uses ferritin as a substrate for its degradation (11). Ferritin consists of a ferritin light chain (FTL) and ferritin heavy chain (FTH). Both FTL and FTH are key indicators of cellular iron homeostasis. The decrease in FTH1 levels marks a decrease in ferritin in the inert form and an increase in active cell-free iron. Abundant ferritin is a key factor controlling ferroptosis sensitivity, and iron is released into unstable iron pools after ferrotinophagy, ultimately resulting in cells that are more sensitive to ferroptosis (17). Nuclear receptor coactivator 4 (NCOA4) is a ferrotinophagy-specific receptor that induces ferritin transfer to autophagosomes and ferrotinophagy (18). Autophagy-related gene 5 (ATG5) and autophagy-related gene 7 (ATG7) mediate ferroptosis by promoting ferrotinophagy, ultimately facilitating increased intracellular iron content and lipid peroxidation (19–21). Ferrotinophagy participates in the pathological process of EBI after SAH. In a study by Liang et al. (11), it was reported that when SAH occurs, ferrotinophagy is accompanied by decreased FTH1 and decreased ferritin in the inert form, and active cell-free iron was increased, eventually leading to iron death. After inhibiting the expression of ATG5, ferrotinophagy was inhibited, the concentration of active iron decreased, and LPO was decreased. Concurrently, ferroptosis-protecting protein content was observed. For example, there is an increase in the expression of glutathione peroxidase 4 (GPX4), and this in turn alleviates ferroptosis induced by SAH and improves the prognostic indicators of SAH. Additionally, studies examining hemorrhagic stroke have demonstrated that the degradation of ferritin and the increase in iron content for various reasons are key causes of brain damage and that the iron chelator desoxamine can alleviate brain damage, thus suggesting that iron overload is an important trigger factor of ferroptosis and providing new insights into the neuroprotective effect of iron chelators (22). These studies also provide a basis for further research focused on ferroptosis in the context of EBI. This is expected to improve the degree of ferroptosis in SAH by regulating ferrotinophagy. These studies not only suggest that SAH causes neuronal ferroptosis by activating ferrotinophagy but also suggest that regulating ferrotinophagy and maintaining iron homeostasis may provide clues for the prevention of EBI (11). It is worth mentioning that autophagy can also mediate the production of lysosomal ROS and can increase the susceptibility of cells to ferroptosis (23, 24). Overall, ferrotinophagy mediates ferroptosis and is anticipated to become a new breakthrough point for the clinical treatment of EBI after SAH.

With the occurrence of SAH, many erythrocytes enter the subarachnoid region and the concentration of extracellular iron ions increases rapidly. Extracellular iron is primarily composed of Fe^{3+} ions. First, Fe^{3+} must bind to transferrin (TRF) and then bind to transferrin receptors (TFR) to form a ternary complex that transports Fe^{3+} into cells across the membrane. Fe^{3+} entering cells form endosomes. Six-Transmembrane Epithelial Antigen of Prostate 3 (STEAP3) reduces Fe^{3+} to Fe^{2+} in endosomes. Fe^{2+} is transported into cells through divalent metal transporter 1 (DMT1, also called SLC11A2), whereas transferrin and transferrin receptors are transported out of the cell. The change in the iron valence is also

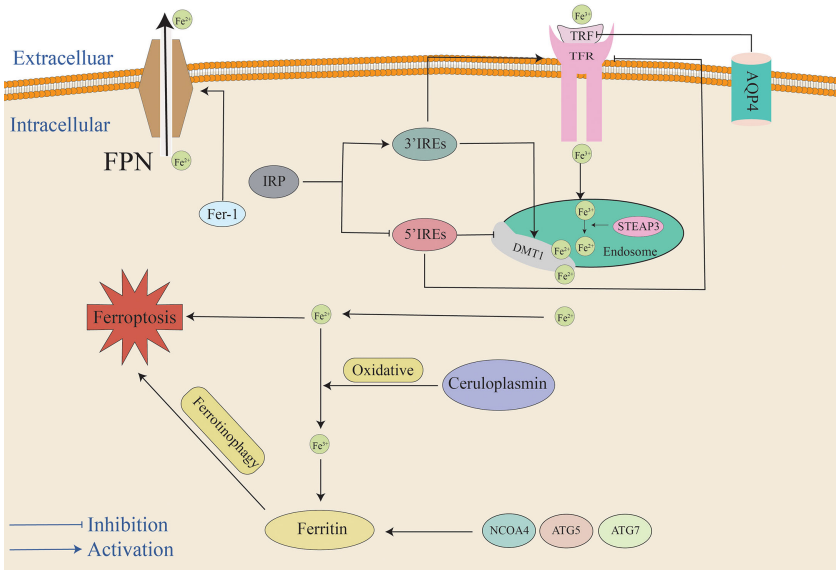

FIGURE 1
Mechanisms of iron metabolism in ferroptosis. After extracellular Fe3+ binds to TRF, it combines with the TFR to constitute a ternary complex on the surface of the cell membrane. AQP4 can inhibit TRF, and the ternary complex enters the cell and forms the endosome. *In vivo*, transmembrane ferroreductase in endosome reduces Fe^{3+} to Fe^{2+}, Fe^{2+} is then transported into cells through DMT1, and IRP regulates the expression of TFR and DMTI by binding to iron response elements at different positions. Fe^{2+} in cells can exist in a free active form or it can be oxidized to Fe3+ by ceruloplasmin to form ferritin. Conversely, ferrotinophagy can also increase intracellular iron content. NCOA4, ATG5, and ATG7 can promote ferrotinophagy. The FPN in the body can transport intracellular iron out of the cell. Elevated intracellular iron levels cause cells to be more susceptible to ferroptosis.: AQP4, aquaporin 4; ATG5, autophagy-related gene 5; ATG7, autophagy-related gene 7; DMT1, divalent metal transporter 1; FPN, ferroportin; Fer-1, ferrostatin-1; IREs, iron-response elements; NCOA4, Nuclear receptor coactivator 4; TFR, transferrin receptors; TRF, transferrin.

beneficial in regard to improving the absorption efficiency of iron ions by cells. A portion of the Fe^{2+} entering the cells is oxidized by ceruloplasmin to Fe^{3+} that combines with apoferritin to form ferritin. It becomes inactive storage iron, and the remaining iron enters the cells as Fe^{2+} (25–28). In a rat model of SAH, it has also been reported that the TFR content is significantly upregulated at 24 h after SAH (29). Yuan et al. also observed that ferritin, TFR, and DMT1 levels increased at 6 h in EBI (30). Zhang et al. reported that the iron metabolism-related proteins hepcidin and DMT1 were upregulated in EBI after SAH. After treatment with the DMT1 inhibitor ebselen, the intracellular iron ion concentration decreased, and the degree of ferroptosis was alleviated. These results indicate that ebselen can inhibit EBI by inhibiting DMT1 to decrease intracellular iron content during this period, and this effectively inhibits ferroptosis (31). Taken together, we speculated that SAH induces the upregulation of iron absorption proteins, thus leading to the accumulation of intracellular iron that in turn promotes ferroptosis.

There are not only iron ion transfer pathways in cells but also iron ion excretion channels. Intracellular iron could also be transported out of the cell through transferrin (FPN) that is the sole known iron exporter that regulates mammalian iron export outside of the cell. Contrary to DMT1 playing a role in increasing intracellular iron content, FPN is an important transporter for reducing intracellular iron content (14). Previous studies have revealed that hepcidin is a regulator of iron metabolism. It induces FPN internalization and degradation by combining with FPN (32) and can also increase the expression of DMT1. Therefore, intracellular iron ions become increased. Zhang et al. observed that the iron metabolism-related proteins hepcidin and DMT1 are upregulated and that FPN and GPX4 are reduced in EBI after SAH, and this ultimately causes lipid peroxidation and ferroptosis (31). A study by Li et al. revealed the content of TFR significantly increased at 24 h after SAH, thus resulting in increased intracellular iron concentration, and they also demonstrated that Ferrostatin-1 (Fer-1) treatment could up-regulate FPN expression, reduce iron levels, reduce lipid peroxidation, inhibit the occurrence of ferroptosis, and improve neurological function (29).

IRP is indispensable for maintaining iron homeostasis. It regulates the gene expression of iron-metabolism-related proteins by binding to RNA stem-loop structures that are known as iron-response elements (IREs) that are present in target mRNAs. By combining with IREs at different sites, IRP can regulate iron storage and export, thereby regulating intracellular iron concentration and maintaining intracellular iron homeostasis. If IRP combines with IRE at the 3'UTR of target mRNAs, the expression of TFR and DMTI increases and the intracellular iron concentration increases, whereas if it binds to the 5'UTR of target mRNAs, it will reduce the intracellular iron ion concentration (16, 33). The functions of IRP in the context of ferroptosis have been confirmed in liver cancer studies. α-enolase 1 (ENO1) is an important glycolytic enzyme. Studies have demonstrated that ENO1 inhibits ferroptosis by degrading the mRNA of IRP1 in cancer cells (34). In a study examining melanoma, after treatment with RSL3 and erastin the expression of RP1 was significantly increased, and this increased the TFRC content and inhibited the expression of FPN and FTH1. It

increases the level of intracellular iron and promotes ferroptosis. When IRP1 is deficient, intracellular iron accumulation is inhibited and cells are less sensitive to ferroptosis (35). Unfortunately, there have been no studies examining the involvement of IRP in ferroptosis after SAH. We believe that IRP may play a significant role in ferroptosis after SAH; however, this requires further verification through follow-up studies.

Along with the primary regulatory routes for iron metabolism that were already mentioned, aquaporin 4 (AQP4) is among the most abundantly expressed aquaporins in the brain. Under physiological conditions, AQP4 is densely expressed in the form of "polar expression" on the endfoot membrane of astrocytes at the junction of the brain parenchyma and cerebrospinal fluid/blood, and it participates in the formation of the glial limiting membrane that exerts a significant impact on maintaining the dynamic water balance in the brain (13, 36). Further research observed that AQP4 exists in the form of orthogonal arrays of particles (OAPs) on the endfoot membrane of astrocytes and that OAPs are the structural basis for AQP4 to perform its efficient water transport function. Under physiological conditions, AQP4 is primarily located in the membranes of astrocyte end-foot membranes. It is closely related to water transport and is essential for preserving the balance of water and electrolytes between the blood-brain/blood-cerebrospinal fluid; however, under pathological conditions such as AQP4 polarity expression disorder, the formation of OAPs is significantly reduced, the efficient water transport function of AQP4 is impaired, and the water balance between the blood brain/blood cerebrospinal fluid is disturbed, ultimately disturbing the internal environment (13, 36). The study observed that within minutes of SAH, blood components quickly entered the subarachnoid area. Destruction of AQP4 polarization in astrocyte foot processes has been demonstrated to be associated with brain edema (37–39). After SAH, the polarization of astrocyte AQP4 was destroyed, and AQP4 was knocked out. This can aggravate brain damage in EBI by causing brain edema, blood-brain barrier disruption, and neuronal death (40–42). Liu et al. reported that AQP4 also participates in ferroptosis. One potential reason for neuronal ferroptosis is the infiltration of transferrin into the brain parenchyma in EBI after SAH. Overexpression of AQP4 can effectively ameliorate AQP4 polarity loss caused by transferrin infiltration and SAH, thus inhibiting ferroptosis and improving disease prognosis.

3 Lipid metabolism and lipid peroxidation

Lipid peroxidation (LPO) is the oxidative deterioration of polyunsaturated fatty acids and lipids. Cell membranes, lipoproteins, and other lipid-containing structures would suffer substantial harm as a result of LPO. LPO can alter the permeability and fluidity of cell membranes, damage DNA and proteins, and affect the normal function of cells, ultimately leading to neuronal death. LPO and anti-oxidation have crucial functions in the metabolic processes occurring within the body. Under normal circumstances, both are in a dynamic balance and maintain the normal progress of many physiological, biochemical, and immune responses in the body. Once this coordination and homeostasis is disturbed and unbalanced, it causes a series of metabolic disorders and decreases immune function, ultimately forming a chain reaction of oxygen free radicals that results in ferroptosis (43).

As a member of the acyl-CoA synthetase long-chain family, acyl-CoA synthetase long-chain family member 4 (ACSL4) is an essential enzyme in fatty acid metabolism. ACSL4 is predominantly expressed in steroid-producing tissues, particularly in the adrenal glands and ovaries. Human ACSL includes ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6, all of which participate in the formation of acyl-CoA from fatty acids (44–46). Although acyl-CoA synthetase long-chain family member 3 (ACSL3) is thought to exert no obvious effect on ferroptosis, in a tumor-related study it was demonstrated that ACSL3-mediated production of monounsaturated fatty acids (MUFAs) limits the oxidation of polyunsaturated fatty acids (PUFAs) and thus inhibits ferroptosis (47), and this also suggests that ACSL3 and ACSL4 may antagonize ferroptosis. Under the action of ACSL4, acyl groups are inserted into PUFAs, and Lysophosphatidylcholine Acyltransferase 3 (LPCAT3) inserts acylated fatty acids into membrane phospholipids. It has been confirmed that phosphatidylethanolamine (PE) containing arachidonic acid (AA) or its derivative epinephrine is a crucial phospholipid that induces cellular lipid peroxidation and ferroptosis (48). In a study by Qu et al., the SAH rat model was used to explore the expression and function of ACSL4 in EBI. This study confirmed that the expression of ACSL4 significantly increased in the brain tissue after brain injury in the early period of SAH. Additionally, they observed that ACSL4 exerted a significant impact on the induction of ferroptosis. Small interfering RNA-mediated inhibition of ACSL4 expression reduces inflammation, BBB damage, oxidative stress, brain edema, and behavioral and cognitive deficits after SAH and increases the number of surviving neurons. They speculated that ACSL4 may cause ferroptosis by mediating lipid metabolism and aggravating brain damage. Additionally, their results revealed that ACSL4 may be utilized as a critical indicator for predicting cell ferroptosis. Reducing the expression of ACSL4 and LPCAT3 is expected to inhibit intracellular lipid peroxide accumulation, and this in turn can inhibit the development of ferroptosis.

The body primarily mediates lipid peroxidation through two pathways after SAH. Additionally, it is worth mentioning that compared to MUFAs, polyunsaturated fatty acid-containing phospholipids (PUFA-PLs) may be a major substrate of lipid peroxidation in ferroptosis in tissues that are thought to be more prone to ferroptosis. The first pathway leading to lipid peroxidation is the non-enzymatic pathway, and this is followed by the enzymatic pathway. Non-enzymatic lipid peroxidation is a free radical-driven chain reaction mediated by the Fenton reaction (49). The Fenton reaction occurs between hydrogen peroxide and Fe^{2+}. It is the primary source of reactive oxygen species (ROS) such as the hydroxyl radical (OH –). OH- is one of the most typical chemical forms of ROS and is a highly flexible water-soluble form of ROS that initiates the oxidation of PUFAs (50, 51). As the first step in a non-enzymatic lipid peroxidation reaction, a diene is removed from the acyl moiety of PUFAs in the PUFA-PLs of the lipid bilayers under the action of OH-. This can result in the generation of a carbon-

centered phospholipid radical (PL•) that subsequently reacts with an oxygen molecule to form a phospholipid peroxyl radical (PLOO•). It can remove hydrogen from other PUFA to form phospholipid hydroperoxides (PLOOHs) or lipid hydrogen peroxides and new PL•. Without GPX4, they can be converted into the corresponding alcohols (PLOHs). Lipid radicals, specifically PLOO•, PLO•, and PLOOHs, react with PUFA-PLs by removing hydrogen atoms and reacting with molecular oxygen, and this leads to the generation of new PLOOHs and lipid peroxidation (49, 50). As a second pathway mediating lipid peroxidation, enzyme-catalyzed lipid peroxidation is regulated by the activity of a family of lipoxygenases (LOXs). LOXs are a class of non-heme iron-containing enzymes that catalyze the production of numerous lipid hydroperoxides from PUFAs, and of these, arachidonic acid lipoxygenase 15 (ALOX15) plays a major role. Gao et al. reported that cepharanthine (CEP) could reduce EBI after SAH in mice by inhibiting ALOX15-mediated ferroptosis of microglia and endothelial cells (10). Tuo et al. observed that ALOX15 inhibitor can minimize the infarct size following ischemic stroke in a mouse middle cerebral artery occlusion (MCAO) model (52). In mouse ischemic and hemorrhagic stroke treatment models, targeted inhibition of ALOX15 has been observed to exhibit important neuroprotective functions (53). In related studies examining melanoma, it was reported that P53 can regulate ferroptosis through the P53-SAT1-ALOX15 pathway. SAT1, a transcriptional target of P53, is a crucial rate-limiting enzyme in polyamine catabolism. ALOX15 induces lipid peroxidation and ferroptosis following SAT1 activation (54). However, Angeli et al. observed that the genetic removal of ALOX15 did not prevent ferroptosis in mouse fibroblasts after GPX4 knockout and that it did not alleviate acute ischemic kidney injury and related lethality *in vivo* (55). This suggests that ALOX15 is the only pathway that leads to lipid peroxidation. As an essential factor in lipid peroxidation, it has been demonstrated that cytochrome P450 exerts a vital function in both membrane phospholipid peroxidation and subsequent ferroptosis, and targeted inhibition of POR exhibits therapeutic potential in regard to protecting cells from ferroptosis (56). However, the role of the POR in SAH requires further verification.

Large amounts of ROS were produced by enzymatic and non-enzymatic reactions (Figure 2). Additionally, many reactive aldehyde by-products are produced such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Reactive aldehydes such as MDA and 4-HNE can covalently modify biomolecules, including amino lipids and proteins, to produce compounds that can aggravate membrane damage and cause ferroptosis (57). This is precisely due to the toxic effects of lipid peroxides and by-products that occur without converting PLOOH and lipid radicals (especially PLOO• and PLO•) into PLOH by GPX4 that reacts to generate PLOOHs by removing the hydrogen atoms and reacting with oxygen molecules. Ultimately, this chain reaction may destroy the integrity of the cell membrane, ultimately mediating cell death (58).

At the molecular level, lipid peroxides are further decomposed into active substances such as MDA and 4-HNE. They can destroy proteins, lipids, and nucleic acids, ultimately resulting in ferroptosis (59). Structurally, extensive peroxidation of lipids causes biofilm thinning and increased bending and results in further oxidation that ultimately leads to unstable membrane and micelle formation, increased membrane density, significantly constricted mitochondria, shrinking mitochondrial cristae or disappearance, and outer mitochondrial membrane rupture with associated electron-dense characteristics. In contrast, the nuclei of the cells remained structurally intact without condensation or chromatin edges. Ferroptosis occurs under the combined influence of these factors (60). Cao et al. observed the presence of ferroptosis by electron lensing in SAH and observed mitochondrial atrophy, membrane density compression, cristae reduction, and outer membrane rupture (2, 29). Through further quantitative analysis, Li et al. reported that the average mitochondrial area in the SAH group was reduced. However, abnormal changes such as mitochondrial contraction and increased membrane density in the SAH + Fer-1 (ferroptosis inhibitor) group were improved. These studies have further confirmed the existence of ferroptosis in SAH, and the morphological changes in the mitochondria of corresponding cells can be improved by treatment with Fer-1 and other ferroptosis inhibitors (29).

4 Antioxidant system

4.1 GSH/GPX4 system

Although there are various pathways that cause lipid peroxidation and ROS generation, diverse antioxidant systems also exist (Figure 3). In the 1950s, Eagle H. et al. confirmed that cysteine is an essential nutrient for many cells, and they observed that cells deprived of cysteine undergo death. The morphology of death differs from that induced by depletion of certain amino acids but possesses a resemblance to the morphology of cell death caused by certain viral infections (61). A study by Bannai et al. further observed that cell death caused by a lack of GSH and cysteine was inhibited by a lipid peroxidation inhibitor (alpha-tocopherol) (62). In 1982, Ursini et al. successfully isolated the enzyme GPX4. As an important antioxidant system, the GSH/GPX4 system is key to cell survival and is the core regulatory protein of ferroptosis. The core mechanism of GPX4 inhibition of lipid peroxidation is the reduction of toxic phospholipid hydroperoxides (PUFAs-OOH) to non-toxic lipid alcohols (PUFAs-OH) in the presence of two molecules of glutathione (GSH) as electron donors, while GSH is oxidized to glutathione disulfide (GSSG) to thereby reduce the accumulation of lipid ROS (63, 64). Wu et al. reported that the induction of ferroptosis by erastin can increase the content of lysosome-associated membrane protein 2a that can promote chaperone-mediated autophagy, thus resulting in the degradation of GPX4 (60, 65). Experiments by Yang et al. demonstrated that RSL3 and DPI7 can directly inhibit the activity of GPX4, thereby causing ferroptosis (66). Liang et al. observed that FIN56 can directly promote GPX4 degradation in tumor-related studies. Additionally, FIN56 combines with squalene synthase, ultimately leading to the exhaustion of endogenous COQ10 to thereby promote ferroptosis (60, 67). Unfortunately, in the EBI after SAH the specific regulation of GPX4 in the process of ferroptosis has not been studied in depth, and it remains unclear if chaperone-

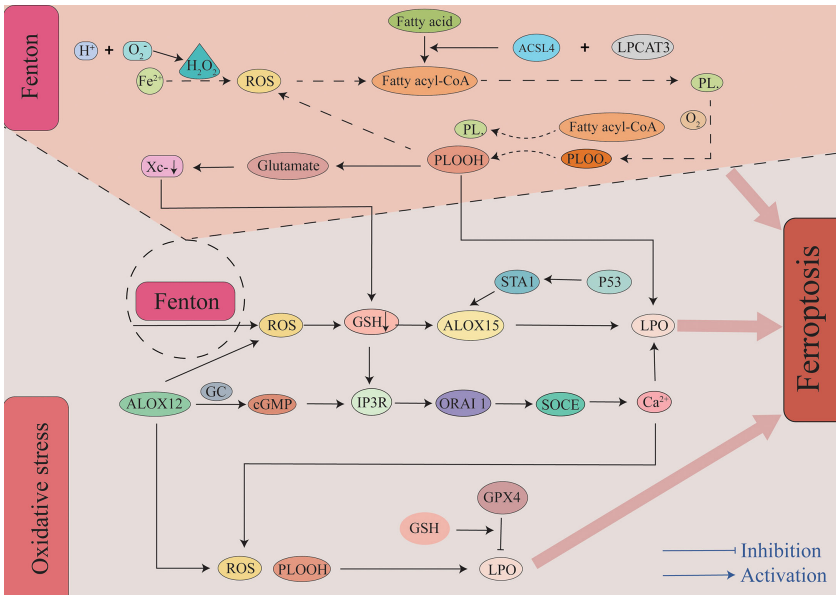

FIGURE 2
The role of lipid peroxidation in ferroptosis. Insertion of PUFAs into membrane phospholipids under the action of ACSL4 and LPCAT3 causes the lipids to be more susceptible to oxidation. There are two primary pathways leading to lipid peroxidation. First, through a non-enzymatic pathway, the Fe^{2+}-mediated Fenton reaction generates a large amount of ROS. A bisallyl hydrogen atom is removed from the PUFA-PLs to form a PL•. It can then react with a molecule of oxygen to constitute a PLOO•. It removes hydrogen from another PUFA to form PLOOH to ultimately lead to the generation of LPO and a new PL•. This forms a vicious cycle that results in a large amount of ROS and LPO. Second, through an enzymatic pathway, ALOX15 oxidizes membrane phospholipids containing PUFAs to generate ROS, and this pathway is also regulated by the P53-STA1 axis. Additionally, metabolite products of ALOX15 and depletion of GSH lead to Ca^{2+} influx, and this in turn leads to the production of ROS. A large amount of ROS and LPO are generated through the above two pathways, and this eventually leads to the occurrence of ferroptosis. ACSL4, acyl-CoA synthetase long-chain family member 4; ALOX15, arachidonic acid lipoxygenase 15; cGMP, cyclic guanosine monophosphate; GC, guanylate cyclase; GPX4, glutathione peroxidase 4; GSH, glutathione; IP3R, inositol triphosphate receptors; LPCAT3, Lysophosphatidylcholine Acyltransferase 3; LPO, lipid peroxidation; ORAI1, calcium release-activated calcium modulator 1; PLOOHs, phospholipid hydroperoxides; ROS, reactive oxygen species; SOCE, store-operated calcium entry.

mediated autophagy, RSL3, and DP17 participate in the adjustment of GPX4. However, this mechanism requires further investigation.

Gao et al. reported that the content of GPX4 was significantly reduced in rat models of EBI after SAH. Overexpression of GPX4 using adenovirus inhibits lipid peroxidation after SAH *in vitro* and *in vivo*, inhibits ferroptosis, and significantly improves brain edema and neurological dysfunction in rats within 24 h of SAH (68, 69). A study by Li et al. reported that GSH concentration and GPX4 activity were significantly reduced in rat cortical brain tissue after SAH. As expected, the ferroptosis inhibitor Fer-1 effectively increased the content of GSH and GPX4. They also observed that Fer-1 could significantly improve erythrocyte-induced accumulation of ROS, thus suggesting that Fer-1 can prevent ferroptosis in EBI by inhibiting neuronal lipid peroxidation. Additionally, Li et al. used flow cytometry to detect the apoptosis rate of neurons and the caspase-3 protein content. They confirmed that Fer-1can significantly minimize the number of dying neurons, while the number of apoptotic neurons is unaffected. Caspase-3 is an important protein in the apoptotic pathway, and its content was obviously elevated in the Hb and control groups. However, the level of caspase-3 in the Fer-1 group was not reduced, and based on this result, this study suggests that the protective mechanism of Fer-1 in EBI is not related to apoptosis (29). Zhang et al. have demonstrated that the content of GPX4 is significantly decreased in the acute phase of intracerebral hemorrhage and that upregulating the expression of GPX4 could save rats. Additionally, GPX4 is a selenium-containing protein, thus indicating that selenium may be associated with ferroptosis. Actually, it is true that in a rat model of cerebral hemorrhage, selenium supplementation to cells or animals can effectively reduce ferroptosis (70–72). The role of N-acetylcysteine (NAC) is as a precursor of cysteine. In the context of hemorrhagic stroke, studies have demonstrated that NAC treatment exerts an anti-ferroptosis effect through the GPX4-GSH axis, and the toxic effect of heme on primary neurons is significantly eliminated, thus indicating a neuroprotective effect for NAC in the context of hemorrhagic stroke (73). Additionally, NAC can effectively alleviate neuronal cell death and promote functional recovery in rat ICH models by neutralizing lipid peroxidation produced by ALOXs (74). Moreover, the multidrug-resistance pump p-glycoprotein (Pgp) was observed on a genetic screen for controllers of ferroptosis susceptibility. It can pump GSH out of cells, thus resulting in increased sensitivity of MDR1/Pgp-expressing cells to ferroptosis; however, this result has not been confirmed in the SAH model (75). SIRT1 is an epigenetic regulator of gene transcription and affects multiple biological functions such as oxidative stress, inflammation, and mitochondrial biogenesis (76). It has been demonstrated that SIRT1 exhibits strong antioxidant ability and neuroprotective effects in EBI after SAH (77). SIRT1 exerts a strong anti-oxidative ability by decreasing the expression of P53 and NF-kappaB (NF-kB) that can mediate the

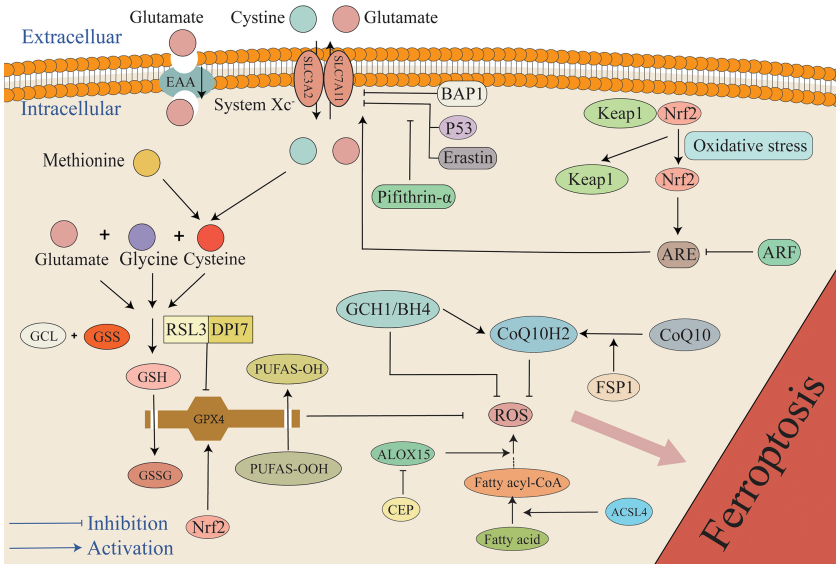

FIGURE 3
The mechanism of the antioxidant system in ferroptosis. The primary antioxidant system is the GSH/GPX4 system that reduces PUFAs-OOH to non-cytotoxic PUFAs-OH, while GSH is oxidized to GSSG. Under the mediation of the Xc- system, cystine enters cells to synthesize cysteine, and glutamate, cysteine, and glycine synthesize GSH. P53, BAP1, and Erastin can inhibit the antioxidant system by inhibiting SLC7A11. Additionally, pifithrin-α can attenuate the inhibition of SLC7A11 by P53. Under the condition of stress, Keap1 and Nrf2 are separated. Under the mediation of Nrf2, ARE increases cellular resistance to ferroptosis by promoting the expression of SLC7A11, but ARF can attenuate the effect of ARE. Furthermore, RSL3 and DPI7 directly inhibit the antioxidant system by inhibiting GPX4. FSP1 can mediate the conversion of oxidized COQ10 to its reduced form CoQ10H2 that can capture ROS, and it is also regulated by the GCH1/BH4 system. Any target that results in a weakened antioxidant system can result in the overload of lipid peroxides, ultimately causing ferroptosis. ARE, antioxidant response elements; BAP1, BRCA1-associated protein 1; BH4, tetrahydrobiopterin; CEP, cepharanthine; GCH1, GTP cyclohydrolase 1; GCL, glutamate-cysteine ligase; GSS, glutathione synthase; Keap1, Kelch ech-associated protein 1; Nrf2, nuclear factor-erythroid 2 related factor 2.

oxidative stress pathway and by upregulating nuclear factor-erythroid 2 related factor 2 (Nrf2) that mediates the antioxidant stress pathway. Studies have reported that SIRT1 activation can inhibit ferroptosis by increasing the contents of GPX4 and ferroptosis suppressor protein 1 (FSP1) after SAH (30).

Similar to GPX4, copper-zinc superoxide dismutase 1 (SOD1) is an important endogenous enzyme that can eliminate superoxide and is an indispensable peroxidase scavenger in the central nervous system (78). SOD1 overexpression alleviates cell damage following SAH (79). In cerebral ischemia, the neuroprotective effect of SOD1 is partially mediated by activation of serine-threonine kinase (AKT) (80). AKT plays a crucial role in the cell death/survival process (81), and it acts downstream of the phosphoinositide 3-kinase pathway and can function under the action of serine phosphorylation (82). AKT activation promotes cell survival and inhibits apoptosis by phosphorylating and inhibiting downstream substrates, including glycogen synthase kinase 3β (GSK3β). Thus, neurons become resistant to apoptotic stimuli (83). Endo et al. demonstrated that SOD1 overexpression could reduce oxidative stress by activating the AKT/GSK3b survival signaling pathway to thereby attenuate acute brain injury after SAH (84). A study reported that the anti-ferroptosis function of polystyrene nanoparticles is partially dependent upon SOD-mediated ROS scavenging (85). Unfortunately, no studies have confirmed the involvement of SOD in the process of ferroptosis in the context of SAH. This aspect deserves further discussion in subsequent studies.

4.2 Xc- system

The antioxidant effect of GPX4 is extremely dependent on GSH, and therefore, the biosynthesis of GSH has also attracted extensive interest. Due to the catalysis of glutamate-cysteine ligase (GCL) and glutathione synthase (GSS), GSH is composed of cysteine, glutamate, and glycine in two stages (86, 87). As raw materials for GSH synthesis, cystine, cysteine, glutamate, and glycine can all affect GSH biosynthesis. Nutrients, including sugars, fats, and amino acids, cannot diffuse directly into cells, and their entry is mediated by specific transporters. Therefore, the components of the amino acids involved in the formation of GSH also require transporters such as the Xc-transporter. The Xc system is a heterodimer transporter formed by a disulfide bond junction that consists of two subunits that include a regulatory subunit composed of solute carrier family 3 member 2 (SLC3A2) and a catalytic subunit composed of solute carrier family 7 member 11 (SLC7A11). The Xc- system promotes the exchange of cystine and glutamate across the cell membrane, where cystine enters the cell and glutamate exits the cell (88). Cystine is reduced to cysteine when transported into the cell. Additionally, another source of cysteine is the reverse transsulfation of methionine (Met) that enters cells through the Xc- system or the ASC system (alanine, serine, and cysteine-preferring) (89). Erastin is a ferroptosis inducer that inhibits Xc expression. Erastin inhibits cystine uptake, thus resulting in the synthesis of the antioxidant GSH that ultimately

leads to cell death due to oxidation (90, 91). Furthermore, it has been demonstrated that P53 can affect the expression of the Xc- system by inhibiting the transcription processes required for this system, thus inhibiting the entry of raw materials into cell and resulting in inhibited GSH synthesis. The reduction of GSH in turn results in a weakened antioxidant capacity of GPX4 and greater susceptibility of cells to ferroptosis (92). Studies have reported that intraperitoneal injection of the P53 inhibitor pifithrin-α can increase the levels of SLC7A11 and GSH in rats, reduce lipid peroxidation, reduce neuronal mitochondrial atrophy, and block ferroptosis after cortical SAH, thus indicating that ferroptosis in EBI after SAH depends at least in part on P53 and that P53 plays a role by mediating the Xc- system (particularly SLC7A11). Inhibition of P53 to reduce ferroptosis exhibits the potential to become a new therapeutic target in EBI after SAH (93). It is worth mentioning that not only is P53 a tumor suppressor that participates in the regulation of ferroptosis, but the BRCA1-associated protein 1 (BAP1) tumor suppressor has also been reported to induce ferroptosis by inhibiting SLC7A11 (94).

The concentration of glutamate inside and outside of the cell also exerts an indispensable effect on the Xc- system, and the difference in the concentrations of glutamate and cystine inside and outside the cell drives its own transmembrane diffusion. Glutamate can be continuously transported through its own transporter (EAA) to maintain a high intracellular concentration of glutamate and exported through the Xc- system, thereby supporting the cellular uptake of cysteine (95). Hydrogen peroxide (H_2O_2) reacts with Fe^{2+} through the Fenton reaction, ultimately producing a large amount of ROS with the accumulation of glutamate (96). Studies have revealed that a high extracellular glutamate content can not only suppress the function of the Xc- system to result in increased cell sensitivity to ferroptosis but can also be mediated by ionotropic glutamate receptors to lead to Ca^{2+} influx that is cytotoxic (97). Glutamate-mediated oxidative stress toxicity and excitotoxicity are important causes of nerve cell damage in neurodegenerative diseases (1). It has been observed that gastrodin can protect HT-22 cells from glutamate-induced ferroptosis through the Nrf2/HO-1 signaling pathway (98). Clinical studies have demonstrated that excitotoxicity is induced by elevated glutamate concentrations and is associated with cerebral vasospasm and ischemic neurological deficiencies after SAH (99). Sun et al. observed that cerebrospinal fluid (CSF) glutamate levels were significantly elevated within 48 hours after SAH, and ifenprodil improved long-term neurological deficits by antagonizing glutamate-induced excitotoxicity (99). However, further investigation is required to determine if glutamate plays a role in SAH by mediating ferroptosis.

4.3 NADPH-FSP1-CoQ10 pathway

CoQ10 is as a crucial component of the mitochondrial electron transport chain that can inhibit lipid peroxidation by trapping free radical intermediates (100). Thus, CoQ10 content plays an indispensable role in the balance of the redox system. CoQ10 depletion renders cells more susceptible to ferroptosis (101). Studies have demonstrated that FSP1 can reduce CoQ10 to its reduced form, CoQ10H2. With the help of NADPH, CoQ10H2 inhibits ferroptosis by trapping lipid peroxy radicals that mediate lipid peroxidation without GPX4 or GSH, and this reveals a novel NADPH-FSP1-CoQ10 pathway that inhibits ferroptosis in parallel with the GPX4/GSH system. Thus, FSP1 is a glutathione-dependent ferroptosis inhibitor (102, 103). Yuan et al. reported that the content of FSP1 and CoQ10 was obviously reduced in both *in vivo* and *in vitro* SAH models, thus suggesting that FSP1-mediated ferroptosis may be involved in EBI after SAH. Additionally, Fer-1 has been demonstrated to increase the content of FSP1, thereby attenuating ferroptosis induced by ferroptosis (30).

Additionally, FSP1 can indirectly influence vitamin E. As a natural antioxidant, vitamin E donates hydrogen atoms to PLOO to form vitamin E radicals (TOC). Immediately thereafter, TOC · can react with other PLOO · to produce a non-radical product, thereby achieving the function of reducing lipid peroxidation products and reducing the seriousness of ferroptosis (104, 105). In a study examining Alzheimer's disease, when GPX4 was knocked out in specific cerebral cortex and hippocampal neurons, mice exhibited significant cognitive disability in the water maze test and hippocampal neuron degeneration. Ferroptosis has been demonstrated to occur. When mice are fed a diet high in vitamin E, the level of neurodegeneration is reduced, thus indicating that vitamin E confers resistance to ferroptosis (105).

In lung cancer studies, plasma-activated medium induces ferroptosis by depleting FSP1. iFSP1 is considered to be the first FSP1 inhibitor discovered, and ferroptosis can be effectively regulated by targeting FSP1. It has been demonstrated that iFSP1 is able to increase sensitivity to ferroptosis in GPX4-KO cancer cells (102). Overall, these results suggest that the potential of FSP1 in ferroptosis is comparable to that of GPX4. Similarly, in ferroptosis, upregulating FSP1 or stabilizing FSP1 may represent a new direction in regard to improving the poor prognosis of SAH, and this also provides potential therapeutic targets for EBI. As a CoQ10 analog, idebenone stabilizes erythrocyte membranes and reduces lipid peroxidation and the severity of cellular damage in a dose-dependent manner. Idebenone has also exhibited a good therapeutic effect in regard to the treatment of retinal ischemia-reperfusion injury. However, a clinical study examining neuroprotective effects in 57 post-stroke aphasia patients revealed that idebenone did not improve the recovery of brain function compared to that of the placebo group. Thus, treatment with idebenone may possess a narrow therapeutic time window during which it can alleviate damage induced by lipid peroxidation, but it does not inhibit neuronal death after stroke. Therefore, the protective effect of idebenone in the context of stroke must be confirmed by further research, and its effect on the prognosis of SAH requires further study (101, 106).

In addition to the NADPH-FSP1-CoQ10 pathway, new research has identified the GCH1-BH4 pathway that can inhibit ferroptosis without CPX4. This pathway involves the GTP cyclohydrolase 1 (GCH1) gene that is the rate-limiting step in tetrahydrobiopterin (BH4) generation. BH4 inhibits ferroptosis by mediating the production of CoQ10H2 and inhibiting lipid peroxidation. A recent study demonstrated that dihydrofolate reductase (DHFR)

can inhibit ferroptosis by regenerating BH4 (107, 108). Kraft et al. observed that the activation of the GCH1/BH4 system can counter lipid peroxidation and alleviate ferroptosis (107).

4.4 The ARF/Keap1/Nrf2 pathway

Nrf2 can be activated by dissociation from Kelch ech-associated protein 1 (Keap1) under various stress conditions. Nrf2 recognizes antioxidant response elements (ARE) and activates a series of downstream antioxidant genes. 1) It can up-regulate the expression of GPX4, SLC7A11, and NADPH (109). Gou et al. confirmed that activation of the AKT/Nrf2/GPX4 pathway could alleviate hypoxic-ischemic brain damage (110). Forsythoside A acts against AD by targeting the Nrf2/GPX4 axis to regulate ferroptosis-mediated neuroinflammation. Additionally, inhibition of the Nrf2/GPX4 pathway can activate NF-κB, thus aggravating neuroinflammation (111). 2) The activity of heme oxygenase-1 (HO-1), an inducible enzyme, is important, as HO-1 is considered a measurable indicator of oxidative stress that oxidizes intracellular heme to carbon monoxide (CO), biliverdin, and Fe^{2+} (112). HO-1 exhibits cytoprotective effects by converting pro-oxidative hemoglobin and heme to the antioxidants bilirubin and biliverdin and may also exacerbate oxidative stress by releasing Fe^{2+} and CO. Therefore, HO-1 may exert a dual effect on the regulation of ferroptosis (113, 114). In a study examining retinal epithelial deformation, HO-1 was observed to induce ferroptosis by mediating the Nrf2/SLC7A11/HO-1 axis and the accumulation of ferrous ions (115). Hu et al. reported that β-caryophyllene activated the Nrf2/HO-1 axis to suppress ferroptosis in cerebral ischemia-reperfusion in rats and improve the degree of brain injury (116). Paradoxically, Wei et al. reported in a colorectal cancer study that activating the PERK/Nrf2/HO-1 axis result in ferroptosis (117). Unfortunately, the mechanism of Nrf2/HO-1 in the process of ferroptosis after SAH is currently poorly understood and is worth exploring. 3) NQO1 is a typical Nrf2 target enzyme (118) that exerts a protective effect against ferroptosis (114). NQO1 possesses both superoxide reductase and ubiquitin reductase activities and plays the role of α-tocopherol quinone reductase to convert endogenous α-tocopherol metabolites to the quinoline type, and this is a potent inhibitor of endogenous lipid peroxidation and ferroptosis (119). 4) Nrf2 plays a crucial role in the regulation of iron metabolism genes, including FTH1, FTL, and FPN1 (120, 121). The iron storage protein FTH1 may reduce active iron concentration and inhibit ferroptosis by converting Fe^{2+} to Fe^{3+} (122).

In addition to the Keap1/Nrf2 pathway, recent studies have revealed that the AMPK/PGC1α/Nrf2 pathway plays a role in ferroptosis after SAH. Puerarin is a flavonoid glycoside extracted from Pueraria roots (123). It has been demonstrated that puerarin possesses neuroprotective functions in the context of various central nervous system diseases. As an antioxidant, puerarin maintains the activity of antioxidant enzymes and protects cells from oxidative stress (124, 125) that can induce cell death. It regulates oxidative stress and mitochondrial function via the AMPK/PGC1α/Nrf2 pathways. The activation of this pathway exerts a critical impact on antioxidant activity in the adjustment of oxidative stress and ferroptosis (126). Previous research has demonstrated that in a rat model of hypoxic-ischemic encephalopathy, promoting AMP-activated protein kinase (AMPK) phosphorylation and upregulating PGC1α expression can exert neuroprotective effects by reducing oxidative stress and neuronal apoptosis. As a major antioxidant regulator, Nrf2 is regulated by the AMPK/PGC1α signaling pathway (127, 128). To explore the mechanism of puerarin in SAH, Huang et al. observed that puerarin reduced oxidative stress and ferroptosis after SAH through activating the AMPK/PGC1α/Nrf2 axis and also improved neurobehavioral disorders to a certain extent (129).

As one of the key regulators of antioxidant stress pathways (130), Nrf2 is normally maintained at low levels through ubiquitination mediated by the tumor suppressor Keap1. Glycogen synthase kinase 3β (GSK3β) is the primary negative regulator of Nrf2 activity, and hyperactivation of GSK3β leads to phosphorylation of specific serine residues in the Neh6 domain of Nrf2 to form a phosphorylated domain for degradation, ultimately resulting in Nrf2 inhibition. Studies have demonstrated that the antioxidant effect of Nrf2 is impaired by upregulation of Keap1 and activation of GSK3β (131). Another negative regulator, BTB domain and CNC homologue 1 (BACH1), inhibits the expression of Nrf2 target genes (e.g., HO1, NQO1, and xCT) by competing with Nrf2 to bind to ARE sequences (132). Namgaladze et al. revealed that silencing of BACH1 reduces labile iron pools and lipid peroxidation and enhances macrophage resistance to ferroptosis (133). Nrf2 was also regulated by ARF. ARFdoes not regulate Nrf2 protein content by interfering with Keap1-mediated ubiquitination but instead suppresses CBD-dependent Nrf2 acetylation that inhibits the expression of NRF2. Conversely, ARFdeletion induces Nrf2 activation and increases cellular resistance to ferroptosis. Additionally, certain miRNAs can alter the susceptibility of cells to ferroptosis by regulating the Nrf2 content. As an important inhibitor of ferroptosis, the function of NRF2 also exhibits other functions. It can also inhibit ROS generation to decrease the susceptibility of cells to ferroptosis (130). In a rat model of transient middle cerebral artery occlusion, Nrf2 concentration increased after 2 h, peaked at 8 h, and decreased between 24 and 72 h (134). The Nrf2 concentration is obviously higher in the penumbra than it is in the core (135), and this may be due to higher oxidative stress in the penumbra (134). TBHQ that can activate Nrf2 can improve Nrf2 activity and significantly reduce brain cell death.

5 Hippo–YAP pathway

Hippo–YAP signaling participates in various biological functions, including cell proliferation and organ size control (136) and is an important pathway in tumorigenesis and development. In a study examining breast tumors, Wu et al. reported that cells grown at high densities tended to be less sensitive to ferroptosis caused by cysteine depletion and GPX4 suppression. This also provided an opportunity for the discovery of the Hippo-YAP pathway in ferroptosis. Further studies have reported that intercellular interactions lead to ferroptosis in tumor cells by

mediating the NF2-YAP pathway and that YAP promotes the transcription of key ferroptosis genes such as ACSL4 and TFRC. They observed that inhibiting the expression of Hippo or promoting the expression of YAP increased the susceptibility of cells to ferroptosis (137). However, paradoxically, a study by Gao et al. that focused on hepatocellular carcinoma reported that YAP/TAZ, as a transcriptional coactivator, formed a complex with TEADs that indirectly bound the TEAD sequence in the SLC7A11 gene promoter, ultimately leading to upregulating the content of SLC7A11 and inhibiting the development of ferroptosis (138). The role of the Hippo-YAP pathway in SAH remains poorly studied.

6 Energy stress AMPK pathway

Energy stress depletes ATP and leads to cell death. Additionally, energy stress and glucose starvation increase ROS production (139, 140). Glucose starvation has been speculated to induce ferroptosis. In contrast, glucose starvation was previously demonstrated to effectively suppress ferroptosis (126). This study reported that this protective effect under energy stress is mediated by the activation of AMPK. When glucose starvation occurs, AMPK is activated, and this inhibits the biosynthesis of PUFAs. The lipid peroxidation drive of PUFAs is critical for ferroptosis (48, 141). Exhaustion of intracellular ATP and corresponding improvement in intracellular AMP concentration during energy stress activates AMPK by binding to AMP. Acetyl-CoA carboxylase 1 (ACC1) and Acetyl-CoA carboxylase 2 (ACC2) are two related enzymes that promote the synthesis of malonyl-CoA from acetyl-CoA and possess the functions of promoting fatty acid synthesis. Activated AMPK inhibits ACC1 and ACC2 that mediate fatty acid synthesis under energy stress, thereby leading to resistance to ferroptosis (126).

7 Relationship between oxidative stress, lipid peroxidation, and ferroptosis

After SAH occurs, the blood components enter the subarachnoid space. Various pathways and oxidative and antioxidant systems regulate the occurrence of ferroptosis (Figure 4), and they also regulate neuronal ischemia and hypoxia, mitochondrial dysfunction, and the production of a large amount of ROS during electron transfer (142, 143). ROS induces a local inflammatory response, thus triggering a downstream inflammatory cascade that causes a near-exponential increase in ROS that ultimately leads to the development of oxidative stress. Additionally, the immune system is activated, and many peripheral inflammatory cells enter the subarachnoid region under the chemotaxis of inflammatory cytokines. The inflammatory cells secrete a variety of inflammatory cytokines, thus forming a vicious cycle that results in the generation of numerous ROS (144). Antioxidant systems such as GSH/GPX4 scavenge ROS, and the imbalance between the generation of ROS and the antioxidant system leads to the accumulation of lipid peroxides and ROS (145, 146) that in turn leads to ferroptosis. Ferroptosis exhibits features that include lipid peroxide accumulation and iron dependence, and it is the most likely form of cell death in response to oxidative stress. Superoxide produced by oxidative stress reacts with H^+ to produce H_2O_2. A large number of erythrocytes entering the subarachnoid space after SAH lead to an increase in the concentration of iron ions, and the Fenton reaction between ferrous iron and H_2O_2 occurs (96), ultimately resulting in the production of highly active OH- with the accumulation of glutamate. The occurrence of lipid peroxidation mediated by ROS species such as OH- causes the accumulation of more lipid peroxidation, while the high extracellular concentration of glutamate inhibits the Xc- system and results in exhaustion of GSH and inhibition of GPX4. It activates ALOXs that use iron as a cofactor and react with membrane phospholipids containing unsaturated fatty acids to generate large amounts of lipid peroxides that further attack and oxidize cell membrane lipids and trigger ferroptosis (147). Concurrently, GSH is depleted by a large amount of ROS, ultimately resulting in the activation of inositol triphosphate receptors (IP3R) to thereby deplete calcium stores in the endoplasmic reticulum and trigger the activation of calcium release-activated calcium modulator 1 (ORAI1). Activation of store-operated calcium entry (SOCE) then leads to a growth in intracellular Ca^{2+} concentration, and this further leads to the production of a large amount of ROS. It not only aggravates oxidative stress, but also leads to ferroptosis due to excess ROS. The change in Ca^{2+} is not only affected by GSH content, but in turn, the changed Ca^{2+} also further depletes GSH by boosting the generation of ROS. The depletion of GSH then leads to the inactivation of GPX4, ultimately causing the accumulation of lipid peroxides that induce ferroptosis (148). Moreover, ALOXs can not only directly react with lipids to produce lipid hydroperoxides, but the metabolites of 12-LOX can also activate soluble guanylate cyclase (GC) to generate cGMP. Activation of ORAI1 and SOCE by cGMP promotes the influx of Ca^{2+} into cells (149, 150) and causes ROS production. Both oxidative stress and ferroptosis are caused by massive accumulation of oxides. GSH plays a major role in anti-oxidation (151). Therefore, both ferroptosis and oxidative stress result in a decrease in GSH that leads to damage to the related antioxidant system. Finally, the production of ROS is greater than its elimination, and the redox system is unbalanced, thus resulting in cytotoxicity. SIRT1 is a III histone deacetylase that can regulate multiple cellular biological processes such as inflammation, oxidative stress, energy metabolism, DNA damage repair, and cell death (152, 153). There is increasing evidence that neuroinflammation is firmly connected to the pathogenesis of a number of neurological diseases (154). Numerous studies have demonstrated that SIRT1 positively affects neuroinflammation-associated disease. For example, Hernández-Jiménez et al. reported that SIRT1 could reduce cerebral ischemia-induced neuroinflammation and neuronal damage by suppressing P53 and NF-κB acetylation (155). The neuroprotective effect of SIRT1 in the context of cerebral ischemia is mediated by multiple mechanisms. After ischemic stress, DNA damage and oxidative stress activate P53 that in turn promotes mitochondrial apoptosis

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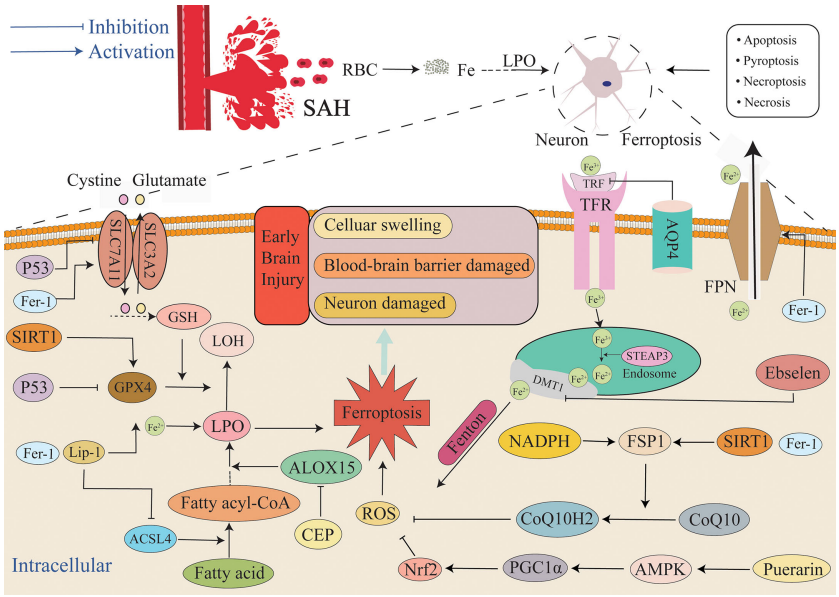

FIGURE 4
Mechanism of ferroptosis in EBI after SAH. After SAH, a large amount of blood and ruptured erythrocyte flow into the subarachnoid space, and ferroptosis, necrosis, apoptosis, necroptosis and pyroptosis can all lead to the death of neurons and other cells. This article focuses on ferroptosis, an iron-independent cell death mechanism characterized by lipid peroxide accumulation that exacerbates EBI. The extracellular environment is primarily Fe^{3+}, and it is primarily combined with TRF and enters into cells through TFR. After entering cells, endosomes are formed, Fe^{3+} is reduced to Fe^{2+} by STEAP3, and Fe^{2+} is diverted into cells via DMT1. Fe^{2+} mediates ROS production by the Fenton reaction. FPN can also reduce the intracellular iron concentration by transporting iron ions out of the cell. Additionally, the GSH/GPX4 and FSP1/COQ10 systems act as the primary antioxidant systems to suppress the production of lipid peroxides, and the imbalance between the oxidation system and the antioxidant system will lead to the accumulation of lipid peroxides and finally lead to ferroptosis. Aquaporin 4 can also reduce iron ion concentration and the severity of ferroptosis by inhibiting TFR. AMPK, AMP-activated protein kinase; DMT1/SLC11A2, divalent metal transporter 1; FSP1, ferroptosis suppressor protein 1; SLC7A11, solute carrier family 7 member 11.

signaling and neuronal death (156–158). SIRT1 inhibition of P53 can suppress apoptosis, promote cell survival, and protect neurons from ischemia-induced cell death (159, 160). SIRT1 gene deletion or pharmacological inhibition increases peri-infarct area (155). Increasing numbers of studies examining SAH have demonstrated that SIRT1 is extensively expressed in the brain and possesses an endogenous neuroprotective function in EBI through regulating oxidative and inflammatory signaling (76, 161–163). SIRT1 activation improves EBI neural function by inhibiting the inflammatory response to oxidative stress, whereas SIRT1 silencing aggravates SAH-induced brain damage. Yuan et al. up-regulated the content of SIRT1 via RSV pretreatment and decreased the content of SIRT1 via SEL pretreatment. The experimental results demonstrated that the artificial overexpression of SIRT1 through RSV mediated the upregulation of GPX4 and FSP1 expression and significantly reduced the concentration of lipid peroxidation, and this significantly alleviated ferroptosis. Furthermore, inhibition of SIRT1 activation via SEL reduced GPX4 and FSP1 concentrations and induced neuronal ferroptosis. Specifically, this suggests that SIRT1 exerts a neuroprotective effect in the context of ferroptosis when the intracellular antioxidant system is activated (30). In conclusion, SIRT1 not only inhibits oxidative stress to a certain extent but also confers resistance to ferroptosis. In summary, it can be observed that ferroptosis and oxidative stress are very similar in many aspects, and ferroptosis cannot simply be considered as an independent cell death pathway and may even be a more particular form of oxidative stress outcome.

8 Crosstalk between microglia, astrocytes and neurons in ferroptosis

After SAH, different cells in the brain play different roles and respond differently with the severity of the disease. Glial cells, comprising astrocytes and microglia, act as vigilant protectors of neurons, working to preserve the integrity of the blood-brain barrier, regulate synaptic activity, and respond to injury within the central nervous system (CNS) (164). Glial cells are known to express a range of iron transporters and iron metabolizing proteins, which are critical for maintaining iron homeostasis and ensuring proper functioning of the brain (165, 166). In order to maintain a balance of iron in the body, it is important for certain cells of the immune system, such as microglia and astrocytes, to play a role. Bind free iron via cytoplasmic and mitochondrial ferritin, thereby reducing extracellular iron concentrations. Astrocyte-neuron interactions protect neurons from iron-mediated cytotoxicity, and circadian regulation of BDNF-mediated Nrf2 activation in astrocytes protects dopaminergic neurons from ferroptosis (167). Microglia are the most sensitive to ferroptosis (168), When microglial iron homeostasis is unbalanced, excessive ROS and inflammation will be produced, accompanied by increased free

iron (169), which will cause cytotoxicity to other cells such as neurons, leading to oxidative stress and ferroptosis (170). The development of single-cell sequencing has significantly contributed to our profound comprehension of the intercellular communication during ferroptosis (171, 172). Through single-cell RNA-sequencing, Chen et al. identified multiple SAH-specific microglial cluster (SMG-C) in a mouse model of SAH, among which the corresponding genes for SMG-C5, SMG-C6, and SMG-C7 were only highly expressed in SAH microglia and not in normal microglia. These SMG-C subgroups were closely associated with neuroinflammation, oxidative phosphorylation, and apoptosis after SAH (173, 174). Additionally, Dang et al. also found that ferroptosis was activated in astrocytes and may be involved in the pathological process of Alzheimer's disease (175). Zhang et al' study identified ten cell types including cholinergic neurons, dopaminergic neurons, glutaminergic neurons, neuronal precursors, microglia, oligodendrocytes Cells and radial glial cells, etc., and revealed ferroptosis as a new mechanism of manganese-induced neurotoxicity (171). Overall, single-cell RNA sequencing can be used to analyze different types of cells in brain tissue, identify cell types and signaling pathways associated with ferroptosis, and investigate the relationship between gene variations or genomic variations in specific cells and ferroptosis, thereby providing clues for potential therapeutic targets for SAH.

9 Detecting indicators of ferroptosis

As ferroptosis is inextricably linked to the EBI of SAH, timely judgment and effective means to inhibit the occurrence of ferroptosis are extremely important in regard to the prognosis of EBI. Ferroptosis can be induced in iron metabolism by detecting intracellular iron content. After a mild traumatic brain injury, iron accumulation in certain regions of the thalamus can indicate the likelihood and severity of future post-traumatic headaches. Specifically, patients who have suffered an acute traumatic brain injury have been found to have higher iron deposition in the left lateral geniculate nucleus compared to healthy controls. This increased iron deposition in the left lateral geniculate nucleus may be indicative of the severity of the injury and could potentially lead to a poorer recovery from post-traumatic headaches (176). Furthermore, research has indicated that serum iron levels at admission can serve as an independent risk factor for delayed cerebral ischemia following SAH (177). The buildup of iron in the brain may be connected to secondary brain injury in individuals with SAH. In patients with poor grade SAH, iron accumulation is often observed in the white matter. Higher levels of intraventricular hemorrhage are correlated with higher levels of iron deposition. Additionally, patients with vasospasm have been found to have higher levels of iron compared to those without vasospasm (176). TFR plays an important role in intracellular iron metabolism. It has been demonstrated that the expression of TFR is significantly positively correlated with the severity of ferroptosis and that apoptosis is not related. Therefore, the TFR is considered as a marker of ferroptosis (178). Ferritin also plays a crucial role in iron homeostasis. FTH1, an important component of cellular ferritin, is a biomarker that reflects intracellular iron homeostasis. In general, higher levels of FTH1 cause cells to be more resistant to ferroptosis (11, 178). This is due to ferroptosis being a result of lipid peroxidation. As an upstream molecule of lipid peroxidation, it has been demonstrated that the expression of ACSL4 is up-regulated, while the expression of other ACSL family members is not upregulated. ACSL4 is a biomarker that predicts ferroptosis sensitivity (179). Additionally, ROS, lipid peroxides, and other oxidation products are significantly correlated with ferroptosis. Metabolites of lipid peroxides such as malondialdehyde (MDA) and 4-HNE are crucial markers of the severity of ferroptosis. Within 2 h after stroke induction, the levels of 4-HNE in the ischemic cerebral cortex were increased (180). Lee et al. further demonstrated that plasma 4-HNE concentration increased in ischemic stroke and became a potential biochemical indicator of ischemic stroke (180, 181). The ability of GPX4 to convert toxic PUFAs-OOH to non-toxic PUFAs-OH in the presence of GSH such as GPX4 and GSH is often considered to be highly correlated with ferroptosis markers. The higher the content of GPX4 and GSH, the less prone they are to ferroptosis (63, 64). However, these indicators are non-specific and can be influenced by many other diseases such as oxidative stress.

10 Outlook and conclusion

Compared to other types of stroke, SAH is a life-threatening cerebrovascular disease that seriously affects the quality of life (182). Although the morbidity and mortality of SAH have declined due to emerging therapies and improvements in clinical management, both remain high (183). As the focus of research has shifted from cerebral vasospasm to EBI, it has been observed that the role of ferroptosis in SAH is particularly important. Experimental and clinical data have demonstrated that ferroptosis is effectively inhibited by regulating iron metabolism, lipid peroxidation, and the CPX4 and Xc systems, and the adverse EBI outcome is improved (2, 10, 11, 29, 31, 68, 93, 184). It has been demonstrated that there are multiple forms of cell death in the context of EBI (185). For example, ferroptosis, necrosis, apoptosis, necroptosis, and pyroptosis that ultimately lead to the poor prognosis of SAH all occur. Therefore, there is crosstalk between ferroptosis and other forms of death that can be synergistic or antagonistic and occur as upstream or downstream reactions. This series of questions is worthy of further investigation. After SAH, the iron ion is one of the initiating factors of ferroptosis, and changes in its concentration are closely related to ferroptosis. Previous studies examining SAH have primarily focused on iron-related transporters and ferrotinophagy. However, IRP is an important target for iron concentration regulation (186, 187), and its role in ferroptosis after SAH has rarely been studied. We speculate that IRP as an important regulatory factor in iron metabolism may exert an indispensable effect on ferroptosis in the context of EBI. Research has revealed that the influx of Ca^{2+} also leads to the generation of ROS (149). Therefore, other inorganic ions such as calcium ions are involved in ferroptosis. An imbalance between the oxidative and antioxidant systems that causes the collection of ROS is the primary cause of ferroptosis. Studies have demonstrated that the GSH/GPX4 system

is the primary antioxidant system, and thus, it is likely that the SOD antioxidant and catalase systems are involved in SAH. Little research has been conducted examining the role of SOD in ferroptosis in EBI. It remains unclear if it can become a new therapeutic target in SAH and improve the lethality and mortality of clinical patients. Currently, in related research examining ferroptosis after SAH, the primary research focuses on the GSH/GPX4, NADPH/FSP1/COQ, and other pathways; however, the Hippo/YAP pathway has exhibited good anti-ferroptosis in tumor-related research (136, 137, 188). It has not yet been verified if it is involved in ferroptosis after SAH. If it is involved, it is worth exploring if the pathway depends upon the GSH/GPX4 pathway. This review provides a plausible conjecture regarding the interrelationship between oxidative stress and ferroptosis in EBI after SAH, but there is growing evidence that there are also interactions between ferroptosis and other types of cell death. It is necessary to fully explore the relationship between various forms of death and ferroptosis and to determine if there is a common pathway. Answering these questions may provide a new therapeutic target for SAH, ultimately improving the poor prognosis of the majority of SAH patients and reducing family and social burden. However, research focused on ferroptosis still faces challenges, as we do not fully understand the mechanisms related to ferroptosis in the context of SAH.

Author contributions

YH and XG contributed to the conception and design of the study. XD, YW, ZH, SW, SZ and CZ organized the database. XD wrote the first draft of the manuscript. YH reviewed and edited. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Repetitive transcranial magnetic stimulation for stroke rehabilitation: insights into the molecular and cellular mechanisms of neuroinflammation

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Stroke is a leading cause of mortality and disability worldwide, with most survivors reporting dysfunctions of motor, sensation, deglutition, cognition, emotion, and speech, *etc.* Repetitive transcranial magnetic stimulation (rTMS), one of noninvasive brain stimulation (NIBS) techniques, is able to modulate neural excitability of brain regions and has been utilized in neurological and psychiatric diseases. Moreover, a large number of studies have shown that the rTMS presents positive effects on function recovery of stroke patients. In this review, we would like to summarized the clinical benefits of rTMS for stroke rehabilitation, including improvements of motor impairment, dysphagia, depression, cognitive function, and central post-stroke pain. In addition, this review will also discuss the molecular and cellular mechanisms underlying rTMS-mediated stroke rehabilitation, especially immune regulatory mechanisms, such as regulation of immune cells and inflammatory cytokines. Moreover, the neuroimaging technique as an important tool in rTMS-mediated stroke rehabilitation has been discussed, to better understanding the mechanisms underlying the effects of rTMS. Finally, the current challenges and future prospects of rTMS-mediated stroke rehabilitation are also elucidated with the intention to accelerate its widespread clinical application.

KEYWORDS

transcranial magnetic stimulation, stroke, rehabilitation, neuroinflammation, microglia, neurotransmitter, neuroimaging technique

1 Introduction

Stroke is one of the leading causes of mortality and disability worldwide, with most survivors reporting a decrease in quality of life, placing heavy financial burden on patients' families and society (1, 2). The stroke can lead to an imbalance in blood supply and thereby induce severe brain damages, result in various dysfunctions, such as motor impairment, dysphagia, cognition impairment, post-stroke pain, depression, *etc* (3). Current best stroke treatment is to minimize initial brain damage and prevent subsequent complications, and then improve function through rehabilitation training (4). Moreover, most stroke miss the best therapeutic window for emergency treatment, such as thrombolysis or thrombectomy, therefore, the rehabilitation is quite important for stroke patients to improve motor, swallowing, and cognitive functions (5, 6). Currently, lots of rehabilitation programs have been applied by rehabilitation specialist to recovery the impaired functions and improve the quality of life. Commonly used rehabilitation programs in clinical practice include: physical therapy, occupational therapy, speech therapy, hyperbaric oxygenation, acupuncture, *etc* (7), which can improve post-stroke dysfunction by stimulating central nervous system (CNS) input through sensorimotor system or by promoting CNS remodeling through intensive training of motor patterns. At present, these traditional rehabilitation programs remain the main methods for post-stroke recovery, which can promote the recovery of various dysfunctions after stroke to a certain extent. However, there are also some shortcomings for these rehabilitation programs, such as slow onset, time-consuming, and poor patient compliance (8). Due to the certain shortcomings of traditional rehabilitation programs, various novel techniques have been utilized for stroke rehabilitation, such as repetitive transcranial magnetic stimulation (rTMS), transcranial direct current stimulation (tDCS), virtual reality, augmented reality, brain-computer interface, and rehabilitation robot, which have shown great stroke rehabilitation effects (9–12).

rTMS, as a noninvasive brain stimulation (NIBS), is a neurostimulation technique that uses a pulsed magnetic field to regulate the membrane potential of neurons, thereby selectively modulating the neural excitability of brain regions (13). rTMS has been applied in neurological and psychiatric diseases, including cognitive impairment, depression, mental disease, and Alzheimer's disease (AD) (14–17). It is painless and easy to operate, making it a valuable tool for providing more precise brain area regulation and prognostic assessment in stroke rehabilitation. The interhemispheric inhibition (IHI) model is the primary theoretical basis for the application of rTMS in stroke rehabilitation (18). This model suggests that the two brain hemispheres are connected by the corpus callosum, which not only transmits information between the hemispheres but also regulates their interactions. Under healthy condition, the excitatory or inhibitory activity between the two hemispheres is balanced through the corpus callosum. However, stroke disrupts the balance of mutual inhibition between the hemispheres, resulting in weakened inhibition of the affected hemisphere on the unaffected hemisphere, and increased inhibition of the unaffected hemisphere on the affected hemisphere (4). Another model is called compensation, which suggests that the neural conduction in the damaged area is disrupted after stroke (19). The neurons and astrocytes in the unaffected hemisphere can form new circuits to compensate for the damaged area, allowing for the recovery of motor function in stroke patients. However, the compensation model cannot be applied when a patient experiences a bilateral stroke (20). A lager number of clinical trials have been conducted to evaluate the potential of rTMS to promote rehabilitation of limb, swallowing, and cognitive functions post-stroke, which showed satisfactory recovery outcomes (21–24).

In this review, the clinical benefits of rTMS for stroke rehabilitation are summarized, including improvements of motor impairment, dysphagia, depression, cognitive function, and central post-stroke pain. Besides, this review will also focus on the molecular and cellular mechanisms underlying rTMS-mediated stroke rehabilitation, especially those related to immune regulation.

2 Clinical benefits of rTMS for stroke rehabilitation

rTMS have been shown to promote effectively rehabilitation of neurological sequelae post-stroke, including motor impairment, dysphagia, cognitive impairment, mental diseases, and neuropathic pain, which will be summarized and discussed in this section (Table 1).

2.1 Motor impairment

Most stroke patients experience upper limb motor impairment, with only 5%-20% of them being able to fully recover their upper limb function (35). Upper limb impairment can significantly affect stoke patients' abilities, leading to a negative impact on their quality of life (36). For example, The loss of upper limb function can severely affect tasks such as eating, dressing, and personal hygiene care, and result in a loss of independence (37). Stroke disrupts the balance between the brain hemispheres, which is an important cause of upper limb motor impairment after stroke (38, 39). Repetitive transcranial magnetic stimulation (rTMS) can modulate cortical excitability and thus recovery the balance post-stroke (40, 41). According to IHI model, there are two main options to using rTMS to promote functional recovery post-stroke: one is to use low-frequency (≤1Hz) rTMS (LF-rTMS) to stimulate the unaffected hemisphere and reduce its excitability, thus decreasing its inhibitory effect on the affected hemisphere; the other is to use high-frequency (≥3Hz) rTMS (HF-rTMS) to stimulate the affected hemisphere and increase its excitability, thereby restoring balance between the hemispheres (42–44). Numerous studies have revealed that rTMS over primary motor cortex (M1) can improve upper limb motor function post-stroke (25, 26, 28, 43, 45, 46). A meta-analysis of Hsu et al. included 392 stroke patients from 18 studies, which suggest that rTMS could promote upper limb motor recovery in stroke patients, especially those with subcortical stroke (47). And

TABLE 1 Main clinical outcomes after rTMS with various characteristics.

Study	Stroke stage	rTMS site	rTMS frequency (Hz)	Intensity (%)	Combined treatment	Clinical outcomes	Outcome measures	References
Aşkın et al. (2017)	Chronic (n=40)	Contra-M1	1	90 RMT	Physical therapy	Improvement of upper limb motor function	FMA, BBT, FIM, FAS	(25)
Lüdemann-Podubecká et al. (2016)	Subacute (n=10)	Contra-PMd	1	110 MT	/	Improvement of motor function of affected hand	JTT, BBT, MEP, CSP, ISP	(26)
Tosun et al. (2017)	Acute/ subacute (n=25)	Contra-M1	1	90 RMT	Physical therapy/ NMES	Improvement of upper limb motor function	BRS, FMA, fMRI, UE-MI, BI, MAS	(27)
Hosomi et al. (2016)	Subacute (n=41)	Ipsi-M1	5	90 RMT	/	Improvement of motor function of paralytic hand	BS, NIHSS, FMA, FIM	(28)
Sasaki et al. (2017)	Acute (n=21)	bilateral leg motor areas	10	90 RMT	/	Improvement of lower limb motor function	BRS, ABMS II	(29)
Choi et al. (2016)	Chronic (n=30)	motor cortical area of the 9th thoracic erector spinae muscles	10	90 RMT	/	Improvement of balance function	BBS, CDP	(30)
Cheng et al. (2014)	Chronic (n=4)	Ipsi-tongue motor cortex	5	90 RMT	/	Improvement of swallowing functions and swallowing related quality of life	SAPP, VFSS, TPA	(24)
Khedr et al. (2009)	Acute (n=26)	Ipsi-oesophageal cortical area	3	100 RMT	/	Improvement in dysphagia	BI, MEP, Grip strength	(31)
Sasaki et al. (2017)	Chronic (n=13)	Region spanning from the dACC to mPFC	10	80 RMT	/	Improvement of apathy	QIDS, AS	(32)
Sharma et al. (2020)	Subacute (n=96)	Contra-M1	1	110 RMT	Physical therapy	Improvement of motor function	HAMD, mBI, mRS, FMA, NIHSS	(33)
Kim et al. (2010)	Chronic (n=18)	Left DLPFC	1, 10	80 MT	/	Improvement of mood	BDI, CPT, mBI	(23)
Yin et al. (2020)	Chronic (n=34)	Left DLPFC	10	80 RMT	/	Improvement of cognitive function and quality of life	ALFF, FC, mBI	(34)

they found that applying LF-rTMS on the unaffected hemisphere might be safer and more effective than HF-rTMS on the affected hemisphere in improving upper limb motor function after stroke. The evidence-based guidelines on the therapeutic use of rTMS, updated by International Federation of Clinical Neurophysiology in 2019, recommended that LF-rTMS over M1 of unaffected hemisphere at the subacute stage of stroke as level A evidence (definite efficacy) could recover hand motor effectively (48). HF-rTMS over the M1 of affected hemisphere at the subacute stage as well as LF-rTMS over M1 of unaffected hemisphere at the chronic stage of stroke were recommended as level B (probable efficacy) and C (possible efficacy) evidences. Consistent with the guideline, a meta-analysis of Mu group including 904 stroke patients from 34 studies indicated that the effectiveness of rTMS on stroke patient present timing-dependent manner: the acute phase > the subacute phase > the chronic phase (49). In addition, rTMS can be combined with other therapies to improve upper limb function after stroke, including occupational therapy (22), virtual reality training (50), action observation (51), and upper-limb training (52), *etc.*

Motor impairment increases the risk of falling due to gait impairments, resulting in limitations in activities of daily living and a lower quality of life (46, 53). Clinicians prioritize the improvement of lower limb motor function and walking ability when treating stroke patients (54, 55). Therefore, the study of rTMS on lower limb motor function also has significant clinical implications. Tung et al. performed a meta-analysis based on 169 stroke patients and found that rTMS could remarkably improve walking speed, lower limb activity, and Fugl-Meyer Assessment lower limb scores (56). Another meta-analysis from Li group indicated that rTMS, especially HF-rTMS over unaffected hemisphere, could significantly improve walking speed of stroke patients (57). In addition, the meta-analysis conducted by Vaz et al. revealed that either HF-rTMS or LF-rTMS combined with other rehabilitation therapies could significantly improve gait speed in

both acute/subacute and chronic stages of stroke (58). In addition to improving walking speed, Choi et al. found that HF-rTMS [10 Hz, 90% resting motor threshold (RMT)] over the trunk motor cortex could remarkably improve the balance function of chronic stroke patients without any side effects (30).

Post-stroke lower limb spasticity impairs gait and balance to reduce speed of walking, thereby increasing the need of wheelchair and caregiver (59). A meta-analysis of Liu et al., including 554 stroke patients from 9 studies, aimed to evaluate the efficacy of rTMS in improving post-stroke lower limb spasticity (60). They revealed that rTMS could decrease Modified Ashworth Scale (MAS) score and elevate Modified Barthel Index (MBI) score, compared with the control group. Further subgroup analysis concluded that LF-rTMS showed a positive effect on lower limb spasticity after stroke, while the HF-rTMS showed no significant effect on the lower limb motor function. Due to the limited studies included in this meta-analysis, the recovery effect of HF-rTMS on lower limb spasticity remains to be studied. Besides, the mechanism by which rTMS improves lower limb spasticity is still unclear and needs to be further explored in future studies.

2.2 Dysphagia

Dysphagia is a common complication in stroke patients, with a prevalence of ~53%, which can result in aspiration pneumonia, malnutrition, electrolyte imbalances, and even death (61). Dysphagia is associated with prolonged hospital stay, poor life quality, elevated mortality, make it imperative to prioritize early intervention for improving swallowing function (62, 63). Current treatments include postural interventions, surgery, botulinum toxin injections and exercise, but these are less effective (64–66). Recently, noninvasive neurostimulation therapies have been found to improve the dysphagia in stroke patients (67). Among the noninvasive neurostimulation therapies, rTMS might be the most effective treatment for dysphagia after stroke, compared to tDCS, pharyngeal electrical stimulation (PES), and surface neuromuscular electrical stimulation (sNMES) (67). For instance, Khedr et al. evaluated the recovery effect of rTMS on the swallowing performance in 26 patients with dysphagia at the acute stage of stroke (31). In rTMS-treated group, the esophageal cortex of the affected hemisphere was received HF-rTMS daily (3 Hz and 120% of RMT) for 5-10 days. The results showed that the rTMS significantly improved the dysphagia of stroke patients for several months. In another study, Verin et al. applied LF-rTMS (1 Hz) on the mylohyoid cortical area of the unaffected hemisphere in patients with dysphagia at the chronic stage of stroke, resulting in a greater improvement in swallowing function and a remarkable decrease in the aspiration score for liquids (68). Besides, HF-rTMS (5 Hz) applied over the tongue region of the motor cortex of the unaffected hemisphere has been shown to improve swallowing performance of stroke patients with chronic dysphagia (24). Although the rTMS has been shown to improve swallowing function in a large number of studies, there is no unified treatment standard for its stimulation site, intensity and treatment duration (69–71). Therefore, it is necessary to conduct large-scale multi-center clinical studies on rTMS for dysphagia, and further develop standardized treatment guideline in the future.

2.3 Depression

Post-stroke depression (PSD) is the most common neuropsychological complication of stroke with an incidence rate of ~33% (72). PSD has been shown to reduce quality of life, affect rehabilitation outcome, and increase mortality rate (32, 73, 74). Current therapies for PSD include pharmacotherapy, psychotherapy and physical therapy, however, some patients do not benefit from these first-line treatments (75–77). Fortunately, several studies indicated that rTMS was expected to improve the neuropsychological disorder (78, 79). And the U.S. Food and Drug Administration (FDA) approved rTMS over left dorsolateral prefrontal cortex for treatment of the major depressive disorder (MDD) in 2008 (80). Thus, a large number of clinical trials have explored the therapeutic efficacy of rTMS on the PSD (81, 82). However, the results from these clinical trials were inconsistent. Thus, a meta-analysis of Shen et al. included 1764 PSD patients from 22 randomized controlled trials (RCTs) studies to explore the therapeutic efficacy of rTMS for PSD (83). The results showed that rTMS could remarkably improve the PSD measured by Hamilton Depression Rating Scale (HAMD). Further safety evaluation showed no statistical difference in withdrawals owing to adverse events. However, most of these results are from single studies with varying degrees of limitations. Therefore, definitive conclusions about the treatment of PSD with rTMS need to be further confirmed by multicenter RCTs. The traditional rTMS protocol requires treatment 5 days per week for more than 4 weeks. Frey et al. proposed an accelerated rTMS strategy to reduce the number of days needed to complete treatment, which can bring convenience to the patients and increase compliancy. They applied HF-rTMS (20 Hz) at 110% RMT over the left dorsolateral prefrontal cortex of the PSD patients 5 sessions per day for 4 days. The results revealed that HAMD of PSD patients remarkably reduced after the accelerated rTMS, which maintained for 3 months. Besides, no significant adverse events associated with rTMS treatment were observed in the study. This study suggested that the accelerated rTMS protocol could be a more convenient adjuvant treatment option for PSD patients.

2.4 Cognitive function

Post-stroke cognitive impairment (PSCI) occurred in nearly 75% of stroke patients (84). Only half of them are able to recovery the cognitive function, whereas the others might develop vascular dementia (85). Since stroke patients with PSCI may experience impaired judgment and memory problems, PSCI can hinder physical recovery (86). Furthermore, prolonged cognitive impairment can significantly affect activities of daily living (ADL), quality of life, and reintegration into the community (87–89). Thus, effective intervention to improve PSCI is a very important part of stroke rehabilitation. The therapies of PSCI included

pharmacological therapy (*e.g.*, acetylcholinesterase inhibitor, memantine, traditional Chinese medicines, *etc.*), cognitive training, risk factor prevention and intervention (90, 91). In recent years, rTMS has been applied to treat cognitive impairment induced by several CNS diseases, including Alzheimer's disease, depression, Parkinson's disease, and bipolar disorder (14, 15, 92, 93). Notably, several studies have found that rTMS also showed positive therapeutic effects on PSCI (23, 34, 94–96). For example, Yin et al. applied 20 sessions of rTMS (10 Hz, 80% RMT) over the left dorsal lateral prefrontal cortex of PSCI patients, which revealed that rTMS could improve cognitive function and ADLs of PSCI patients (34). Moreover, the functional MRI (fMRI) of rTMS-treated patients indicated that the rTMS might activate left medial prefrontal cortex and augmented the functional connectivity to right medial prefrontal cortex and ventral anterior cingulate cortex, resulting in the improvements of cognitive function. These studies suggested that the rTMS can be an important and effective treatment to rescue the cognitive function in PSCI patients, and that functional connectivity (FC) and neural activity in cognition-related brain regions could be crucial indicators to clarify the effect of rTMS on PSCI. In addition, rTMS has exhibited a superior modulating effect in cognitive function compared to other non-invasive stimulation techniques (96). Intermittent theta-burst stimulation (iTBS) as a type of TMS has also been applied to improve the PSCI. Tsai et al. evaluated the therapeutic effect of rTMS (5 Hz, 80% RMT) and iTBS on the PSCI patients, suggesting that both rTMS and iTBS could effectively improve cognitive impairment, including global cognitive function, memory function, and attention (96). Compared with the iTBS group, the rTMS group showed better improvement in attention.

2.5 Central post-stroke pain

Central post-stroke pain (CPSP) is a neuropathic pain syndrome that can occur after a cerebrovascular accident, with an incidence rate of 1~12% (97, 98). The main treatment for CPSP is pharmacology (99). However, the pharmacology has limited pain control and unpleasant side effects. Recently, several studies found that HF-rTMS could relieve CPSP to some extent (100–102). Leung et al. found that rTMS showed great analgesic effect on CPSP (16.7% of visual analog scale score reduction), and rTMS exhibited better improvement in CPSP than peripheral neuropathic pain (101), suggesting that rTMS might be a promising therapeutic tool for CPSP.

FMA, Fugl–Meyer Assessment; BBT, Box and Blocks Test; FIM, Functional Independence Measurement; FAS, Functional Ambulation Scale; rTMS, repetitive transcranial magnetic stimulation; PMd, dorsal premotor cortex; RMT, resting motor threshold; MT, motor threshold; JTT, Jebsen–Taylor Hand Function Test; MEP, motor evoked potential; CSP, cortical silent period; ISP, ipsilateral silent period; NMES, neuromuscular electrical stimulation; fMRI, Functional magnetic resonance imaging; BRS, brunnstrom recovery stage; UE-MI, Upper extremity motricity index; BI, barthel index; MAS, Modified ashworth scale; BS, Brunnstrom stages; NIHSS, National Institutes of Health Stroke Scale; ABMS II, Ability for Basic Movement Scale Revised; ipsi, ipsilateral; contra, contralateral; BBS, Berg Balance Scale; CDP, Computerized dynamic posturogphy; SAPP, Swallowing Activity and Participation Profile; TPA, tongue pressure assessment; VFSS, Videofluoroscopic swallowing study; QIDS, Quick Inventory of Depressive Symptomatology; AS, Apathy Scale; dACC, dorsal anterior cingulate cortex; mPFC, medial PFC; mBI, modified Barthel Index; HAMD, Hamilton Depression Scale; mRS, modified Rankin Scale; DLPFC, dorsolateral prefrontal cortex; BDI, Beck Depression Inventory; CPT, Continuous Performance Test; ALFF, amplitude of low-frequency fluctuation; FC, functional connectivity.

3 The molecular and cellular mechanisms of neuroinflammation underlying rTMS-mediated stroke rehabilitation

Despite the confirmed value, the regulatory mechanism *via* which TMS shows the beneficial effects on stroke rehabilitation remain unclear. Recent studies have shown that the basic mechanisms might be involved in regulation of neurotransmitters release, immune cells, and cytokines (Figure 1) (102–105).

3.1 Neurotransmitters

Neurotransmitters as chemical messengers are released from a neuron to excite or inhibit other neurons (106). Neurotransmitters, including glutamate, gamma-aminobutyric acid (GABA), glycine (Gly), and acetylcholine (ACh), play an important role in chemical synapses of the CNS. Thus, the abnormal metabolism or release of the neurotransmitters can result in synaptic dysfunction, impaired neurogenesis, impairment of cognitive function, depression, memory deficits and CPSP, *etc* (107–109). Numerous studies have shown that stroke could induce abnormal release of neurotransmitters (107, 110). Therefore, modulation the neurotransmitters might be a rational approach to promote the stroke rehabilitation.

A number of studies have shown that rTMS could alter the release and expression of neurotransmitters in the CNS, which might be the underlying mechanisms of rTMS-based stroke rehabilitation (102, 105, 111–114). As an excitatory neurotransmitter, glutamate-mediated excitotoxicity is a crucial mechanism resulting in post-stroke injury (115). GABA and glycine are major inhibitory neurotransmitters in the brain (116). Ikeda et al. revealed that application of rTMS (20 Hz) were able to regulate the mRNA expression levels of several neurotransmitter-related genes, including GABAergic, glutamatergic, and glycinergic neurotransmission systems in mouse cerebellum and brain stem, suggesting that rTMS can modulate the activity of neurons and synaptic plasticity *via* regulating the levels of neurotransmitters (111, 114). Zangen et al. revealed that TMS over the frontal or caudal cortex of the healthy rat brain elevated the extracellular dopamine and glutamate levels in the nucleus accumbens (117). However, LF-rTMS

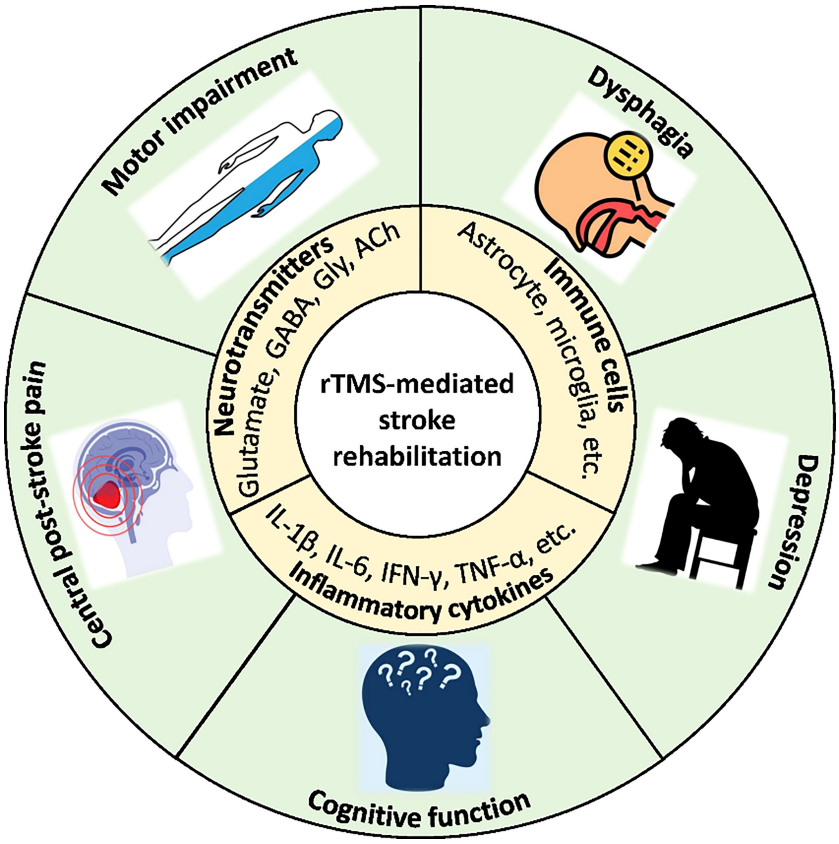

FIGURE 1
Scheme of neuroinflammation mechanisms of rTMS-mediated stroke rehabilitation.

(1 Hz) on the primary motor cortex of healthy human could not influence the excitatory (glutamate) neurotransmitter but decrease the inhibitory (GABA) neurotransmitter in both ipsilateral and contralateral motor cortices (112). Moreover, Chen et al. reported that rTMS (10 Hz) at the affected M1 and rTMS (1 Hz) at the unaffected M1 could reduce the GABA content in M1 of ischemic stroke patients, which was associated with the improvement of motor function (105). Several studies have shown that decreased GABAergic neurotransmission in the CNS might be a main cause of chronic neuropathic pain, such as CPSP (118). Decrease of inhibitory GABAergic tone was found at the level of dorsal spinal cord, somatosensory cortex, and thalamus sensory nuclei, which led to neuronal hyperactivity in the sensorimotor cortex (119). Besides, the neuronal hyperactivity related to deafferentation pain was caused by abnormal recruitment of N-methyl-D-aspartate (NMDA) receptors (120). Lefaucheur et al. found that HF-rTMS (10 Hz) on CPSP patients could significantly increase intracortical inhibition (ICI) which could reflect GABAergic neurotransmission function (113). Overall, the CPSP was associated with the imbalance between GABAergic and glutamatergic transmission in the CNS, which could be restored through rTMS-mediated GABAergic neurotransmission regulation.

Cholinergic neurons can synthesize and release the Ach when they are excited. Ach is an excitatory neurotransmitter and plays a key role in learning and memory processes (121). Li group treated the vascular dementia (VD) rats with rTMS (0.5 Hz, 1.33T) for 30 days and found that the rTMS treatment could remarkably enhance the acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activities, and increase the density of cholinergic neurons (122). The learning and memory deficits of VD rats was also significantly improved, the underlying mechanism of which might be associated with recovery of cholinergic nervous system activity in CA1 region of the hippocampus. Furthermore, brain-derived neurotrophic factor (BDNF), a neurotransmitter modulator, can promote the migration and proliferation of neural stem cells, and further enhance stroke recovery. Luo et al. revealed that HF-rTMS could activate BDNF/tropomyosin-related kinase B (TrkB) signaling pathway and antiapoptotic pathways to increase number of BrdU+ NESTIN+ cells, decrease Bcl-2 expression and elevate Bax expression, leading to the improvement of the cognitive impairment of rats with ischemic stroke (123). In addition, Cambiaghi et al. found that HF-rTMS was able to enhance dendritic complexity and spine number of neurons through BDNF and calcium-dependent signaling pathways (124).

3.2 Immune cells

Previous researches on the mechanism of rTMS were mainly concentrated on the effects on neurons. The HF-rTMS enhances neuronal excitatory at the affected hemisphere, while LF-rTMS decreases neuronal excitatory at the unaffected hemisphere (125). Nevertheless, little is known about the influence of rTMS on the other neural cells, such as astrocyte and microglia.

Astrocytes are the most abundant glial cells and play an important role in the maintenance of the blood-brain barrier (BBB) and CNS homeostasis (126). The astrocytes are activated after stroke and further polarized into two distinct phenotypes: neurotoxic (pro-inflammatory) type A1 and neuroprotective (anti-inflammatory) type A2. A1 astrocytes can secrete inflammatory cytokines and neurotoxic mediators to induce neuroinflammation and further aggravate brain damage, while A2 astrocytes mainly secrete anti-inflammatory cytokines and nerve growth factor to promote neuroregeneration and exert neuroprotective functions. In addition, astrocytes have neurotransmitter receptors and ion channels, which play a crucial role in synaptic neurotransmission (127). Therefore, rTMS might be able to alter cell membrane potential and further regulate the function of astrocytes (128, 129).

rTMS with different frequencies has been shown to regulate the expression of GFAP (a cytoskeletal marker of astrocytic reactivity) and the density of GFAP-positive astrocytes in various *in vivo* brain injury or disease model, such as cortical stab injury, Parkinson's disease, *etc* (130–132). Moreover, Clarke et al. found that rTMS (1 or 10 Hz) over the culture mouse cortical astrocytes could alter the expression of genes and proteins related to calcium signaling and inflammation, such as intercellular adhesion molecule 1 (Icam1), stromal interaction molecule 1 (Stim1), and ORAI calcium release-activated calcium modulator 3 (Orai3), *etc* (133).

Astrocyte modulation has also been indicated as one mechanism for rTMS-mediated stroke rehabilitation. Hong et al. evaluated the effects of rTMS on astrocyte polarization in *in vitro* and *in vivo* cerebral ischemic/reperfusion injury model (134). They revealed that the rTMS (10 Hz) could decrease the concentration of pro-inflammatory cytokine TNF-α, increase anti-inflammatory cytokine IL-10, and regulate astrocytic polarization (from A1 to A2 astrocytes) after ischemic/reperfusion injury. Besides, the astrocyte culture medium collected from rTMS-treated astrocytes could remarkably reduce ischemic/reperfusion injury-induced neuronal apoptosis. In addition, the rTMS could suppress the excessive astrocyte-vessel interactions and promote the vasculature-associated A1 to A2 astrocyte switch in the peri-infarct cortex after stroke, which was beneficial to vessel and BBB protection post-stroke (103). Further mechanistic studies displayed that rTMS significantly upregulated the expression level of platelet-derived grow factor receptor beta (PDGFRβ) which mediated the interactions of A2 astrocytes and their adjacent vessels, and that the angiogenesis-associated factors (TGFβ and VEGF) in A2 astrocytes were remarkably increased. Overall, these results suggested that rTMS could reduce neuroinflammation, inhibit neuronal apoptosis, and protect the vasculature by regulating astrocyte polarization, thus promoting stroke rehabilitation.

Microglia are the resident immune cells of the CNS, which are distributed in the gray matter and white matter, accounting for ~10% of the number of cells in the brain (135). Microglia maintain a resting state with certain migration and swallowing ability when not activated. They are able to monitor the CNS microenvironment and remove the necrotic neurons timely, thereby maintaining the CNS homeostasis (136). Once activated, microglia can polarize into two distinct phenotypes: the pro-inflammatory M1 and the anti-inflammatory M2 (137). In addition, microglia are early participants in post-stroke neuroinflammation and play an important role in the post-stroke recovery stage (138, 139). Shifting the balance of microglial polarization towards the anti-inflammatory M2 phenotype has been shown to exhibit neuroprotective effects in cerebral stroke (140–142). However, the influence of rTMS on the microglia has been largely unexplored. The number or state of microglia in motor cortex of healthy rats was not changed following application of chronic LF-rTMS (1 Hz) (143). Nevertheless, application of HF-rTMS on the Mongolian gerbils with cerebral ischemia has been shown to significantly increase the number of activated microglia and the expression of Iba1 in the hippocampus (144). Zong et al. applied HF-rTMS (50 Hz) over the affected hemisphere of rat photothrombotic (PT) stroke model, which significantly improved behavioral functions and infarct volume post-stroke (104). Microglial over-activation has been shown to affect neuroinflammation, leading to neuronal death. The immunofluorescence results displayed that Iba1 immunoactivity was obviously increased in the peri-infarct region of PT stroke rat, which could be remarkably rescued by rTMS treatment. Besides, they found that rTMS could effectively induce a M1 to M2 switch in microglial phenotypes, as evidenced by downregulation and upregulation of proteins related to M1 activation (CD74, CD32, and CD86) and the M2 phenotype (CD206, IL-10, and IL-4), respectively. Furthermore, the reactive microglia can release signals to promote astrocytic activation and polarization (145). The A1 to A2 switch in astrocytic phenotypes was also found in rTMS-treated PT stroke rat (103). Luo et al. revealed that rTMS could induce the anti-inflammatory M2 phenotype of microglia and facilitate the generation of anti-inflammatory cytokines (146). Moreover, they found that neural stem cells cultured with medium from rTMS-treated microglia presented decreased apoptosis and increased neuronal differentiation. In addition, Chen et al. revealed that HF-rTMS could regulate the Janus kinase 2 (JAK2)-signal transducer and transcription 3 (STAT3) pathways, and further inhibit microglial activation as well as promote the switch of microglia toward the neuroprotective M2 phenotype, resulting in alleviation of ischemic white matter damage and improvement of cognitive impairment (147). Taken together, rTMS may regulate the microglial polarization and further modulate the inflammatory microenvironment, thereby promoting neurogenesis and improving stroke rehabilitation.

3.3 Inflammatory cytokines

Previous studies have shown that the expression levels of inflammatory cytokine, including interleukin (IL)-1β, IL-2, IL-6, IL-10, IL-17a, interferon (IFN)-γ, transforming growth factor beta (TGF-β), and tumor necrosis factor alpha (TNF-α), in brain tissues and peripheral blood were significantly changed after stroke (148, 149). Among these cytokines, IL-1β, IL-2, IL-6, TNF-α, and IFN-γ are pro-inflammatory, while the IL-10 and TGF-β belong to anti-inflammatory cytokines. IL-10 were reported to be secreted by several cell types after stroke, including M2 microglia, macrophages, regulatory T cells, and B lymphocytes. TGF-β, a factor secreted by A2 astrocytes which regulates cell growth and differentiation, can inhibit the activation of inflammatory/immune cells and the release of various inflammatory mediators, thereby promoting tissue repair after injury (150). Inflammatory cells, including microglia, astrocytes, and infiltrating peripheral immune cells (neutrophils, monocytes and macrophages, *etc.*), in the ischemic lesions after acute ischemic stroke can release a large number of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, *etc*, which can cause neuronal damage and upregulate the levels of selectin and ICAM-1 to increase the permeability of cerebral vascular endothelium (151, 152). Moreover, these pro-inflammatory cytokines can also recruit peripheral neutrophils, macrophages, and lymphocytes, which further amplifies the neuroinflammation, eventually causing a vicious cycle of pro-inflammatory. Additionally, the occurrence of PSD is closely related to the imbalance of serum levels of inflammatory cytokines, such as IL-2, IL-10, IL-17a and IFN-γ (153). Among various mechanisms of rTMS-mediated stroke rehabilitation, regulation of inflammatory cytokine may be one of the important mechanisms. Cha et al. revealed that HF-rTMS on the affected dorsolateral prefrontal cortex (DLPFC) of stroke patients could significantly reduce the mRNA level of pro-inflammatory cytokines (IL-1β, IL-6, TGF-β, TNF-α) in blood samples, indicating the anti-inflammatory effect of rTMS (154). Besides, the reduction of IL-6 was strongly correlated with increase of auditory verbal learning test (AVLT, r=-0.928) and complex figure copy test (CFT, r=-0.886). Another study on the ischemic stroke also found that the rTMS were able to decrease the serum levels of IL-6, IL-8, and TNF-α (155). Furthermore, the rTMS treatment could suppress the M1 microglia polarization *via* let-7b-5p/HMGA2/NF-κB pathway, and further decrease the TNF-α level but elevate the IL-10 concentration, promoting the anti-inflammatory effect (155). iTBS treatment could remarkably reduce the high concentrations of IL-1β, IFN-γ, TNF-α, and IL-17A as well as increase the IL-10 level in brain tissues of cerebral ischemic mice (156). In addition, the pro-inflammatory cytokines could affect the synaptic plasticity, which might further influence cognitive function and behavior (157–159). For example, the TNF-α, produced by microglia and peripheral immune cells, could affect long-term potentiation (LTP) of excitatory neurotransmission at higher concentration (160, 161). Therefore, the regulation of cytokine concentrations in serum and brain tissues is an important mechanism of rTMS-mediated rehabilitation, which could also be used as indicators to evaluate the effect of stroke rehabilitation.

4 The roles of imaging techniques in rTMS-mediated stroke rehabilitation

Imaging techniques play an important role in understanding the mechanisms underlying the effects of rTMS and in guiding the optimization of rTMS protocols. First, these imaging techniques could be applied to identify the optimal target. Specifically, imaging techniques such as fMRI and TMS mapping can help identify the regions of the brain that are affected by the stroke and the regions that need to be targeted by rTMS for optimal recovery (162). Secondly, the imaging techniques can be used to assess the treatment effects of rTMS on brain function and connectivity of stroke patients. For example, fMRI can be utilized to measure changes in brain activity, while diffusion tensor imaging (DTI) can be used to detect changes in white matter integrity (163, 164).Li et al. utilized resting-state fMRI (rs-fMRI) to explore the effect and mechanism of rTMS on PSCI patients (95). They revealed that the rTMS (5 Hz, 100% RMT) could significantly improve cognitive functions measured by Minimum Mental State Examination (MMSE) and Montreal cognitive assessment (MoCA). They also found that FC and neural activity in several cognition-related brain regions were significantly regulated by rTMS therapy. These imaging results indicated that the rTMS showed effective impact on the PSCI patients to improve their cognitive function. Besides, the imaging techniques can be applied to personalize rTMS treatment for individual patients. For instance, fMRI can be used to identify regions of the brain that are still functionally active after stroke, and then rTMS can be targeted to these regions to enhance recovery (165). In addition, the imaging techniques can be also utilized to predict the outcomes of rTMS-mediated stroke rehabilitation. The diffusion MRI can be used to predict motor function recovery after stroke, which could also guide rTMS treatment (166). Overall, imaging techniques are an important tool in rTMS-mediated stroke rehabilitation, providing valuable information on the underlying mechanisms of stroke recovery and helping to optimize rTMS protocols for individual patients.

5 Prospects and challenges

rTMS is a novel technique that can regulate neurotransmitters, activation/polarization of immune cells (astrocytes and microglia), and inflammatory cytokines in the brain, thereby affecting brain function and improving post-stroke dysfunctions. Moreover, rTMS combined with other traditional rehabilitation programs can present a synergistic effect and further enhance the rehabilitation effect of stroke patients.

According to previous safety evaluation, although the incidence rate is low, rTMS could induce several side effects, including epilepsy, syncope, short-term hearing loss, headache pain, dizziness, toothache, and paresthesia, *etc* (167–172). Thus, the rTMS should be applied within the range of treatment parameters according to the guideline. There are many stimulation parameters of rTMS, including coil type, coil position, stimulation frequency,

stimulation time, stimulation interval, and total stimulation volume, *etc*, which might be the main reason for the heterogeneity of clinical treatment effects and basic research results. Due to the obvious differences in different rTMS stimulations and clinical outcome evaluation indicators, it has brought great difficulties to the study of optimal stimulation parameters. In some clinical studies, patients in control group did not receive reliable sham stimulation, therefore, it is difficult to determine the relative efficacy of placebo in rTMS. Generally, the sample size of current clinical studies of rTMS-mediated stroke rehabilitation is still insufficient. Therefore, randomized, double-blind, large sample size, and placebo-controlled clinical studies should be performed in the future to further clarify the clinical value of TMS. In addition, LF-rTMS and HF-rTMS are more clinically applied at present. TBS has fewer clinical applications, but it requires lower stimulation intensity, fewer pulses, and produces longer-lasting cortical excitability compared to the formers. Thus, TBS might be a promising approach for stroke rehabilitation, which needs to further explored in the future study.

Recent study has shown that LF-rTMS on the frontal cortex could reduce the local inhibition and disrupt feedforward and feedback connections, whereas, the LF-rTMS on the occipital cortex could enhance the local inhibition and increase forward signaling. A more thorough understanding of the mechanism underlying the rTMS-mediated CNS regulation allow us to better apply rTMS to clinical treatment. However, the current mechanism research is still in the preliminary stage, thus, future studies need to further explore the regulatory mechanism of rTMS on the CNS, especially the regulation of immune inflammation.

Author contributions

RS wrote the manuscript. PY drew the pictures. CC and HC revised the manuscript. RS and PY collected the literature. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Advances in the application of neuroinflammatory molecular imaging in brain malignancies

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The prevalence of brain cancer has been increasing in recent decades, posing significant healthcare challenges. The introduction of immunotherapies has brought forth notable diagnostic imaging challenges for brain tumors. The tumor microenvironment undergoes substantial changes in induced immunosuppression and immune responses following the development of primary brain tumor and brain metastasis, affecting the progression and metastasis of brain tumors. Consequently, effective and accurate neuroimaging techniques are necessary for clinical practice and monitoring. However, patients with brain tumors might experience radiation-induced necrosis or other neuroinflammation. Currently, positron emission tomography and various magnetic resonance imaging techniques play a crucial role in diagnosing and evaluating brain tumors. Nevertheless, differentiating between brain tumors and necrotic lesions or inflamed tissues remains a significant challenge in the clinical diagnosis of the advancements in immunotherapeutics and precision oncology have underscored the importance of clinically applicable imaging measures for diagnosing and monitoring neuroinflammation. This review summarizes recent advances in neuroimaging methods aimed at enhancing the specificity of brain tumor diagnosis and evaluating inflamed lesions.

KEYWORDS

magnetic resonance imaging, positron emission tomography, neuroinflammation, brain tumor, metastasis

1 Introduction

The most prevalent brain tumors comprise meningiomas, gliomas—particularly glioblastomas (GBMs)—and intracranial metastases originating from various cancers (1). The treatment approach for intracranial metastases involves a combination of chemoradiotherapy and neurosurgery (2). Meningiomas are predominantly benign and are typically managed by surgical resection (3). GBM represents the primary malignancy in the central nervous system (CNS) and is known for its aggressive nature, with limited benefits derived from advanced therapeutic strategies (4). Therefore, early diagnosis and

accurate monitoring through neuroimaging are crucial for managing brain tumors. Neuroimaging is an indispensable aspect of clinical practice, offering valuable insights into brain tumors.

The advancement of computed tomography (CT) and magnetic resonance imaging (MRI) has enabled more precise diagnoses, improved clinical monitoring, and enhanced accuracy in prognostic prediction (1). In particular, neuroimaging has played a crucial role in diagnosing and treating brain tumors. By combining molecular pathology and histopathology, significant progress has been made in classifying various types of brain tumors. Precision neuro-oncology integrates tumor-specific neuroinflammation and distinct protein alterations (5). However, the translation of the precision oncology paradigm to neuro-oncology faces several significant challenges, including the criteria for assessing therapeutic effects and the need for accurate monitoring approaches. Advanced neuroimaging techniques currently offer potential solutions to overcome these challenges. The interactions between brain tumors and immune responses are critical focal points (6, 7). Consequently, current immunotherapeutic strategies have been developed to target specific immune cells or inflammatory mediators within the tumor microenvironment (TME) (8). Neuroinflammation plays a crucial role in the progression of brain tumors. GBM, in particular, is characterized by tissue necrosis accompanied by heightened neuroinflammation. Additionally, immunosuppressive neuroinflammation and induced necrosis also contribute to treatment resistance and poor prognosis (5, 9). However, immunotherapies also affect imaging phenotypes in clinical practice. Therefore, it is essential to gain a better understanding of the inflammatory factors and their associated neuroimaging characteristics.

In this review, the key aspects of neuroimaging in diagnosing and distinguishing brain tumors from inflammatory-associated lesions were emphasized. This information is immensely valuable for gaining a better understanding of imaging characteristics and common patterns that aid in diagnosis. Furthermore, a comprehensive understanding of the inflammatory mechanisms within the TME and their corresponding imaging features facilitates the development of noninvasive prognostic and predictive imaging strategies in clinical practice.

2 Current limitations in neuroimaging in the context of brain malignancy

The clinical diagnosis, evaluation, and monitoring of therapeutic effects in patients with brain malignancies heavily rely on neuroimaging techniques. There is a wide range of neuroimaging options available for clinical use. Structural MRI is a useful choice for identifying and classifying tumors and guiding surgical strategies. Additionally, PET imaging is predominantly used to assess tissue metabolism, providing valuable information about cancer cell proliferation and early-stage tumor detection (10). PET is a type of molecular imaging technique that is suitable for detecting specific molecules, such as choline, fluorodeoxyglucose, methionine, and phenylalanine (11, 12). Thus, the critical mechanism of PET-based neuroimaging involves defining and tracing the targeting molecules that are specific or sensitive to immune cells, including microglia and macrophages.

The efficiency of current neuroimaging techniques is widely acknowledged; however, there are still several limitations requiring further improvement. For instance, distinguishing relapsed brain tumors, brain metastasis, or inflammatory and necrotic lesions poses a significant challenge in clinical treatment, as both tumors and metastatic sites exhibit similar intratumoral textures on MRI (13, 14). The application of PET techniques also faces various challenges. The low permeability of biotracers across the blood-brain barrier (BBB) and systemic plasma binding affect the imaging results. Additionally, inherent characteristics of the neuroimaging technique itself impose certain limitations. The most commonly used PET probe for neuroimaging, 2-deoxy-2-18F-fluoro-D-glucose, is based on glucose metabolism (15). Glucose transporters are highly expressed not only in tumor cells but also the inflamed areas (16). Importantly, glucose metabolism plays a crucial role in inflammatory responses, leading to false-positive results in tumor diagnosis. Therefore, in this review, the recent advances in neuroimaging approaches aimed at improving the evaluation of brain tumors and brain metastases combined with neuroinflammatory response information have been discussed. The first radiotracer used for neuroinflammation is based on an 18-kDa protein named translator protein (TSPO), a peripheral benzodiazepine receptor (17). TSPO is a mitochondrial transmembrane protein predominantly located in macrophages and microglia (18). Accumulative evidence indicated that TSPO up-regulation can be observed in pro-inflammatory and immunosuppressive conditions. Previous studies have shown that TSPO expression can be up-regulated in response to neuroinflammation and tumor malignancy (19). Additionally, TSPO can also be found in neoplastic glioma cells. Consequently, over 13 unique TSPO radiotracers have been developed for use in malignant brain pathologies, enhancing tumor-to-background brain signals (20). Furthermore, TSPO-based PET imaging shows promise in delineating clinically important neuroinflammatory tumor features (21). Therefore, the establishment of novel neuroimage techniques is urgently required for patients with brain tumors.

3 Brain tumors as neuroinflammatory diseases: tumor immune microenvironment

Neuroinflammation plays a crucial role in brain TME, resulting in carcinogenesis and tumor progression. In particular, primary brain malignancies, such as GBMs, are characterized by significant immunosuppressive neuroinflammation and necrosis, resulting in resistance to chemotherapy and poor clinical outcomes (22). Tumor cells produce and secrete various immunomodulatory substances, including interleukin (IL)-10, IL-1β, galectin-1, and transforming growth factor-β, which regulate the behavior of infiltrating immune

cells and establish a pro-tumoral microenvironment (23, 24). Among the components of the pro-tumoral microenvironment, tumor-associated macrophages (TAMs) are particularly prominent (6, 25). Neuroinflammation induces immunosuppressive changes and further enhances the proliferation, migration, and therapeutic resistance of tumor cells (26).

3.1 Immune component of the TME in CNS malignancies

Under normal conditions, the CNS is protected by the BBB, which renders it immune-privileged and shields it from the influence of systemic inflammation. However, immune surveillance and inflammatory regulation in the CNS are mediated by resident immune cells and infiltrating peripheral cells. The microenvironment within brain tumors comprises a diverse array of cellular and immune components, which significantly affect the growth, drug resistance, and recurrence of primary and metastatic brain tumors (27, 28).

First and foremost, it has been reported that astrocytes can be immediately infiltrated around brain tumors, exhibiting varying morphology and cellular functions (29). Active astrocytes, notably, have the ability to promote the invasive capacity and drug resistance of tumor cells, thereby enhancing tumor growth and survival, while suppressing the therapeutic efficiency in clinical treatment (30). Conversely, several studies have also reported the anti-tumor function of astrocytes. It has been demonstrated that specific astrocyte-derived exosomes comprising micro-ribonucleic acid (RNA) can significantly inhibit tumor growth (31). Additionally, microglia, macrophages, endothelial cells, and pericytes play key roles in tumor growth and neuroinflammation, potentially serving as clinical imaging features (32). Accumulative evidence illustrates the critical roles of microglia and macrophages in tumor malignancy, accounting for more than 30% of the cell populations (33). Microglia derived from the yolk sac precursor cells during embryonic development are resident phagocytic cells, while macrophages are derived from the bone marrow-derived monocytes (BMDMs) and migrate into the brain parenchyma. Lineage-tracing experiments in brain metastases have demonstrated the infiltration of microglia and BMDMs in such malignancies. Collectively, microglia and macrophages are widely considered to be associated with tumor malignancy, referred to as TAMs (34).

The tumor vasculature primarily comprises endothelial cells, playing a critical role in tumor growth and facilitating immune reactions within the TME through the release of various neuroimmune substances, including IL-1β, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (35, 36). Additionally, pericytes are localized around the vascular vessels and are responsible for tumor vascularization and maintaining BBB integrity (37). The tumor vasculature comprises established vasculature and neo-vasculature, with the latter being significantly enhanced under the regulation of local hypoxia, metabolic demands, and vascular endothelial growth factor stimulation (38). Furthermore, the tumor vasculature exhibits abnormalities in integrity and function. Previous studies have shown a higher prevalence of dysfunctional vasculature within the tumor region, while the peritumoral vasculature resembles normal blood vessels. Following the intravenous administration of a gadolinium-based contrast agent (GBCA), the GBCA enters the extracellular space in the brain parenchyma through this abnormal vasculature, resulting in hyperintensity on T1-weighted magnetic resonance (MR) images (39). However, tumor cells might still be present in the non-enhancing regions around the areas of enhancement. Therefore, the presence or absence of tumor cells is not directly associated with the compromised BBB, increasing the difficulty of clinical diagnosis.

3.2 The interactions between the immune responses and brain tumors

The CNS possesses a unique immune monitoring system that involves meningeal or brain parenchymal lymphatic vessels and immune surveillance. TAMs can be classified into pro-inflammatory (M1) or anti-inflammatory (M2) types based on their cellular function and morphological features, which are associated with various processes such as the inflammatory cascade, immune activation, angiogenesis, tissue remodeling, and tumor survival. This classification framework allows for a better understanding of the multifaceted roles of TAMs (40). M1 TAMs are responsible for pathogen recognition and tumor killing. On the contrary, M2 TAMs primarily regulate inflammatory reactions and establish a favorable TME through the release of anti-inflammatory cytokines, thereby promoting tumor survival and growth (41).

Furthermore, the dysregulated adaptive immune reactions within the TME also contribute to tumor growth. The function and prognostic predictive value of adaptive immune cells in brain tumors have gained considerable attention. A previous clinical study has shown a correlation between prolonged survival in patients with GBM and the infiltration of the cluster of differentiation CD 8^+ T lymphocytes, whereas regulatory T cells are associated with worse prognosis in patients (42, 43). The adaptive immune responses are profoundly influenced by immune checkpoints, which regulate the immune response to self-antigens and contribute to tumor control. Accumulating evidence indicates that various tumors, including CNS malignancies and metastases, evade recognition and surveillance by the immune system through immune checkpoint regulation (44).

Evidence has revealed that therapeutic mobilization of innate and adaptive immune responses can be employed to induce immune-mediated tumor cell death. As a result, a wide array of immunotherapeutic strategies has been established, targeting the interaction between tumor cells and immune cells. These strategies include immune checkpoint inhibition, engineered chimeric antigen receptor T cells, oncolytic viral therapies, and vaccines (45, 46). The underlying mechanisms of immune checkpoint inhibition and vaccines involve the activation of T lymphocytes by eliminating inhibitory signals. Additionally, tumor antigens are presented to antigen-presenting cells, such as dendritic cells (47). These cells are then administered to patients with GBMs after being stimulated with tumor antigens, triggering adaptive immune

activation. Based on this approach, vaccine-based immunotherapies use tumor-associated antigens to enhance cytotoxic effects through the enhancement of immune reactions. Due to the critical role of immune components within the TME, neuro-inflammation-based imaging techniques may be a potentially effective approach in the diagnosis of patients with brain tumors.

4 Novel neuroimaging approaches for evaluating brain tumor recurrence

Despite the current efficiency of existing neuroimaging techniques in brain tumor detection, there is still a huge challenge in distinguishing recurrent brain tumors, solitary brain metastases and inflammatory and necrotic lesions (48). It has been revealed that conventional neuroradiological techniques struggled to distinguish recurrent tumors and inflammatory alterations, due to their similarly-showing imaging in MRI. Therefore, a better understanding of novel approaches to diagnose recurrent brain tumors may present great clinical potential in the follow-up of patients after anti-tumor therapies. With the rapid advances in neuroimaging, the direct labelling of critical immune cells has emerged as a potential option for neuroinflammation imaging with clinical relevance. Given that TAMs are considered the predominant immune cells within the brain TME, significant efforts have been made to label TAMs based on accumulating evidence. Furthermore, MRI plays an essential role in clinical assessment and monitoring, making novel approaches using MRI for neuroinflammation imaging likely to be applicable in clinical practice. In conventional MRI, GBCAs are commonly used to examine contrast dynamics and identify CNS malignancies. However, it is noteworthy that ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles possess a unique capability to detect neuroinflammation by being taken up by immune cells (49). One such USPIO nanoparticle is ferumoxytol, which has been extensively studied in patients with brain tumors (49). Previous clinical studies have indicated that ferumoxytol exhibits a preferential accumulation in inflamed sites (50). Similarly, an experimental study revealed that ferumoxytol is predominantly observed in activated astrocytes, microglia, and macrophages but not in tumor cells. This underscores the potential of ferumoxytol-enhanced MRI for neuroinflammation imaging in patients with brain tumors (51). Currently, ferumoxytol has received Food and Drug Administration approval for clinical use. In cases where the administration of GBCA is restricted due to clinical conditions such as GBCA allergy or renal failure, ferumoxytol is considered an alternative contrast agent (52). Additionally, ferumoxytol has demonstrated potential advantages over GBCA in the assessment of neuroinflammation. Its plasma half-life is approximately 14-21 h, allowing delayed localization within TAMs or the neovascular space (53). This prolonged intravascular signal persistence contributes to the observed parenchymal signal during delayed imaging (54). A prospective pilot study involving patients with gliomas revealed that delayed ferumoxytol imaging captures TAM signals within the TME. Furthermore, there is a positive correlation between susceptibility and relaxation and the number of macrophages (54).

The interpretation of T1-weighted ferumoxytol contrast-enhanced MRI depends on clinical conditions (55). During the diagnostic phase, similar imaging characteristics can be observed in primary or metastatic brain tumors using either GBCA or ferumoxytol-enhanced MRI. However, after chemoradiotherapies, these enhancing images might differ. Specific immunotherapies can induce neuroinflammation, leading to gadolinium enhancement and T2 hyperintensity on MRI. Although the enhanced signals resemble those of solid tumors, they subsequently undergo spontaneous regression, known as pseudoprogression. Psuedoprogression can be observed in approximately 30% of patients with GBMs after chemotherapy or radiotherapy. Given the underlying mechanisms of immunotherapies, neuroinflammation induced by immunotherapies is more likely to be detected than that induced by chemoradiotherapies. Therefore, distinguishing pseudoprogression from actual tumor growth becomes crucial. Additionally, iron nanoparticle imaging requires specific attention. The prolonged clearance of iron nanoparticles should be given careful consideration in clinical practice (56). A previous study has revealed an increased level of methionine in metastatic tumor tissues (57, 58). Furthermore, elevated levels of the neuroinflammatory marker PBR-TSPO can be observed in necrotic sites and quantified using a specific PET biotracer. Therefore, PET biotracers based on ^{11}Carbon (C)-methionine have been used in patients with metastatic brain tumors to accurately diagnose tumor recurrence (59, 60). In one clinical trial, ^{11}C-methionine-based PET biotracers correctly diagnosed tumor recurrence, as confirmed by pathological examination, in seven patients. In comparison, ^{11}C-PBR28-based PET biotracers only identified three lesions, highlighting the reliability of ^{11}C-methionine-based biotracers for tumor recurrence detection. Thus, ^{11}C-methionine is considered a reliable marker for tumor recurrence compared with ^{11}C-PBR28 PET (61). PET imaging provides significant benefits in advanced photon techniques, particularly in treatment planning and the sparing of normal tissue for skull base meningioma using advanced photons and protons. Additionally, incorporating ^{68}Gallium-DOTATOC-PET information has a substantial impact on target volumes (62). Moreover, the combination of Multiparametric ^{18}F-FET PET/MRI improves the therapeutic effectiveness by distinguishing between tumor progression and therapy-related alterations (63). According to another clinical study, combined dynamic and static ^{18}F-FET PET/CT parameters can be used in differentiating radiation necrosis from recurrent tumor after cyberknife robotic radiosurgery (64). Effective imaging techniques are still required to be explored in future studies.

5 Neuroimaging approaches for evaluating brain tumors and radiation-induced necrosis

Currently, MRI (Table 1), PET, and single-photon emission CT (SPECT) are the predominant imaging techniques for the noninvasive assessment of neuroinflammation (87). Among them, SPECT can detect gamma rays emitted by radioactive isotopes used

TABLE 1 Magnetic resonance imaging (MRI)-based neuroimaging applied in brain tumors.

Imaging approach	Application	Image features	Author	Year
Proton magnetic resonance (MR) spectroscopy	To differentiate tumor recurrence from radiation necrosis	Increased lactate/creatine (Cr) ratio and decreased choline (Cho)-containing compounds/Cr ratio in necrosis or all the major metabolites were completely diminished.	Kamada et al.	1997 (65)
Two-dimensional (2D) chemical shift imaging MR spectroscopy	To differentiate tumor recurrence from radiation necrosis	Diagnostic spectra can be obtained in 97% of the patients. The Cho/N-acetylaspartate (NAA) and Cho/Cr ratios are the best numeric discriminators.	Weybright et al.	2005 (66)
T2-weighted dynamic susceptibility-weighted contrast material-enhanced (DSC) MRI	To differentiate tumor recurrence from radiation necrosis	Significantly higher relative peak height (rPH) and relative cerebral blood volume (rCBV) and lower relative percentage signal recovery (PSR) values in patients with recurrent glioblastomas (GBMs) than in patients with radiation necrosis.	Barajas et al.	2009 (67)
Proton MR spectroscopy	To differentiate tumor recurrence from radiation necrosis	Increasing Cho levels in patients with radiation necrosis.	Nakajima et al.	2009 (68)
MR spectroscopy and MR perfusion	To differentiate tumor recurrence from radiation necrosis	Cho/NAA and Cho/Cr ratios and rCBV more accurately differentiate between necrosis and recurrent tumors.	Chuang et al.	2016 (69)
Diffusion tensor imaging (DTI) and calculation of the apparent diffusion coefficient (ADC)	To differentiate tumors from radiation abscesses	Hyperintense signal changes in abscesses. The combination of DTI and dynamic susceptibility contrast perfusion-weighted imaging improves the differentiation between tumors and brain infections.	Bink et al.	2005 (70)
Calculation of the ADC	To differentiate tumors from radiation abscesses	The accuracy of ADC ratios in discriminating brain abscesses from cystic or necrotic neoplasms is high and can be further improved with the use of T2 rim characteristics.	Fertikh et al.	2007 (71)
MR spectroscopic imaging	To differentiate tumors from radiation abscesses	Metabolite ratios and maximum Cho/Cho-n, Cho/Cr, and Cho/NAA ratios of the contrast-enhancing rim could differentiate abscesses from brain tumors.	Lai et al.	2008 (72)
MR spectroscopy	To differentiate high-grade gliomas from metastases	Intratumoral Cr is suggestive of a glioma. The absence of Cr indicates metastasis.	Ishimaru et al.	2001 (73)
Phase difference enhanced imaging (PADRE) in MRI	To differentiate high-grade glioma from metastases	Evaluation of peritumoral areas on color PADRE helps differentiate GBMs from metastases	Doishita et al.	2018 (74)
Computational-aided quantitative image analysis (T2-weighted/susceptibility-weighted/contrast-enhanced T1-weighted MRI)	To differentiate high-grade gliomas from metastases	Computational-aided quantitative analysis of MRI improves diagnostic accuracy while differentiating GBM from metastases.	Petrujkić et al.	2019 (75)
Resonance imaging texture analysis	To differentiate high-grade gliomas from metastases	The peritumoral edema was higher than the edema surrounding the metastatic tumor.	Skogen et al.	2019 (76)
Combining arterial spin labelling perfusion (ASL)- and DTI-derived metrics	To differentiate high-grade gliomas from metastases	A combination of ASL- and DTI-derived metrics of the peritumoral part helps differentiate between GBMs and brain metastasis	Abdel et al.	2019 (77)
Multilayer perceptron (MLP) models with non-enhancing T2 hyperintense regions	To differentiate high-grade gliomas from metastases	A trained multi-class MLP model using parameters from preoperative MR images could help differentiate between GBMs, brain metastases, and central nervous system lymphomas.	Swinburne et al.	2019 (78)
Calculation of the ADC	To differentiate high-grade gliomas from metastases	Higher homogeneity and the inverse difference moment in GBMs compared with metastases	Zhang et al.	2019 (79)
Evaluate the rCBV gradient in the peritumoral brain zone (PBZ)	To differentiate high-grade gliomas from metastases	The rCBV gradient derived from DSC MRI in the PBZ is an efficient parameter to differentiate GBMs from brain metastases.	She et al.	2019 (80)
Machine learning method based on texture parameters in MRI	To differentiate high-grade gliomas from metastases	Based on the texture parameters in MRI, the performance of the machine learning method was superior to that of the univariate method while differentiating GBMs from brain metastases.	Tateishi et al.	2020 (81)
2D texture features extracted from MRI	To differentiate high-grade gliomas from metastases	High accuracy employing a set of 2D texture features to discriminate between GBMs and brain metastases.	Ortiz-Ramón et al.	2020 (82)

(Continued)

TABLE 1 Continued

Imaging approach	Application	Image features	Author	Year
Three-dimensional T1-weighted (3DT1) MR images with the machine learning classifier	To differentiate high-grade gliomas from metastasis	The proposed diagnostic support system based on radiomics features extracted from post-contrast 3DT1 MR images helps differentiate solitary metastases from GBMs.	de Causans et al.	2021 (83)
MR spectroscopy	To differentiate high-grade gliomas from metastases	No difference in the ADC values and ratios, as well as standard deviation values and ratios between GBMs and brain metastases.	Beig Zali et al.	2021 (84)
A deep learning-based model based on MRI	To differentiate high-grade gliomas from metastases	An efficient deep learning-based model was established and validated using MR images.	Shin et al.	2021 (85)
MRI-based machine learning decision	Identification of medulloblastoma subgroups	MRI-based machine learning helps identify clinically relevant molecular pediatric medulloblastoma subgroups	Zhang et al.	2022 (86)

in clinical imaging. By employing various radiotracers, SPECT enables precise evaluation of metabolic and molecular processes, such as glucose utilization, nucleoside and amino acid transporter expression, and protein and deoxyribonucleic acid (DNA) synthesis (88). SPECT has been extensively used to investigate the molecular neurodegeneration mechanisms in drug addiction and to enhance therapeutic strategies with minimal adverse effects. Furthermore, SPECT plays a valuable role in neuroscience research, particularly in neurodegeneration and neuro-oncology (89).

However, conventional neuroradiological techniques have several limitations. The increased contrast enhancement observed in MRI could lead to difficulty in distinguishing between radiation necrosis and tumor recurrence, as the lesions appear similar. Currently, radiotherapy and stereotactic radiosurgery are commonly used for treating brain metastasis. However, the incidence of radiation-induced necrosis poses a significant challenge in clinically differentiating between metastasis and radiation-induced necrosis. A previous study has demonstrated that conventional MRI has a specificity of 75% and a sensitivity of only 44% in distinguishing between tumors and inflamed necrotic tissue (90, 91).

Neuroinflammation and necrosis are prominent side effects of radiotherapies for treating brain malignancies. A previous retrospective study reported that the rate of radiation-induced necrosis in patients with GBM ranged from 2.5% to 30% (92, 93). This necrotic tissue disrupts the vasculature and perivascular parenchyma, resulting in an abundance of neuroinflammation. The compromised BBB facilitates peripheral immune cell infiltration and brain edema, thereby amplifying the inflammatory response initiated by activated and infiltrated immune cells. Currently, proton MR spectroscopic imaging is employed to assess cellular metabolism by detecting the distribution of proton metabolites, including creatine, lactate, lipid, and especially choline, which is particularly elevated in cell populations exhibiting enhanced cell proliferation. This advanced MRI technique allows for a detailed evaluation of tissue biochemical composition and blood perfusion, providing valuable information for distinguishing solid tumor tissues and necrotic regions. In a previous clinical trial involving 29 consecutive patients, the feasibility and utility of two-dimensional (2D) chemical shift imaging MR spectroscopy were investigated, demonstrating that increased ratios of choline content can differentiate brain tumors from necrotic tissues with an accuracy of up to 97% (66). The choline/N-acetylaspartate and choline/phosphocreatine ratios are particularly effective discriminators in necrotic lesions. However, it is important to note that a transient increase in choline levels can be observed in patients with radiation-induced necrosis, resulting in a false-positive diagnosis of tumor recurrence when using proton MR spectroscopy (68). Another study focusing on patients undergoing high-dose radiotherapy revealed that elevated lactate/creatine ratios and decreased metabolites are more commonly observed in patients with radiation necrosis than in those with recurrent GBM (65).

A retrospective study revealed significant differences in the mean, maximum and minimum relative peak height, and relative cerebral blood volume between patients with GBM and those, as detected by T2-weighted dynamic susceptibility-weighted contrast material-enhanced MRI (67). Furthermore, lower recovery values were observed in recurrent GBM compared with radiation necrosis. Additionally, a meta-analysis of 13 studies demonstrated that the detection of choline/N-acetylaspartate and choline/phosphocreatine ratios, along with relative cerebral blood volume (rCBV) using MR spectroscopy and MR perfusion, significantly improves accuracy in diagnosing primary or metastatic brain tumors (69).

PET scan, as a widely applied noninvasive neuroimaging approach, is useful for imaging neuroinflammation (Table 2). According to a previous study, PET scans exhibit a specificity of 69% and a sensitivity of 92% in differentiating between tumor recurrence and radiation necrosis, surpassing nuclear MR spectroscopy for choline/N-acetylaspartate and choline/creatine ratios at various thresholds (98). Another experimental study conducted on orthotopic GBM rat models demonstrated the excellent ability of PET with ^{18}Fluorine (F)-fluorodeoxyglucose and ^{18}F-fluoroethyltyrosine to distinguish primary GBMs from necrosis (96). Furthermore, a meta-analysis of six studies suggested that ^{11}C-choline PET is the most accurate diagnostic approach for distinguishing tumor relapse from necrosis in patients with glioma (97). Additionally, a retrospective study with long-term follow-up revealed that ^{11}C-choline PET/CT outperform MRI and ^{18}F-fluorodeoxyglucose in evaluating tumor recurrence or radiation-induced necrosis (94). Furthermore, a previous clinical study involving 50 patients demonstrated that ^{11}C-methionine PET outperforms ^{11}C-choline and ^{18}F-fluorodeoxyglucose PET in distinguishing primary GBMs from necrosis (95). Additionally, the L-type amino acid transporter 1 tumor-specific PET tracer,

TABLE 2 Positron emission tomography (PET)-based neuroimaging applied in brain tumors.

Imaging approach	Application	Image features	Author	Year
^{11}Carbon (C)-choline PET/computed tomography (CT)	To differentiate recurrent GBMs from radiation necrosis	^{11}C-choline PET/CT exhibits higher sensitivity and specificity while differentiating recurrent brain tumors from necrosis compared with ^{18}Fluorine (F)-fluorodeoxyglucose (FDG) PET/CT and MRI.	Tan et al.	2011 (94)
^{11}C-methionine PET	To differentiate recurrent GBMs from necrosis	^{11}C-methionine PET exhibits higher sensitivity and specificity while differentiating recurrent brain tumors from necrosis compared with ^{11}C-choline or ^{18}F-FDG PET.	Takenaka et al.	2014 (95)
^{18}F-FDG delayed PET	To differentiate GBMs from radiation necrosis	^{18}F-fluorodeoxyglucose delayed PET helps differentiate GBMs from radiation necrosis	Bolcaen et al.	2015 (96)
^{11}C-choline PET	To differentiate recurrent GBMs from radiation necrosis	^{11}C-choline exhibits high diagnostic accuracy while differentiating recurrent tumors from radiation-induced necrosis in gliomas.	Gao et al.	2018 (97)
^{11}C-methionine PET	To differentiate high-grade gliomas from metastasis	^{11}C-methionine was a more reliable recurrent tumor marker than ^{11}C-peripheral benzodiazepine receptor 28.	Tran et al.	2020 (61)
^{68}Galium-DOTATOC-PET	Helps in treatment planning for skull base meningiomas with advanced photons and protons	The addition of 68Ga-DOTATOC-PET information during treatment planning for skull base meningiomas significantly affects target volumes.	Stade et al.	2018 (62)
PET scan	To differentiate tumor recurrence from radiation necrosis	PET scan had the best sensitivity and specificity, for choline/N-acetylaspartate and choline/creatine ratios across different thresholds.	Menoux et al.	2017 (98)
^{18}F-FET PET/MRI	To distinguish between tumor progression and therapy-related alterations	Multiparametric ^{18}F-FET PET/MRI improves the therapeutic effectiveness by distinguishing between tumor progression and therapy-related alterations.	Brendel et al.	2022 (63)
^{18}F-FET-PET/CT	To differentiate recurrent GBMs from radiation necrosis	Combined dynamic and static 18F-FET PET/CT parameters can be used in differentiating radiation necrosis from recurrent tumor after cyberknife robotic radiosurgery.	Lim et al.	2022 (64)

^{18}F-2-fluoroethyl-L-phenylalanine, is also a superior option compared with ^{18}F-fluorodeoxyglucose PET in clinical differentiation, as it exhibits low sensitivity to neuroinflammation. However, the high rate of false-positive and false-negative results in PET scans remains a significant limitation in clinical practice. Therefore, there is an urgent need for improved neuroimaging techniques based on the aforementioned methods to achieve accurate diagnosis and clinical evaluation. Currently, advanced approaches offer substantial benefits in evaluating neuroinflammation through immune substance labelling, assessing BBB integrity *via* contrast agent leakage, and identifying inflammatory consequences combined with phenotypic imaging patterns and imaging genomics.

6 Neuroinflammatory molecular-based imaging strategies for brain metastases

Brain metastasis is more prevalent than primary brain tumors, mainly due to the limited therapeutic efficacy against various primary cancer, such as breast, lung, and colorectal cancers. The prognosis for patients with brain metastasis is significantly compromised, underscoring the importance of early detection and accurate diagnosis. The TME plays a crucial role in the development of brain metastasis. Notably, the cancer stem cells (CSCs) are the predominant population involved in mediating metastasis, while neuroinflammation also plays a decisive role. Inflamed regions within the brain parenchyma facilitate the adhesion of peripheral tumor cells to activated endothelial cells, initiating invasion and metastasis. Tissue lesions caused by brain metastasis contribute to the establishment of neuroinflammation, characterized by persistent activation of astrocytes and microglia, increased production and release of pro-inflammatory substances, compromised BBB permeability, and infiltration of immune cells. Consequently, a diverse array of immune cells and inflammatory factors promote the progression of brain metastasis, exhibiting high heterogeneity depending on the origin of the primary malignancy and the specific brain site involved. Similar to GBMs, hypoxia-induced stimulation triggers pro-inflammatory substance expression in CSCs, further promoting a pro-inflammatory phenotype in GBM. These inflammatory factors contribute to tumor growth and metastasis in GBM (99, 100).

Distinguishing between primary brain tumors and brain metastasis using neuroimaging remains a significant challenge in clinical diagnosis. Both primary and metastatic brain malignancies exhibit similar peritumoral hyperintensities and intratumoral texture on MRI. Previous studies have shown limited differences in apparent diffusion coefficient (ADC) measurements between primary brain tumors and brain metastasis. However, several studies have revealed that GBMs exhibit higher homogeneity and inverse difference moment than brain metastasis (79). Regarding peritumoral edema, MRI demonstrates greater heterogeneity of

peritumoral edema in GBMs compared with that in metastasis, with high sensitivity and specificity of 80% and 90%, respectively (76). Additionally, the use of 2D texture features extracted from MRI images enables fast and noninvasive discrimination between GBM and brain metastases (82). Machine learning algorithms have gained significant attention in neuroimaging applications to improve the accuracy of clinical diagnosis. Quantitative analysis of MRI using machine learning and deep learning-based models facilitates the differentiation between primary and metastatic malignancies, emphasizing the significance of texture feature analysis (81, 85). Furthermore, texture features extracted from post-contrast three-dimensional T1-weighted MR images, optimized by machine learning classifiers, have demonstrated high diagnostic performance and generalizability in differentiating solitary brain metastasis from GBM with high diagnosis performance and generalizability (83). In pediatric medulloblastoma (MB), radiogenomics combined with MRI-based machine learning offers an opportunity for MB risk stratification. Studies have reported the beneficial use of MRI-based machine learning in identifying four clinically relevant molecular pediatric MB subgroups (86).

A previous study demonstrated that extracting texture features from post-contrast diffusion tensor imaging (DTI) MRI contributes to distinguishing between primary and metastatic brain tumors, offering high performance and generalizability in clinical diagnosis. Moreover, combining arterial spin labelling perfusion and DTI has shown significant clinical value (77). However, no differentiation in ADC values and ratios, as well as standard deviation values and ratios, was observed between GBMs and brain metastasis (84). In computational-aided quantitative analysis of MRI images (T2-weighted/susceptibility-weighted/contrast-enhanced T1-weighted MRI), high accuracy was achieved in differentiating GBMs from metastases, emphasizing the significance of texture features rather than fractal-based features in clinical practice (75). Trained multi-class multilayer perceptron models using non-enhancing T2 hyperintense regions can differentiate glioblastoma, brain metastasis, and CNS lymphoma with modest diagnostic accuracy, resulting in an approximately 19% increase in diagnostic yield (78).

The integration of DTI, neurite orientation dispersion, intracellular or extracellular volume fraction, and metabolite analysis with neuroimaging techniques have promoted accurate clinical discrimination. Furthermore, the rCBV in the peritumoral brain zone (PBZ) distinguishes GBMs from metastases. Moreover, the CBV gradient or color map obtained from phase difference enhanced imaging in the PBZ also serves as an effective approach for distinguishing GBMs from metastasis. Significantly higher rCBV ratios and rCBV gradient were observed in the PBZ of GBMs compared with brain metastasis (80). Intratumoral proton MR spectroscopy reveals high levels of creatine in primary brain malignancies, particularly GBMs, whereas the absence of intratumoral creatine suggests the presence of metastatic brain malignancies (73). Definite lipid signals indicate tumor tissue necrosis, while the absence of lipid signals might rule out metastasis. Currently, the evaluation of peritumoral areas using color phase difference enhanced imaging, a novel phase-related MRI technique, also aids in the differentiation between GBM tumors and metastases (74). The prognosis for patients with brain metastasis is compromised, therefore, early detection and accurate diagnosis of brain metastasis are critical in clinical management.

7 Neuroinflammatory molecular imaging to distinguish CNS malignancies from intracranial infections

In neuroimaging, brain abscess manifests as expansile, rim-enhancing masses surrounded by edema, which can resemble necrotic malignant tumors, particularly GBMs (101). Consequently, lesions caused by brain infections, particularly brain abscesses, are often misdiagnosed as brain tumors due to their similar MRI appearance and characteristics. However, brain abscesses and GBMs could cause nonspecific headaches in the absence of fever, focal neurologic deficits, and epileptic seizures (102). Therefore, the development of rapid and accurate diagnostic techniques is necessary to distinguish between brain abscesses and malignancies. Pathological examination reveals that the enhancing rim of GBMs comprises infiltrated tumor cells, whereas the enhancing rim of pyogenic abscesses comprises inflammatory components such as neutrophils, macrophages, and lymphocytes (103, 104). Therefore, the choline/creatine ratio of the rim-enhancing lesion in abscesses is expected to be lower than that in GBMs. During MRI, the ADC and diffusion-weighted imaging provide valuable information for clinical diagnosis. Previous studies have reported that abscesses exhibit hyperintense signal changes on diffusion-weighted imaging, while GBMs demonstrate varying degrees of hyperintense to hypointense signal conversion. Significant differences have been observed in the choline/creatine, choline/N-acetylaspartate, and choline/choline-n ratios within the contrast-enhancing rim, allowing for differentiation between abscess and GBMs (71). Furthermore, combining dynamic susceptibility contrast perfusion-weighted imaging and DTI has shown improved efficacy in distinguishing inflammatory lesions compared with using a single neuroimaging technique. Research has indicated that choline levels in the ring-enhancing portion of abscesses are significantly lower compared with that of brain tumors (70). Moreover, a subsequent clinical study has demonstrated the significant role of MR spectroscopic imaging in discriminating abscesses from brain tumors. Metabolite ratios and maximum choline/choline-n, choline/creatine, and choline/N-acetylaspartate ratios within the contrast-enhancing rim could effectively differentiate abscesses from brain tumors (72).

8 Conclusion

Imaging genomics has emerged as a technique for evaluating neuroinflammation, involving the use of novel imaging biomarkers that capture DNA and RNA patterns associated with the biology or immune states of cancers. The imaging features are closely associated with gene expression patterns, mutations, and protein

modifications (105). In the context of GBMs, imaging genomics has been employed, and several biomarkers have been established, such as the isocitrate dehydrogenase 1 mutation status and immunoreactivity. A radiogenomic profiling of 60 patients with GBM demonstrated positive correlations between CD68, CSF1 receptor, CD33, CD4, and CBV (106). Thus, imaging genomics holds the potential for bridging the gap between neuroimaging and tumor diagnosis in clinical practice.

In the era of immunotherapy and precision oncology, focusing solely on isolated imaging of brain tumor is not enough to establish predictive biomarkers and define neuroinflammation. Therefore, novel MRI and PET scans based on the tumor-associated neuroinflammation have attracted great attention. Secondary surgery towards recurrent brain tumors always accompanies elevating surgical risk and high therapeutic costs, therefore, it is important for accurate discrimination between tumor recurrence and radiation-induced necrosis. Multi-parametric MRI presents versatile imaging information and is considered an effective and useful imaging approach in clinical diagnosis. Accumulative evidence emphasized the clinical value of the apparent diffusion coefficient, volume transfer constant, and relative cerebral blood volume in the distinction of tumor cancer, radiation-induced necrosis, and other brain diseases in daily neuro-oncological practice. PET scans present a unique function in determining tumor microenvironment, assessing drug delivery, and evaluating therapeutic effects. Although the application of PET scans presents great advantages in clinical use, high economic costs and restricted devices limit its generalization. The development of neuroimaging and the combination of novel MRI contrast agents and PET radiotracers and imaging genomic techniques enable the evaluation of neuroinflammatory components and the improvement of accurate diagnosis and clinical discrimination. Of note, there are still several limitations requiring further improvement in neuroinflammation-based neuroimaging to minimize the false-positive diagnosis of tumor recurrence and misdiagnosis with necrosis or intracranial infection. In conclusion, the efforts on such noninvasive neuroinflammation imaging towards accurate diagnosis and personalized therapeutic efficacy monitoring will help the establishment of precision oncology strategies for patients with brain tumors or other malignancy.

Author contributions

WD and ZQ designed the study; WD wrote the draft; ZQ and NW revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Association between a four-parameter inflammatory index and all-cause mortality in critical ill patients with non-traumatic subarachnoid hemorrhage: a retrospective analysis of the MIMIC-IV database (2012-2019)

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Background: Non-traumatic subarachnoid hemorrhage (SAH), primarily due to the rupture of intracranial aneurysms, contributes significantly to the global stroke population. A novel biomarker, pan-immune-inflammation value (PIV) or called the aggregate index of systemic inflammation (AISI), linked to progression-free survival and overall survival in non-small-cell lung cancer and mortality in Coronavirus Disease 2019 (COVID-19) patients, has surfaced recently. Its role in non-traumatic SAH patients, however, remains under-researched. This study aims to determine the relationship between PIV and all-cause mortality in non-traumatic SAH patients.

Methods: A retrospective analysis was conducted using data from the Medical Information Mart for Intensive Care (MIMIC-IV) database to examine the association between PIV and all-cause mortality in critically ill patients with non-traumatic SAH. PIV measurements were collected at Intensive Care Unit (ICU) admission, and several mortality measures were examined. To control for potential confounding effects, a 1:1 propensity score matching (PSM) method was applied. The optimal PIV cutoff value was identified as 1362.45 using X-tile software that is often used to calculate the optimal cut-off values in survival analysis and continuous data of medical or epidemiological research. The relationship between PIV and short- and long-term all-cause mortality was analyzed using a multivariate Cox proportional hazard regression model and Kaplan-Meier (K-M) survival curve analysis. Interaction and subgroup analyses were also carried out.

Results: The study included 774 non-traumatic SAH patients. After PSM, 241 pairs of score-matched patients were generated. The Cox proportional hazard model, adjusted for potential confounders, found a high PIV (≥ 1362.45) independently

associated with 90-day all-cause mortality both pre- (hazard ratio [HR]: 1.67; 95% confidence intervals (CI): 1.05-2.65; P = 0.030) and post-PSM (HR: 1.58; 95% CI: 1.14-2.67; P = 0.042). K-M survival curves revealed lower 90-day survival rates in patients with PIV ≥ 1362.45 before (31.1% vs. 16.1%%, P < 0.001) and after PSM (68.9% vs. 80.9%, P < 0.001). Similarly, elevated PIV were associated with increased risk of ICU (pre-PSM: HR: 2.10; 95% CI: 1.12-3.95; P = 0.02; post-PSM: HR: 2.33; 95% CI: 1.11-4.91; P = 0.016), in-hospital (pre-PSM: HR: 1.91; 95% CI: 1.12-3.26; P = 0.018; post-PSM: 2.06; 95% CI: 1.10-3.84; P = 0.034), 30-day (pre-PSM: HR: 1.69; 95% CI: 1.01-2.82; P = 0.045; post-PSM: 1.66; 95% CI: 1.11-2.97; P = 0.047), and 1-year (pre-PSM: HR: 1.58; 95% CI: 1.04-2.40; P = 0.032; post-PSM: 1.56; 95% CI: 1.10-2.53; P = 0.044) all-cause mortality. The K-M survival curves confirmed lower survival rates in patients with higher PIV both pre- and post PSM for ICU (pre-PSM: 18.3% vs. 8.4%, P < 0.001; post-PSM:81.7 vs. 91.3%, P < 0.001), in-hospital (pre-PSM: 25.3% vs. 12.8%, P < 0.001; post-PSM: 75.1 vs. 88.0%, P < 0.001), 30-day (pre-PSM: 24.9% vs. 11.4%, P < 0.001; post-PSM:74.7 vs. 86.3%, P < 0.001), and 1-year (pre-PSM: 36.9% vs. 20.8%, P < 0.001; P = 0.02; post-PSM: 63.1 vs. 75.1%, P < 0.001) all-cause mortality. Stratified analyses indicated that the relationship between PIV and all-cause mortality varied across different subgroups.

Conclusion: In critically ill patients suffering from non-traumatic SAH, an elevated PIV upon admission correlated with a rise in all-cause mortality at various stages, including ICU, in-hospital, the 30-day, 90-day, and 1-year mortality, solidifying its position as an independent mortality risk determinant. This study represents an attempt to bridge the current knowledge gap and to provide a more nuanced understanding of the role of inflammation-based biomarkers in non-traumatic SAH. Nevertheless, to endorse the predictive value of PIV for prognosticating outcomes in non-traumatic SAH patients, additional prospective case-control studies are deemed necessary.

KEYWORDS

pan-immune-inflammation value (PIV), subarachnoid hemorrhage (SAH), mortality, biomarkers, MIMIC-IV, propensity score matching (PSM)

Introduction

Non-traumatic subarachnoid hemorrhage (SAH) represents a critical medical condition, primarily attributable to the rupture of intracranial aneurysms, contributing to 2–7% of overall stroke cases (1). The disease burden correlated with non-traumatic SAH is indeed profound and perhaps underappreciated. Remarkably, half of the non-traumatic SAH incidence arises in individuals aged below 60, with approximately one-third of these patients losing their lives before hospital admission. Consequently, immediate interventions in the intensive care unit (ICU) become indispensable for the remaining patients (1, 2). Regrettably, even with the execution of the best management protocols in the ICU, the in-hospital mortality rates associated with non-traumatic SAH remain alarmingly high (3). Consistent epidemiological studies have manifested the escalated prevalence of non-traumatic SAH, registering in-hospital mortality rates as high as 40% (1). Considering the potentially fatal implications of non-traumatic SAH, there is a pressing requirement for cost-effective, non-invasive diagnostic tools that can identify individuals at an elevated risk of mortality, thereby enabling timely implementation of preventative measures to reduce the likelihood of fatal consequences.

Systemic inflammation is hypothesized to play a significant role in the onset and progression of non-traumatic subarachnoid hemorrhage (SAH) due to its common risk factors and inflammatory profiles shared with certain inflammatory diseases (4, 5). The exact pathophysiological pathways through which inflammation catalyzes non-traumatic SAH's evolution are yet to be comprehensively elucidated; however, they are postulated to encompass leukocytosis and platelet aggregation (6–8). It has been observed that elevated platelet aggregation and systemic inflammation are directly related to early brain injury and contribute to non-traumatic SAH's pathogenesis. Additionally, inflammatory and thrombotic processes have been identified as crucial players in the underlying pathophysiological mechanism (7,

9), thereby amplifying the predisposition to delayed cerebral ischemia (DCI) subsequent to non-traumatic SAH.

The potential of systemic inflammatory indices derived from complete blood count (CBC) tests, such as the neutrophil/lymphocyte ratio (NLR), derived-NLR, platelet/lymphocyte ratio (PLR), and monocyte/lymphocyte ratio (MLR), has recently been highlighted. These indices, with their cost-effective and accessible nature, demonstrate considerable predictive utility across a variety of disorders, including non-traumatic SAH (10–16). Further, the systemic inflammation response index (SIRI) and systemic immune-inflammation index (SII) offer innovative, comprehensive biomarkers derived from three distinct blood cell counts. SIRI employs the absolute values of peripheral neutrophil, monocyte, and lymphocyte counts (N×M/L) (17), while SII uses counts of platelets, neutrophils, and lymphocytes (N×P/L) (18). Both SIRI and SII have shown valuable predictive capacity regarding clinical outcomes and severity in SAH patients (19–23). The pan-immune-inflammation value (PIV), also called the aggregate index of systemic inflammation (AISI), is computed by multiplying the counts of neutrophils, monocytes, and platelets, followed by dividing the result by the lymphocyte count (N×M×P/L). These four types of blood cells reflect different inflammatory and immune pathways in the body and can provide a more comprehensive reflection of the body's inflammatory status. PIV was widely applied in studies of cancer that two meta-analyses have demonstrated that PIV is a prognostic biomarker of overall survival and progression-free survival in cancer patients (24, 25), while AISI is usually used in studies of COVID-19 (26–28) as well as malignant conditions, including non-small-cell lung cancer (29), esophageal cancer (30), and prostate cancer (31). In fact, these two indices are same but with different names and they shared the same formula. The cells incorporated in this four-parameter inflammatory index calculation are crucial for maintaining equilibrium in the immune system, which safeguards the body against pathogens and diseases. Yet, these cells can also generate pro-inflammatory substances linked with various inflammatory diseases (32, 33).

Considering the widespread utilization of PIV and the heavy burden of stroke globally, we hypothesize that PIV has a similar predictive ability for mortality in non-traumatic SAH patients as that in COVID-19 and caner patients. Consequently, this study was designed with the aim of identifying the relationship between PIV and all-cause mortality in patients suffering from non-traumatic SAH. Besides, we hope the findings will provide new insight into how to manage non-traumatic SAH patients for clinicians.

Methods

Data source

This study employed a retrospective cohort design, utilizing the Medical Information Mart for Intensive Care (MIMIC-IV) database (version 2.2) (34). MIMIC-IV, a publicly accessible critical care database, is renowned for its extensive clinical data on patients treated in intensive care units. Acknowledged as one of the most voluminous and frequently engaged databases in intensive care medicine, it offers crucial resources for research and analytic purposes. With a wide spectrum of ICU-related data, MIMIC-IV serves as an invaluable tool for investigating critical care outcomes, predictive modeling, clinical decision support, and additional research areas. Permission to use the MIMIC-IV database in this study was obtained from the Massachusetts Institute of Technology and the Institutional Review Board of Beth Israel Deaconess Medical Center (BIDMC, Boston, MA, USA).

Ethical considerations and data privacy

To ensure ethical standards and patient privacy, the data used in this study underwent de-identification, with all precautions taken to uphold patient confidentiality. The author, Yong-Wei Huang, successfully completed the "Protecting Human Research Participants" web-based course offered by the National Institutes of Health (Record ID: 12150448) and was thereby authorized to access the MIMIC-IV database for data extraction. Given the de-identified nature of the data, the Beth Israel Deaconess Medical Center's ethical committee waived the requirement for informed consent.

Study population, variable extraction, and outcomes

The MIMIC-IV database contained data for a total of 180,733 individuals admitted to the ICU between 2012 and 2019. Out of these, 2,937 patients were identified as having non-traumatic subarachnoid hemorrhage (SAH) based on ICD-9 and ICD-10 codes (ICD-9 code 430 and ICD-10 codes I60, I600 to I6012, I6000 to I6002, I6020 to I6022, I6030 to I6032, and I6050 to I6052). For this investigation, only patients aged 18 years and above were initially considered, and data from their initial ICU stay were gathered. Exclusion criteria encompassed patients with missing values for neutrophils, platelets, monocytes, or lymphocytes post ICU admission, those with an ICU stay duration of less than 24 hours, and those with a negative survival time. Subsequently, a total of 774 patients met the inclusion standards and were incorporated in the final analysis (Figure 1).

The primary variable of interest in this study was the first blood routine obtained post admission to the ICU, which was viewed as the primary exposure factor. All variables used were extracted from the MIMIC-IV database using Structured Query Language (SQL) with PostgreSQL. The extraction process covered five main components: demographic variables, clinical severity upon admission, vital signs, comorbidities, and laboratory variables. To handle any missing data, the predicted mean matching method was utilized to impute values in the dataset. There were 124 missing values in the matching variables (Supplemental Table 1). The missing values in the matching variables occurred primarily due to missing completely at random. We conducted a comparison between the results obtained using the predicted mean matching method for imputing missing values and the results from a complete case analysis. The results were completely consistent.

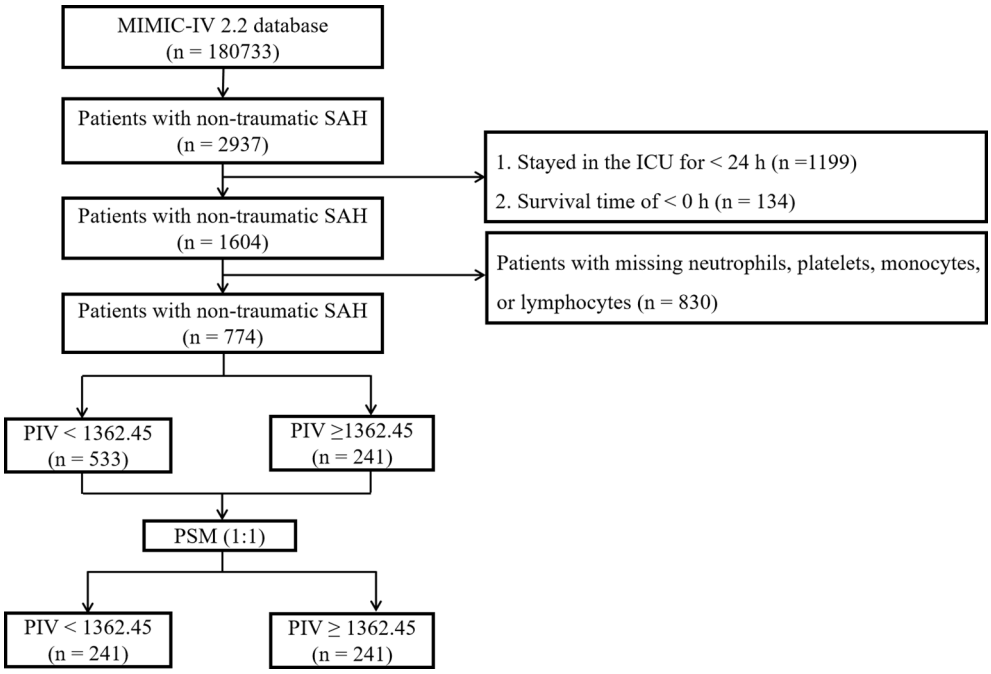

FIGURE 1
A flowchart for the patient selection process.

The study's primary endpoint was 90-day mortality, while secondary endpoints comprised ICU mortality, in-hospital mortality, 30-day mortality, and 1-year all-cause mortality. Importantly, the 90-day mortality was defined as the death within 90 days after SAH rather than a simple dead status at the 90th days from the ICU admission. The definitions of secondary endpoints are similar to this.

For an exhaustive list of the extracted variables, refer to Table 1.

Propensity score matching

Due to the inherent limitations of the retrospective study design, the patient selection process might have introduced selection bias and potential confounding factors. To mitigate these issues, we conducted a propensity score matching (PSM) analysis, which aimed to minimize the impact of bias and confounders. The PSM analysis involved creating a logistic regression model to compute propensity scores, subsequently utilized to match patients based on several variables. These variables, incorporated for calculating propensity scores, encompassed age, sex, ethnicity, GCS, SAPS II, SBP, DBP, MBP, temperature, heart rate, RR, SpO2, Hypertension, diabetes mellitus, CHF, COPD, sepsis, RF, liver diseases, malignancy, WBC count, hemoglobin, glucose, chloride, creatinine, BUN, vasopressor, ventilation, and oxygen. The PSM analysis employed a 1:1 nearest neighbor matching algorithm with a caliper of 0.1. To evaluate the balance between the two groups, we calculated absolute standardized differences (ASDs) pre and post-matching. ASDs below 0.10 implied a well-balanced distribution of characteristics between the matched groups.

Statistical analysis

Continuous variables were presented as median with interquartile range (IQR), and their differences were analyzed using the t-test or Mann-Whitney U-test. Categorical variables were reported as counts with proportions and compared using the Chi-square test or Fisher's exact test. To determine the optimal cutoff value for the PIV in relation to 90-day mortality, we employed X-tile software (Version 3.6.1, Yale University School of Medicine) that is often used to calculate the optimal cut-off values in survival analysis and continuous data of medical or epidemiological research. Consequently, PIV was dichotomized into two groups using the pre-determined optimal cutoff value. Further, the choice of optimal cut-off point that maximized the risk ratio can be found in Supplemental Figure 1, as well as the relationship of PIV ≥1362.45 and the distribution of PIV. We assessed the proportional hazards assumption (PHA) using both graphical methods and statistical tests. Graphically, we employed Kaplan-Meier (K-M) curves to visualize the stability of hazard ratios (HRs) in survival analysis. For statistical tests, we performed the Schoenfeld residuals test and the Grambsch-Therneau test to formally assess the PHA assumption. In our study, we had censored data, meaning that some patients did not experience the event of interest by the end of the observation period. To handle censored data, we followed the standard practice in Cox regression by treating censored individuals as having experienced no events during the observation period. The time-to-event variable in our analysis was the time from ICU admission until the occurrence of the death at endpoint time of interest. Univariate and multivariate analyses of prognostic factors were performed

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TABLE 1 The detailed extracted variables in MIMIC-IV database.

Items	Composition
Demographic variables	Age, sex, ethnicity
Clinical severity on admission	GCS, SAPS II
Vital signs	SBP, DBP, MBP, temperature, heart rate, respiratory rate, SpO_2
Comorbidities	Hypertension, diabetes mellitus, CHF, COPD, sepsis, RF, liver diseases, malignancy
Laboratory variables	WBC count, neutrophils count, monocytes count, lymphocytes count, platelets count, hemoglobin, glucose, chloride, creatinine, BUN
Treatments	Vasopressor, ventilation, oxygen
Clinical outcomes	ICU, in-hospital, 30-day, 90-day, and 1-year all-cause mortality

GCS, Glasgow Coma Scale; SAPS II, Simplified Acute Physiology Score; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; SpO2, percutaneous oxygen saturation; CHF, congestive heart failure; COPD, chronic pulmonary disease; RF, renal failure; WBC, white blood cell; BUN, blood urea nitrogen; ICU, intensive care unit.

using the Cox proportional hazards model to identify independent predictors of 90-day, ICU, in-hospital, 30-day, and 1-year mortality following non-traumatic SAH. The results were reported as HRs with 95% confidence intervals (CIs). We conducted subgroup analyses to investigate the impact of PIV on mortality within different subgroups. Stratification was carried out using a Cox regression model based on age (< 70 and ≥ 70 years), gender, hypertension, diabetes mellitus, liver disease, malignancy, and RF. Additionally, PIV was divided into four equal-interval quartiles to explore the relationship between varying PIV levels and all-cause mortality, with the first quartile serving as the reference group. To explore non-linear relationships, we employed restricted cubic splines (RCS). Smooth curve fitting and generalized additive models were used to investigate the threshold effect of PIV on the all-cause mortality in critical ill patients with non-traumatic SAH and identify the inflection point. All statistical analyses were two-sided, and p-values less than 0.05 were deemed statistically significant. The software used for analyses included R statistical software (R version 4.2.2, R Foundation for Statistical Computing), SPSS Statistics 26 (IBM, Chicago, IL, USA), and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Baseline characteristics of subjects

The study included a total of 774 out of 2937 non-traumatic SAH patients who received ICU treatment. There were 401 (51.8%) males and 373 (48.2%) females. The median age of the entire cohort was 62 (IQR, 51–76). Patients were divided into two groups based on the PIV optimal cutoff value determined by X-tile software, resulting in a low PIV group (< 1362.45) and a high PIV group (≥ 1362.45). Before propensity score matching, patients with higher PIV were found to have a higher SAPS II score, heart rate, and respiratory rate, as well as higher rates of sepsis, RF, ventilation, and vasopressor, elevated levels of WBC count, neutrophils, monocytes, platelets, glucose, BUN, and short- and long-term all-cause mortality. Further described, patients with PIV ≥ 1362.45 had a higher risk of various adverse outcomes compared to those in the PIV < 1362.45 group. They experienced a higher rates of ICU mortality (18.3% vs. 8.4%%, P < 0.001), in-hospital mortality (24.9% vs. 11.4%%, P < 0.001), 30-day mortality (25.3% vs. 12.8%, P < 0.001), 90-day mortality (31.1% vs. 16.1%%, P < 0.001), and 1-year mortality (36.9% vs. 20.8%%, P < 0.001). Nevertheless, no significant differences were observed between high PIV group and low PIV group in LOS ICU (P = 0.174) and LOS hospital (P = 0.052). More detailed results can be found in Table 2. It is worth noting that there was a rather high number of patients with missing inflammation markers, which were excluded from analyses. In order to further assess the possible bias introduced by excluding these patients, we analyzed the baseline characteristics of included individuals and excluded individuals, and most of the baseline characteristics between them were of no difference. Hence, we put the related statistical results in Supplemental Table 2.

Univariate and multivariate Cox regression models of PIV with mortality in patients with non-traumatic SAH before propensity score matching

To elucidate the potential relationship between the PIV and mortality outcomes in patients suffering from non-traumatic SAH, both univariate and multivariate Cox regression models were executed, with PIV categorized in binary. In the initial model without adjustments, a heightened PIV (≥ 1362.45) was demonstrably linked with escalated risks of mortality at various intervals: 90-day (HR = 2.07, 95% CI: 1.52-2.82, P < 0.001), within the ICU (HR = 1.95, 95% CI: 1.29-2.97, P = 0.002), during hospitalization (HR = 1.91, 95% CI: 1.34-2.73, P < 0.001), 30-day (HR = 2.16, 95% CI: 1.53-3.04, P < 0.001), and at the 1-year (HR = 1.97, 95% CI: 1.49-2.60, P < 0.001) for all causes of mortality. When adjusting for confounding variables such as age, gender, and ethnicity in multivariate model 1, the patient group exhibiting a PIV ≥ 1362.45 persisted in showing an elevated risk for mortality at the previously mentioned time points. An additional multivariate model (model 2), which incorporated further potential confounders (P < 0.05) identified in Table 2, further identified that a heightened PIV was independently linked with increased mortality risk across the aforementioned time intervals. Detailed data are presented in Table 3.

The Kaplan-Meier (K-M) survival curves distinctly illustrated higher mortality rates at 90-day, ICU, in-hospital, 30-day, and 1-year intervals in patients with a PIV value ≥ 1362.45, as opposed to those with PIV < 1362.45 (31.1% vs. 16.1%, P < 0.001; 18.3% vs. 8.4%, P < 0.001; 25.3% vs. 12.8%, P < 0.001; 24.9% vs. 11.4%, P < 0.001; 36.9% vs. 20.8%, P < 0.001). More results can be found in Figure 2.

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TABLE 2 Baseline characteristics before propensity score matching.

Variable	Total (n=774)	PIV		*P* value
		< 1362.45 (n = 533)	≥ 1362.45 (n = 241)	
Demographics				
Age, years	62 (51-76)	61 (51-75)	66 (51-78)	0.158
Men, n (%)	401 (51.8%)	266 (49.9%)	135 (56.0%)	0.115
Ethnicity, n (%)				0.007
Asian	27 (3.5%)	14 (2.6%)	13 (5.4%)	
White	450 (58.1%)	310 (58.2%)	140 (58.1%)	
Black	45 (5.8%)	40 (7.5%)	5 (2.1%)	
Others	252 (32.6%)	169 (31.7%)	83 (34.4%)	
Clinical severity				
GCS	15 (14-15)	15 (14-15)	15 (14-15)	0.146
SAPS II	31 (24-39)	30 (24-37)	33 (25-41)	0.002
Vital signs				
SBP, mm Hg	132 (115-146)	132 (115-146)	132 (117-144)	0.912
DBP, mm Hg	72 (62-83)	72 (62-82)	72 (61-84)	0.806
MBP, mm Hg	88 (78-100)	88 (78-100)	88 (79-100)	0.833
Temperature	36.9 (36.6-37.2)	36.8 (36.6-37.1)	36.9 (36.6-37.2)	0.882
Heart rate	82 (71-94)	81 (70-93)	85 (73-98)	0.027
Respiratory rate	18 (15-21)	18 (15-21)	19 (16-22)	0.046
SpO_2	99 (96-100)	98 (96-100)	99 (96-100)	0.092
Comorbidities				
Hypertension, n (%)	380 (49.1%)	255 (47.8%)	125 (51.9%)	0.300
Diabetes mellitus, n (%)	146 (18.9%)	100 (18.8%)	46 (19.1%)	0.915
CHF, n (%)	9 (1.2%)	8 (1.5%)	1 (0.4%)	0.287
COPD, n (%)	59 (7.6%)	37 (6.9%)	22 (9.1%)	0.288
Sepsis, n (%)	367 (47.4%)	224 (42.0%)	143 (59.3%)	<0.001
Malignancy, n (%)	92 (11.9%)	64 (12.0%)	28 (11.6%)	0.877
RF, n (%)	437 (56.5%)	287 (53.9%)	150 (62.2%)	0.029
Liver disease n (%)	98 (12.7%)	68 (12.8%)	30 (12.5%)	0.904
Laboratory parameters				
WBC counts, 10^9/L	11 (8.3-14)	10.1 (7.5-13.1)	13.2 (10.3-16)	< 0.001
Neutrophils counts, 10^9/L	7.7 (4.8-11.5)	5.8 (4-8.3)	12.7 (10.5-15.7)	< 0.001
Lymphocytes counts, 10^9/L	1.4 (0.9-2)	1.6 (1-2.2)	1 (0.7-1.4)	< 0.001
Monocytes counts, 10^9/L	0.7 (0.5-0.9)	0.6 (0.4-0.8)	1 (0.7-1.3)	< 0.001
Platelets counts, 10^9/L	195 (159-240)	187 (153-225)	219 (175-268)	< 0.001
Hemoglobin (g/L)	11.9 (10.6-13.1)	11.9 (10.6-13)	11.9 (10.7-13.2)	0.315
Glucose (mmol/L)	127 (106-151)	123 (104-147)	134 (114-161)	< 0.001
Chloride	104 (101-107)	104 (101-107)	104 (100-107)	0.305

(Continued)

TABLE 2 Continued

Variable	Total (n=774)	PIV		*P* value
		< 1362.45 (n = 533)	≥ 1362.45 (n = 241)	
Creatinine	0.8 (0.7-1)	0.8 (0.6-1)	0.8 (0.7-1)	0.077
BUN	14 (10-20)	14(10-19)	15 (11-21)	0.005
PIV	722.4 (292.8-1626.8)	403.3 (200.3-758.9)	2366.8 (1728.4-3455.5)	< 0.001
Treatment				
Ventilation, n (%)	531 (68.6%)	346 (64.9%)	185 (76.7%)	0.001
Oxygen, n (%)	474 (61.2%)	330 (61.9%)	144 (59.8%)	0.567
Vasopressor, n (%)	197 (25.5%)	116 (21.8%)	81 (33.6%)	< 0.001
Clinical Outcomes				
LOS ICU, day	4 (2-10)	4 (2-9)	4 (1-13)	0.174
LOS Hospital, day	9 (4-18)	9 (5-16)	11 (4-22)	0.052
In-hospital mortality, n (%)	121 (15.6%)	61 (11.4%)	60 (24.9%)	< 0.001
ICU mortality, n (%)	89 (11.5%)	45 (8.4%)	44 (18.3%)	< 0.001
30-day mortality, n (%)	129 (16.7%)	68 (12.8%)	61 (25.3%)	< 0.001
90-day mortality, n (%)	161 (20.8%)	86 (16.1%)	75 (31.1%)	< 0.001
1-year mortality, n (%)	200 (25.8%)	111 (20.8%)	89 (36.9%)	< 0.001

PIV, pan-immune-inflammation value; GCS, Glasgow Coma Scale; SAPS II, Simplified Acute Physiology Score; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; SpO2, percutaneous oxygen saturation; CHF, congestive heart failure; COPD, chronic pulmonary disease; RF, renal failure; WBC, white blood cell; BUN, blood urea nitrogen; LOS ICU, Length of ICU stay; LOS hospital, Length of hospital stay.

TABLE 3 Univariate and multivariate Cox regression models of PIV with mortality in patients with non-traumatic SAH before propensity score matching.

Outcome	Unadjusted		Model 1		Model 2	
	HR, 95% CI	P value	HR, 95% CI	P value	HR, 95% CI	P value
ICU mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥1362.45)	1.95 (1.29-2.97)	0.002	1.62 (1.05-2.50)	0.029	2.10 (1.12-3.95)	0.021
In-hospital mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.91(1.34-2.73)	< 0.001	1.57 (1.08-2.29)	0.018	1.91(1.12-3.26)	0.018
30-day mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	2.16 (1.53-3.04)	< 0.001	1.86 (1.30-2.67)	<0.001	1.69 (1.01-2.82)	0.045
90-day mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	2.07 (1.52-2.82)	< 0.001	1.84 (1.33-2.54)	< 0.001	1.67 (1.05-2.65)	0.030
1-year mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.97 (1.49-2.60)	<0.001	1.74 (1.30-2.32)	< 0.001	1.58 (1.04-2.40)	0.032

Model 1: Unadjusted.
Model 2: Adjusted age, gender, and ethnicity.
Model 3: Adjusted all variables of P < 0.05 in Table 2.

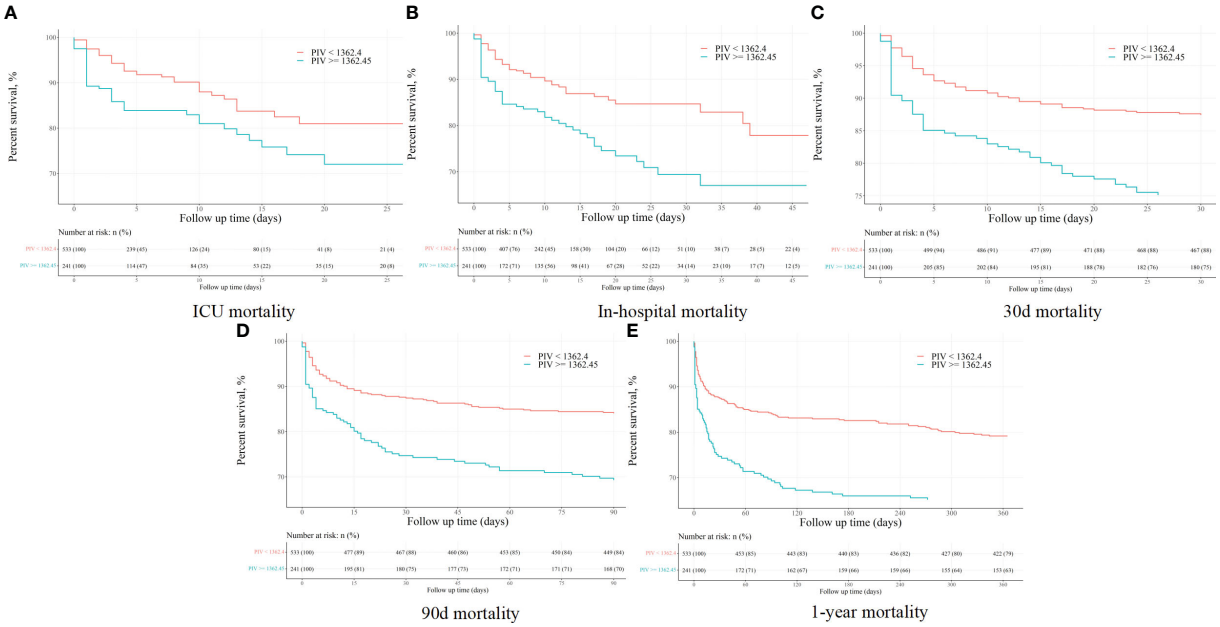

FIGURE 2
The K-M survival plots of **(A)** ICU, **(B)** in-hospital, **(C)** 30-day, **(D)** 90-day, and **(E)** 1-year mortality before propensity score matching.

Relationship between the PIV and all-cause mortality in non-traumatic SAH patients after propensity score matching

In an effort to normalize the disparity in baseline features between the patient groups with low and high PIV, a 1:1 PSM analysis was executed, leading to the formation of 241 matched patient pairs. The demographics, comorbidities, majority of laboratory parameters, metrics, and treatments exhibited balance between the two cohorts post-PSM, as delineated in Table 4. However, elements such as neutrophil count, monocyte count, platelet count, and lymphocyte count that directly contribute to the PIV were not considered as matching variables. The efficacy of the matching process was evaluated by determining the absolute standardized differences (ASD) both prior to and following PSM, as illustrated in Figure 3.

After PSM, discernible disparities continued to exist between the two cohorts concerning 90-day (31.1 vs. 19.1%, P = 0.002), ICU (18.3 vs. 8.7%, P = 0.002), in-hospital (24.9 vs. 12.0%, P < 0.001), 30-day (25.3 vs. 13.7%, P = 0.001), and 1-year (36.9 vs. 24.9%, P < 0.001) all-cause mortality rates. Meanwhile, no significant variances were evident in length of stay (LOS) in the ICU (P = 0.265) and in the hospital (P = 0.433) (Table 4). Additionally, outcomes of the multivariate Cox regression analyses in patients following PSM manifested that a PIV ≥ 1362.45 retained its status as an independent prognosticator of increased mortality in the ICU (HR = 2.33, 95% CI: 1.11-4.91, P = 0.016), during hospitalization (HR = 2.06, 95% CI: 1.10-3.84, P = 0.034), at 30-day (HR = 1.66, 95% CI: 1.11-2.97, P = 0.047), 90-day (HR = 1.58, 95% CI: 1.14-2.67, P = 0.042), and 1-year (HR = 1.56, 95% CI: 1.10-2.53, P = 0.044) time points (Table 5). Moreover, K-M survival curves contrasting the two groups highlighted that even after PSM, patients with a PIV value ≥ 1362.45 consistently demonstrated significantly diminished survival rates at 90-day (68.9 vs. 80.9%, P < 0.001), within the ICU (81.7 vs. 91.3%, P < 0.001), during hospital stay (75.1 vs. 88.0%, P < 0.001) 30-day (74.7 vs. 86.3%, P < 0.001), and 1-year (63.1 vs. 75.1%, P < 0.001) intervals compared to patients with a PIV value < 1362.45 (Figure 4).

Subgroup analysis for the PIV on clinical outcomes in patients with non-traumatic SAH

Subgroup analyses were conducted to examine the association between SIRI and 90-day all-cause mortality in patients with AIS based on age (< 70 and ≥ 70 years), gender, hypertension, diabetes mellitus, liver disease, malignancy, and RF. The results revealed a consistent relationship between increasing PIV and higher 90-day all-cause mortality across all subgroups (Figure 5). All the stratification factors did not significantly affect the relationship between PIV and 90-day all-cause mortality.

Regression cubic splines

Besides, we further analyzed the original data when they were regarded as continuous variables. And we found that there are no statistical differences between different subgroups in the full-adjusted model 2 in short- and long-term all-cause mortality. Nevertheless, statistical differences between different subgroups were found in model 1 in terms of long-term all-cause mortality. In the unadjusted model, we found that statistical differences between different subgroups were found in terms of long-term all-cause mortality, except for ICU mortality. We described the related statistical results in Supplemental Table 3 and Figure 2. To explore non-linear relationships, we employed restricted cubic

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TABLE 4 Baseline characteristics after propensity score matching.

Variable	Total (n=482)	PIV		*P* value
		< 1362.45 (n = 241)	≥ 1362.45 (n = 241)	
Demographics				
Age, years	66 (52-78)	65 (52-78)	66 (51-78)	0.434
Men, n (%)	265 (55.0%)	130 (53.9%)	135 (56.0%)	0.647
Ethnicity, n (%)				0.028
Asian	12 (2.5%)	9 (3.7%)	3 (1.2%)	
White	18 (3.7%)	13 (5.4%)	5 (2.1%)	
Black	286 (59.3%)	146 (60.6%)	140 (58.1%)	
Others	166 (34.4%)	73 (30.3%)	93 (38.6%)	
Clinical severity				
GCS	15 (14-15)	15 (14-15)	15 (14-15)	0.326
SAPS II	32.5 (25-40)	32 (26-39)	33 (25-41)	0.152
Vital signs				
SBP, mmHg	132 (117-146)	132 (116-147)	132 (117-144)	0.915
DBP, mmHg	72 (62-83)	71 (62-83)	72 (61-84)	0.383
MBP, mmHg	88 (78-100)	89 (77-100)	88 (79-100)	0.511
Temperature (°C)	36.9 (36.6-37.2)	36.9 (36.6-37.1)	36.9 (36.6-37.2)	0.063
Heart rate	83 (72-95)	81(71-93)	85 (73-98)	0.091
Respiratory rate	18 (15-21)	18 (15-21)	19 (16-22)	0.041
SpO_2	99 (96-100)	98 (96-100)	99 (96-100)	0.003
Comorbidities				
Hypertension, n (%)	235 (48.8%)	110 (45.6%)	125 (51.9%)	0.172
Diabetes mellitus, n (%)	99 (20.5%)	53 (22.0%)	46 (19.1%)	0.430
CHF, n (%)	4 (0.8%)	3 (1.2%)	1 (0.4%)	0.315
COPD, n (%)	47 (9.8%)	25 (10.4%)	22 (9.1%)	0.645
Sepsis, n (%)	252 (52.3%)	109 (45.2%)	143 (59.3%)	0.002
Malignancy, n (%)	59 (12.2%)	31 (12.9%)	28 (11.6%)	0.097
Renal failure, n (%)	292 (60.6%)	142 (58.9%)	150 (62.2%)	0.455
Liver disease n (%)	56 (11.6%)	26 (10.8%)	30 (12.5%)	0.569
Laboratory parameters				
WBC counts, 10^9/L	11.6 (9.2-14.3)	10.1 (8-12.8)	13.2 (10.3-16)	<0.001
Neutrophils counts, 10^9/L	10.4 (7.7-13.4)	8.1 (6-10.2)	12.7 (10.5-15.7)	<0.001
Lymphocytes counts, 10^9/L	1.1 (0.8-1.6)	1.2 (0.8-1.6)	1 (0.7-1.4)	0.017
Monocytes counts, 10^9/L	0.8 (0.6-1.1)	0.7 (0.5-0.8)	1 (0.7-1.3)	<0.001
Platelets counts, 10^9/L	203 (165-254)	192 (161-240)	219 (175-268)	<0.001
Hemoglobin (g/L)	11.9 (10.7-13.2)	11.9 (10.7-13.1)	11.9 (10.7-13.2)	0.619
Glucose (mmol/L)	129 (109-155)	125 (105-148)	134 (114-161)	<0.001
Chloride	104 (100-107)	104 (100-106)	104 (100-107)	0.793

(Continued)

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TABLE 4 Continued

Variable	Total (n=482)	PIV		*P* value
		< 1362.45 (n = 241)	≥ 1362.45 (n = 241)	
Creatinine	0.8 (0.7-1)	0.8 (0.7-1)	0.8 (0.7-1)	0.922
BUN	15 (11-21)	14 (10-21)	15 (11-21)	0.351
PIV	1362.5 (789-2366.8)	789 (581-1018.3)	2366.8 (1728.4-3455.5)	<0.001
Treatment				
Ventilation, n (%)	344 (71.4%)	159 (66.0%)	185 (76.8%)	0.009
Oxygen, n (%)	295 (61.2%)	151 (62.3%)	144 (59.8%)	0.677
Vasopressor, n (%)	136 (28.2%)	55 (22.8%)	81 (33.6%)	0.166
Clinical Outcomes				
LOS ICU, day	4 (1-12)	4 (2-12)	4 (1-13)	0.265
LOS Hospital, day	10 (5-20)	9 (5-18)	11 (4-22)	0.433
In-hospital mortality, n (%)	89 (18.5%)	29 (12.0%)	60 (24.9%)	< 0.001
ICU mortality, n (%)	65 (13.5%)	21 (8.7%)	44 (18.3%)	0.002
30-day mortality, n (%)	94 (19.5%)	33 (13.7%)	61 (25.3%)	0.001
90-day mortality, n (%)	121 (25.1%)	46 (19.1%)	75 (31.1%)	0.002
1-year mortality, n (%)	149 (30.9%)	60 (24.9%)	89 (36.9%)	< 0.001

splines (RCSs). Smooth curve fitting and generalized additive models were used to investigate the threshold effect of PIV on all-cause mortality in critically ill patients with non-traumatic SAH and identify the inflection point. A non-linear correlation was detectable between PIV and the propensity of ICU, in-hospital, 30-day, 90-day, and 1-year mortality before and after PSM, and the detailed statistical results can be found in Figure 6.

Discussion

Given its broader cell type inclusion compared to other indices (SII or SIRI), PIV is perceived to be a superior indicator of inflammation, but it remains underutilized in the clinical sphere. The effectiveness of PIV has been tested in diverse scenarios, like being a predictive marker for small cell lung carcinoma (35),

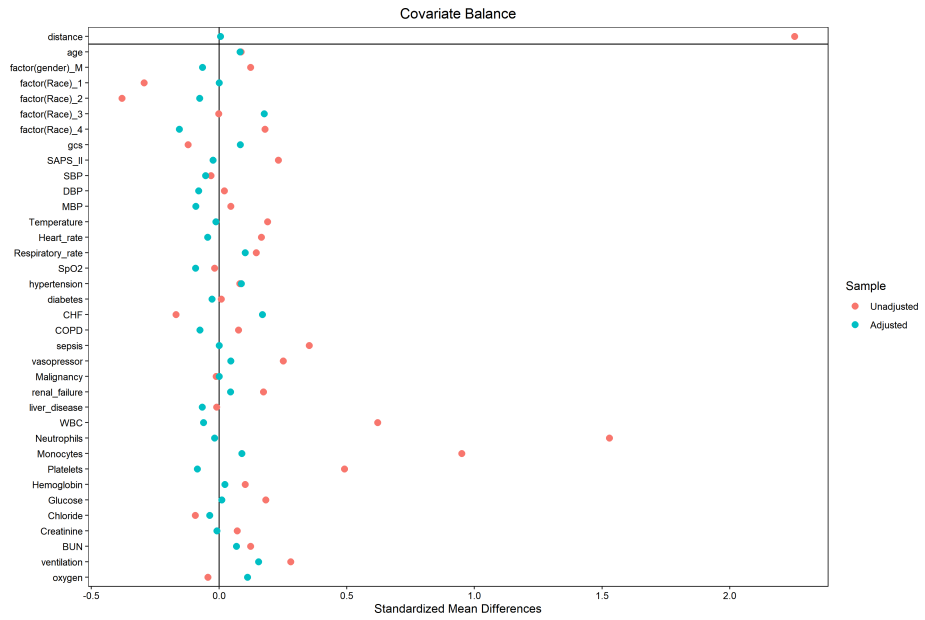

FIGURE 3
The absolute standardized differences for variables used to match the two groups.

TABLE 5 Univariate and multivariate Cox regression models of PIV with mortality in patients with non-traumatic SAH after propensity score matching.

Outcome	Unadjusted		Model 1		Model 2	
	HR, 95% CI	P value	HR, 95% CI	P value	HR, 95% CI	P value
ICU mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.90 (1.15-3.15)	0.013	1.92 (1.15-3.23)	0.014	2.33 (1.11-4.91)	0.016
In-hospital mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.83 (1.18-2.82)	0.007	1.82 (1.17-2.85)	0.008	2.06 (1.10-3.84)	0.034
30-day mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.92 (1.27-2.91)	0.002	1.96 (1.29-3.00)	0.002	1.66 (1.11-2.97)	0.047
90-day mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.67 (1.17-2.39)	0.005	1.73 (1.20-2.50)	0.003	1.58 (1.14-2.67)	0.042
1-year mortality						
PIV (< 1362.45)	1 (Ref)		1 (Ref)		1 (Ref)	
PIV (≥ 1362.45)	1.62 (1.17-2.23)	0.004	1.71 (1.23-2.38)	0.002	1.56 (1.10-2.53)	0.044

Model 1: Unadjusted.
Model 2: Adjusted age, gender, and ethnicity.
Model 3: Adjusted all variables of P < 0.05 in Table 2.

determining the risk of extended hospitalization post-elective thoracic surgery (36), and as an estimator for severity and ICU necessity in COVID-19 patients (37). Another piece of research has underscored the prognostic value of PIV in forecasting adverse outcomes for idiopathic pulmonary fibrosis patients (38). PIV quantifies inflammation by integrating various cell types participating in the immune response, such as neutrophils, lymphocytes, and platelets, along with monocyte counts. These

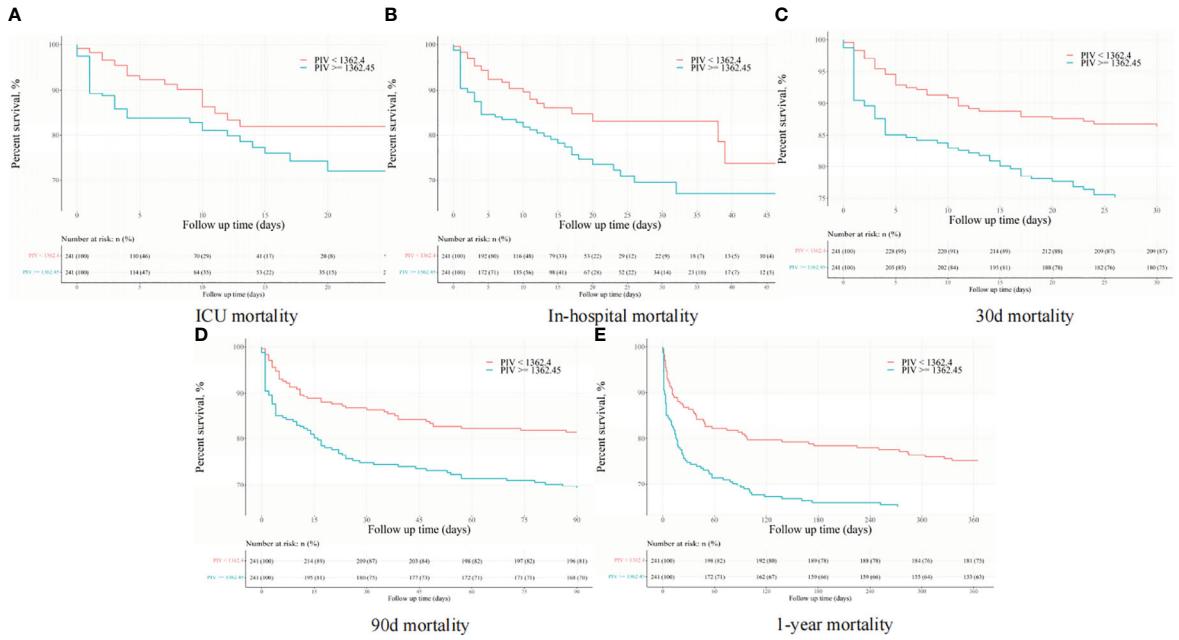

FIGURE 4
The K-M survival plots of **(A)** ICU, **(B)** in-hospital, **(C)** 30-day, **(D)** 90-day, and **(E)** 1-year mortality after propensity score matching.

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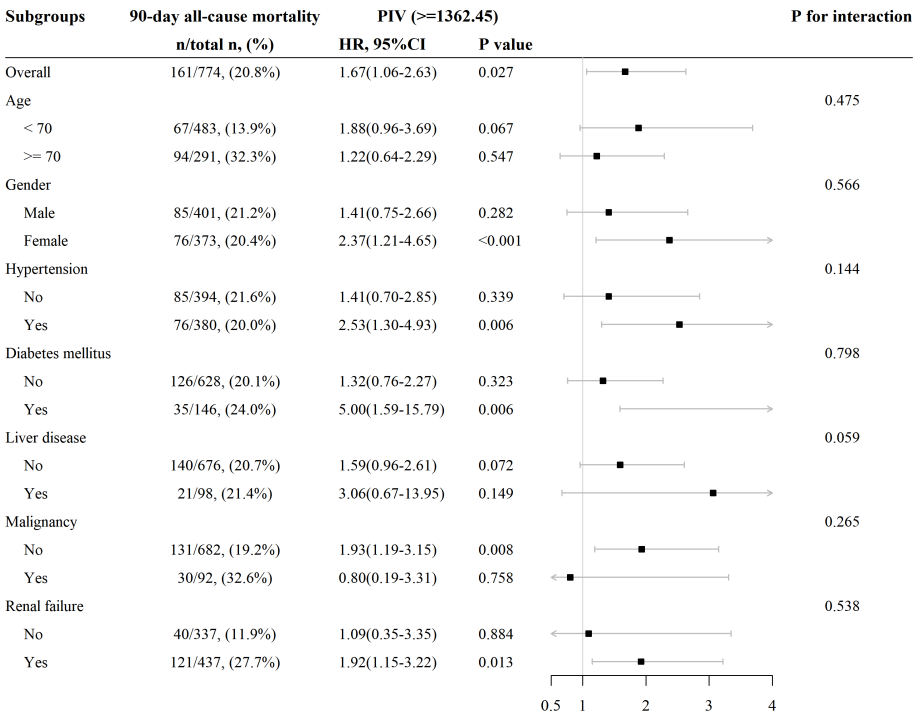

FIGURE 5
Subgroup analysis for the effect of PIV on 90-day all-cause mortality in patients with non-traumatic SAH.

cells are involved in the production of proinflammatory compounds like cytokines, chemokines, enzymes, and reactive oxidative species that fuel inflammation and instigate certain diseases (32). PIV, as a potential aggregate index, may assess the systemic inflammatory state and assess the balance between boosting and inhibiting inflammatory reactions. PIV is a simple biomarker that can be extracted from serum and is appropriate for long-term surveillance. Our study, for the first time, demonstrated that PIV plays a critical role in inflammation of non-traumatic SAH and is correlated with all-cause mortality.

It is becoming increasingly apparent that SAH is not solely a condition of the central nervous system. Rather, it has systemic implications, impacting not only the CNS, but also the cardiac and respiratory systems, as well as triggering systemic inflammatory responses (39–42). A growing body of research examining the interplay of inflammatory reactions and immune system dysregulation following non-traumatic SAH has indicated a significant involvement of the autonomic nervous system. Specifically, these studies point toward an upregulated sympathetic activity emanating from the hypothalamic-pituitary-adrenal axis (43). This heightened activity stimulates systemic inflammation, given that significant clusters of immune cells are responsive to catecholamines and cortisol (40, 43). Neutrophils, the preeminent leukocyte subtype in the human body, are crucial in mitigating both acute and chronic inflammatory reactions, undertaking phagocytic roles, and orchestrating the dissemination of anti-inflammatory agents (40, 44). On the other hand, lymphocytes have an essential part in both the instigation and resolution phases of inflammatory processes, with their functional state being either stimulated or inhibited based on diverse signaling pathways. The infiltration of lymphocytes contributes significantly to the initiation and escalation of inflammatory reactions, underlying the tissue deterioration and functional anomalies observed in inflammatory diseases (40, 41). Additionally, monocytes, another white blood cell variant, participate in the immune response and the onset of inflammation (40, 42). An escalated platelet to lymphocyte ratio (PLR) is deemed a negative prognostic indicator in the context of inflammatory disorders, given that an upsurged platelet count may lead to a reduction in lymphocyte count, a condition known as lymphopenia (43, 44). Hypothetically, neutrophils and lymphocytes could have a more pronounced influence on the pathogenesis of non-traumatic SAH compared to other cell types, and the assessment of multiple cellular categories in PIV evaluations might lead to an apparent dilution of their influence. While an elevated PIV proved beneficial in identifying patients at risk of mortality, the utilization of a solitary PIV measurement may not serve as an efficient instrument for gauging risk subsequent to a non-traumatic SAH. It is imperative to contemplate additional evaluation methodologies to corroborate the clinical significance of PIV within the non-traumatic SAH patient population.

The primary merit of our investigation lies in its reliance on extensive, real-world evidence, and the development of analogous cohorts via group matching, thus making strides toward diminishing bias resulting from confounding variables. Within the confines of our study, PIV exhibited commendable predictive prowess for mortality at any intervals. However, our study wasn't without its limitations. First, we eliminated patients missing crucial data, such as WBC subtypes, potentially leading to a selection bias.

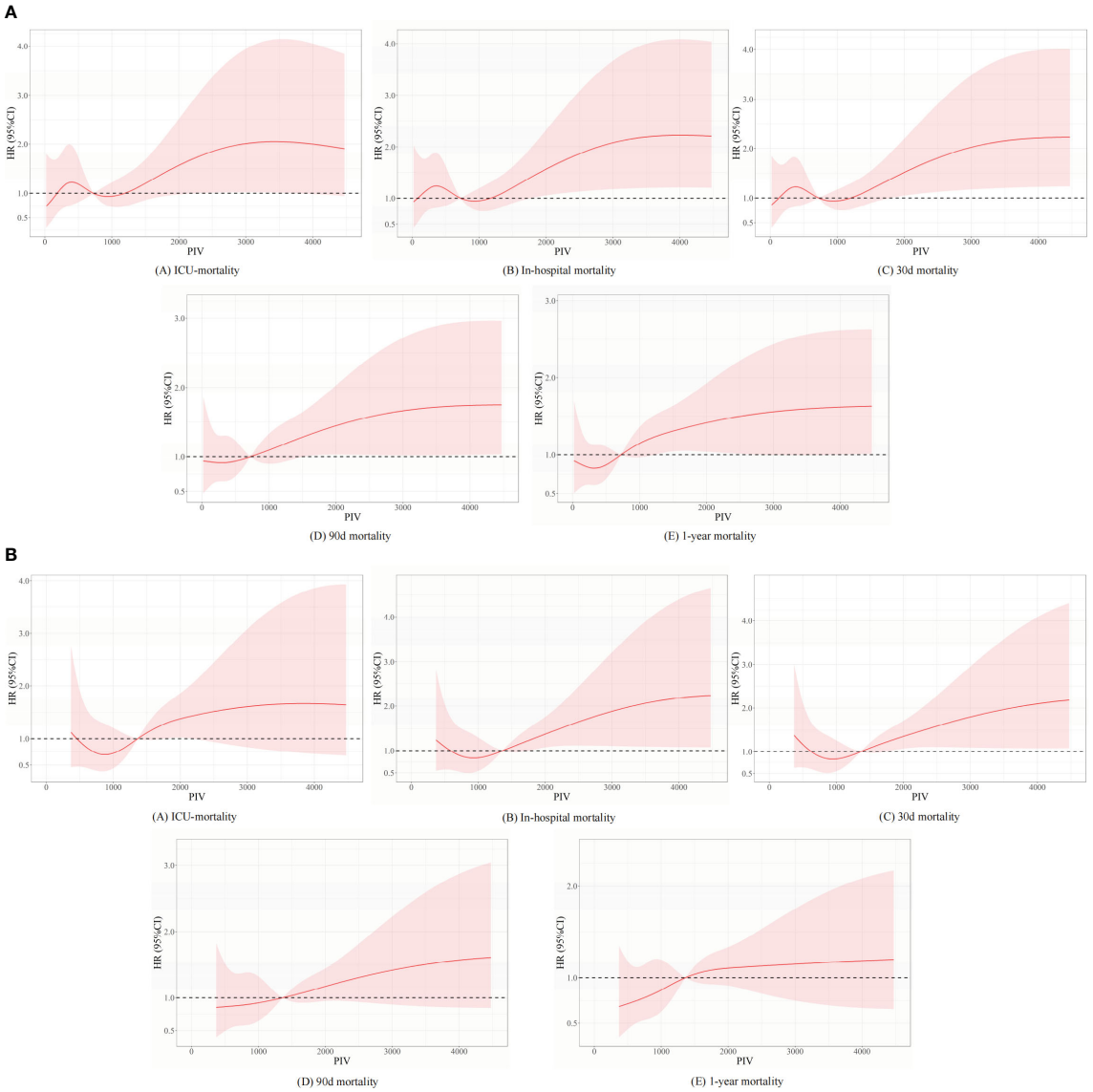

FIGURE 6
Underlying non-linear correlations between PIV and short- and long-term all-cause mortality **(A)** before and **(B)** after propensity score matching.

Second, the study was confined to a single-center, and thus the predictive capacity of PIV for non-traumatic SAH warrants additional validation across diverse populations and geographical locales. Third, due to the retrospective data aggregation, variables were not uniformly disseminated among the groups, although PSM analysis was deployed to lessen disparities between cohorts. Fourth, given that the ICD code is a definitive diagnosis, immediate complications like stunned heart syndrome, pneumonia were excluded from our study, which may result in an inflated PIV due to severe exacerbation of cerebral perfusion and tissue necrosis. Hence, acknowledging these constraints is crucial when appraising the outcomes of this investigation, and future studies stand to gain by corroborating and augmenting these findings. Additional research is warranted to shed light on the underlying interplay between immune-related cellular entities and non-traumatic SAH patients, and to examine the potential shortcomings of utilizing systemic inflammatory response indices for inflammatory condition assessment.

Conclusion

In critically ill patients suffering from non-traumatic SAH, an elevated PIV upon admission correlated with a rise in all-cause mortality at various stages, including ICU, in-hospital, the 30-day, 90-day, and 1-year mortality, solidifying its position as an independent mortality risk determinant. Nevertheless, to endorse the predictive value of PIV for prognosticating outcomes in non-traumatic SAH patients, additional prospective case-control studies are deemed necessary.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by The Institutional Review Boards of Massachusetts Institute of Technology (Cambridge, MA, USA) and Beth Israel Deaconess Medical Center (Boston, MA, USA). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because according to national legislation and institutional guidelines, written informed consent was not necessitated for this study.

Author contributions

Y-WH conceptualized and planned the study. Y-WH and YZ were responsible for the initial drafting of the manuscript. Y-WH, YZ, and X-SY handled the collection and analysis of clinical data. Both Z-PL and Y-WH contributed to the statistical methodologies applied in the study and offered constructive suggestions. Z-PL and X-SY critically reviewed the study and partook in the interpretation of the results. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1235266/full#supplementary-material

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Takinib inhibits microglial M1 polarization and oxidative damage after subarachnoid hemorrhage by targeting TAK1-dependent NLRP3 inflammasome signaling pathway

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Transforming growth factor-β-activated kinase 1 (TAK1) positively regulates oxidative stress and inflammation in different diseases. Takinib, a novel and specific TAK1 inhibitor, has beneficial effects in a variety of disorders. However, the effects of takinib on early brain injury (EBI) after subarachnoid hemorrhage (SAH) and the underlying molecular mechanisms remain unknown. Our study showed that takinib administration significantly inhibited phosphorylated TAK1 expression after SAH. In addition, takinib suppressed M1 microglial polarization and promoted M2 microglial polarization. Furthermore, blockade of TAK1 by takinib reduced neuroinflammation, oxidative damage, brain edema, and neuronal apoptosis, and improved neurological behavior after SAH. Mechanistically, we revealed that TAK1 inhibition by takinib mitigated reactive oxygen species (ROS) production and ROS-mediated nod-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome activation. In contrast, NLRP3 activation by nigericin abated the neuroprotective effects of takinib against EBI after SAH. In general, our study demonstrated that takinib could protect against EBI by targeting TAK1-ROS-NLRP3 inflammasome signaling. Inhibition of TAK1 might be a promising option in the management of SAH.

KEYWORDS

subarachnoid hemorrhage, early brain injury, takinib, TAK1, NLRP3

Abbreviations: 8-OhdG, 8-hydroxydeoxyguanosine; AMPK, AMP-activated protein kinase; CNS, central nervous system; EBI, early brain injury; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; NF--κB, nuclear factor-κB; NLRP3, nod-like receptor pyrin domain-containing protein 3; ROS, reactive oxygen species; SAH, subarachnoid hemorrhage; TAK1, Transforming growth factor-β-activated kinase 1.

1 Introduction

Subarachnoid hemorrhage (SAH) remains a life-threatening disease with poor prognosis. How to improve the clinical outcome after SAH is still a tough challenge. Multiple pathophysiological processes occurring after SAH, including early brain injury (EBI), cerebral vasospasm, and delayed cerebral ischaemia (1). Till now, mounting evidence has demonstrated that the neurological outcome after SAH is seriously influenced by early brain injury (EBI) (2–4). Multiple cellular mechanisms are involved in the EBI pathophysiology after SAH. Among them, activation of inflammatory response and oxidative damage contribute greatly to the development of EBI (5–7). Thus, a potential therapy for improving the prognosis after SAH is intervening neuroinflammation and oxidative stress.

Transforming growth factor-β-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase (MAPK) family, has a wide range of biological functions (8–10). It has demonstrated that TAK1 activation could strongly elicit pro-inflammatory cytokines release and drive microglial toward a proinflammatory phenotype (11–14). In addition, TAK1 mediates reactive oxygen species (ROS) generation to aggravate oxidative damage in different diseases models (15, 16). In central nervous system (CNS), TAK1 is mainly expressed in neurons and could regulate a variety of intracellular signaling pathways, including nuclear factor-κB (NF-κB), AMP-activated protein kinase (AMPK), and nod-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome (17–19). Since these molecular mechanisms contribute to EBI pathophysiology, targeting TAK1 might be an effective treatment for SAH.

Takinib, a novel and specific TAK1 inhibitor, has been recently evaluated in a variety of disease models (20–22). Previous studies have demonstrated that takinib could inhibit inflammation and apoptosis by suppression of TAK1-mediated signaling pathway (20, 21). However, whether takinib could protect against EBI after SAH and the potential molecular mechanisms remain unclear. Hence, this study aimed to investigate the possible role of takinib in EBI after SAH and its underlying mechanisms.

2 Materials and methods

All the experimental procedures were approved by the Animal Ethics Review Committee of Beijing Friendship Hospital and carried out in accordance with the National Institutes of Health guidelines. A total of 190 rats (weighing 250 to 300 g) were used in our study. Among them, 154 rats underwent SAH insults and 8 rats were excluded due to mild SAH grading score (less than 8) and intracerebral hematoma. All rats were numbered consecutively and randomization was conducted by using the website Randomization (http://www.randomization.com).

2.1 SAH model

The SAH model was performed according to our previous studies (23, 24). Briefly, rats were anesthetized with avertin (200 mg/kg). After anesthetization, rats were operated in a stereotactic frame. A hole was drilled into the skull in the midline 7.5 mm anterior to the bregma. SAH animals were injected with 0.35 ml of nonheparinized fresh autologous arterial blood from the femoral artery through the burr hole under aseptic conditions. Sham group rats underwent the same procedures, but were injected with 0.35 ml physiological saline. The basal temporal lobe adjacent to the clotted blood were collected for evaluation. After recovering from anesthesia, rats were housed in their cages and were free to food and water.

2.2 Experiment design

In the first set of experiments, the dose-dependent effects of takinib on TAK1 activation were examined. Total of 70 rats were randomly assigned into 5 groups, including sham + vehicle (n = 12), SAH + vehicle (n = 15, 3 rats died), SAH + 0.1 mM takinib (n = 15, 3 rats died), SAH + 0.3 mM takinib (n = 14, 2 rats died), and SAH + 0.9 mM takinib (n = 14, 2 rats died). Western blotting and double immunofluorescence staining were conducted to show the protein expression and cellular distribution of TAK1 in the brain cortex.

In the second set of experiments, we further explored the effects of takinib on microglial activation, ROS overproduction, and the subsequent brain damage after SAH. A total of 83 rats were randomly assigned into 4 groups, including sham + vehicle (n = 18), SAH + vehicle (n = 22, 4 rats died), SAH + 0.9 mM takinib (n = 21, 3 rats died), and SAH + 0.9 mM takinib + nigericin (n = 22, 4 rats died). Western blotting, double immunofluorescence staining, biochemical estimation, brain edema, and neurological behavior were conducted to determine the effects of takinib on EBI after SAH and the possible mechanisms.

In the third set of experiments, we investigated the effects of takinib on histopathological change and neurological behavior at day 3 after SAH. A total of 29 rats were randomly assigned into 4 groups, including sham + vehicle (n = 6), SAH + vehicle (n = 8, 1 rats died), SAH + 0.9 mM takinib (n = 7, 1 rats died), and SAH + 0.9 mM takinib + nigericin (n = 8, 1 rats died). Nissl staining and neurological behavior were performed. The experiment design and timeline were shown in Supplementary Figure 1.

2.3 Drug administration

Takinib (Selleck) was dissolved in dimethyl sulfoxide (DMSO) and diluted in physiologic saline at concentrations of 0.1, 0.3, and 0.9 mM. Various doses of takinib (10 μl) or vehicle was administered into the left lateral ventricle at 30 min post SAH insults. Nigericin (Selleck, 2 μg) was dissolved in 2 μl ethanol and physiologic saline and was administered to rats by intracerebroventricular route 2 h before SAH construction. For the intracerebroventricular administration, rats were put on a stereotactic frame. Coordinates for the intracerebroventricular injection were 1.5 mm posterior and 1.0 mm lateral to bregma, and 4.5 mm below the dural layer. The doses of takinib and nigericin were selected according to previous studies (20, 25).

2.4 Neurological scoring

Neurological function was evaluated by using a neurological severity scoring system as previously reported (26). Six test subscores were included in this scoring system. The high scores represented a relative normal neurological function. In addition, the rotarod test was conducted to measure motor function. The duration on the rotarod was recorded for statistical analysis (1, 27).

2.5 Brain water content

Brain water content was determined by using a wet/dry method (28). At 24 h following SAH, rats were deeply anesthetized with an overdose of avertin and the brains were separated into three parts, including cerebrum, cerebellum and brain stem. The wet weight was recorded. Each part of brain was dried at 80°C for 72 h and weighed again (dry weight). Brain water content was calculated by [(wet weight − dry weight)/wet weight] × 100%.

2.6 Western blotting

Western blotting was conducted according to previous studies (23, 29). In brief, the protein samples were separated by SDS-PAGE gel. And then they were transferred to nitrocellulose membrane. Afterward, the membranes were incubated with primary antibodies against TAK1 (1:1000, Cell Signaling), p-TAK1 (1:1000, Cell Signaling), NLRP3 (1:200, Santa Cruz Biotechnology), ASC (1:200, Santa Cruz Biotechnology), caspase-1 (1:200, Santa Cruz Biotechnology), cleaved caspase-1 (1:200, Santa Cruz Biotechnology), 3-nitrotyrosine (1:2000, Abcam), and β-actin (1:3000, Bioworld Technology) overnight at 4°C. After that, appropriate secondary antibodies were incubated at room temperature. Membranes were then exposed by ECL reagent.

2.7 Immunofluorescence

Immunofluorescence staining was conducted according to previous studies (23, 24). In brief, brain sections were blocked with 5% donkey serum. And then they were incubated overnight at 4°C with primary antibodies against p-TAK1 (1:100, Cell Signaling), CD16/32 (1:100, BD Biosciences), CD206 (1:100, Invitrogen), NLRP3 (1:50, Santa Cruz Biotechnology), 8-hydroxydeoxyguanosine (8-OhdG) (1:100, Abcam), IL-1β (1:100, Abcam), NeuN (1:200, EMD Millipore), and Iba-1 (1:50, Santa Cruz Biotechnology). Next, the brain sections were incubated with appropriate secondary antibodies. The fluorescently stained cells were visualized and photographed under a fluorescence microscope.

2.8 TUNEL staining

TUNEL staining (Beyotime) was conducted in line with the operation instructions. In brief, the brain sections were incubated with primary antibody against NeuN overnight. Afterward, the slides were incubated with TUNEL reaction mixture. Sections were visualized and photographed under a fluorescence microscope.

2.9 Malondialdehyde detection

The level of malondialdehyde (MDA) in brain samples were examined according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). MDA was determined at the wavelength of 535 nm using a spectrophotometer.

2.10 Statistical analysis

All values were expressed as means ± s.d. GraphPad Prism 8.02 was used to conduct statistical analysis. All data were tested for normality via Shapiro–Wilk test. Measurements were analyzed with one-way ANOVA followed by Tukey's *post-hoc* test. $P < 0.05$ was verified as statistically different.

3 Results

3.1 Effects of takinib on TAK1 activation after SAH

Takinib is a novel and highly selective TAK1 inhibitor. However, the influence of takinib on TAK1 activation after SAH remains obscure. Western blotting was conducted to detect the expression of p-TAK1 and TAK-1 after SAH. The results showed that doses of 0.3 mM and 0.9 mM takinib, but not 0.1 mM takinib, markedly inhibited p-TAK1 expression as compared with SAH + vehicle group (Figures 1A, B). There was no significant difference among all experimental groups in total TAK1 expression (Figure 1C). It has been demonstrated that TAK1 is mainly expressed in neurons. Consistent with previous studies, double immunofluorescence revealed that TAK1 activation was mainly distributed in neurons after SAH. In contrast, takinib treatment at 0.3 mM and 0.9 mM could significantly suppress TAK1 activation in neurons (Figures 1D, E). Since 0.9 mM takinib had the maximal effects, we used this dose in the subsequent experiments.

3.2 Influence of takinib on NLRP3 inflammasome signaling after SAH

TAK1 has been verified as a key regulator of NLRP3 inflammasome activation. However, whether takinib could modulate NLRP3 inflammasome signaling after SAH remains unknown. In this experiment, we evaluated the effects of takinib on NLRP3 inflammasome signaling after SAH. As shown, western blotting data revealed that SAH insults significantly induced the protein levels of p-TAK1, NLRP3, ASC, cleaved caspase1, IL-1β, and IL-18, all of which were reversed by takinib treatment (Figures 2A–H). However, in addition to p-TAK1, other molecular targets alterations by takinib were counteracted by

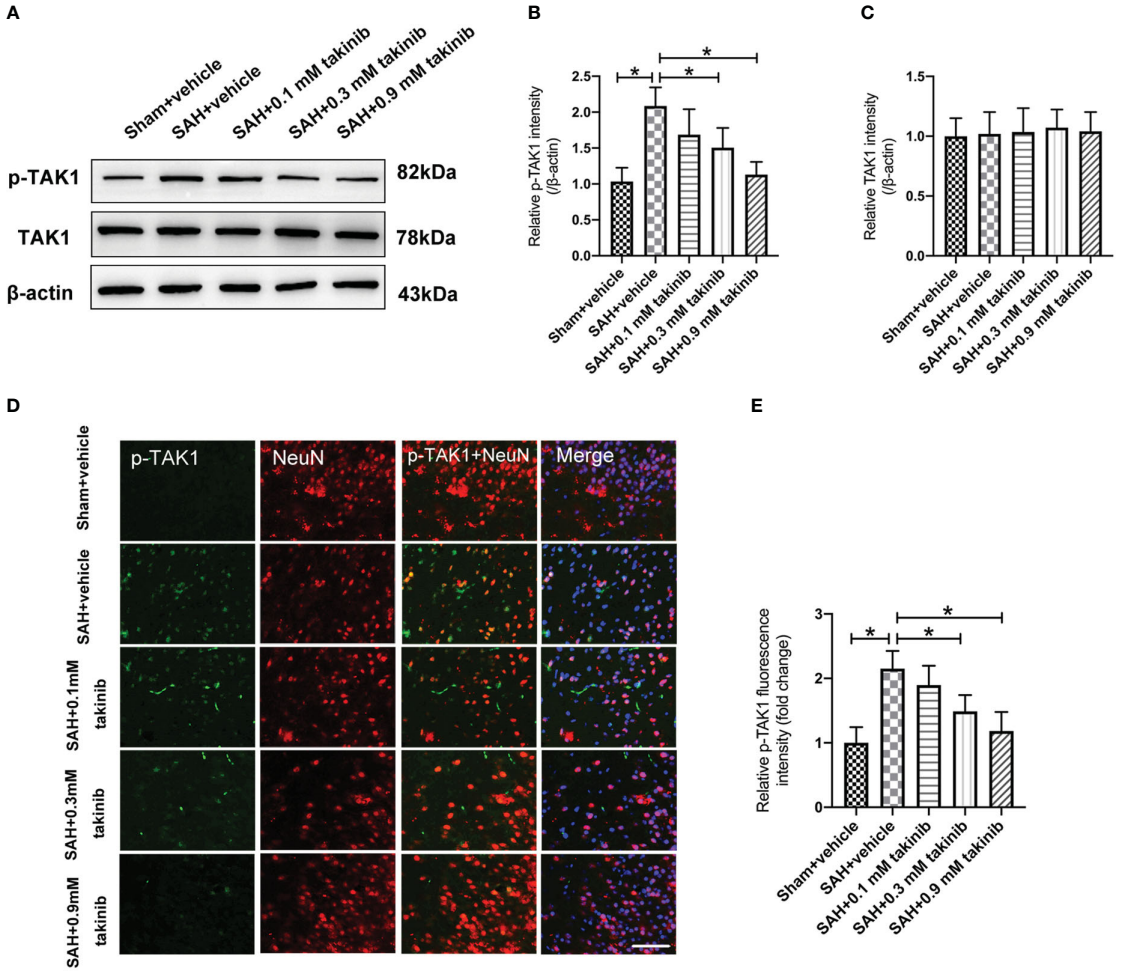

FIGURE 1
Dose-response effects of takinib on TAK1 activation after SAH. **(A)** Representative western blot bands and quantitative analyses of p-TAK1 **(B)** and TAK1 **(C)** after SAH (n = 6 each group). **(D)** Representative photomicrographs and quantification **(E)** of p-TAK1 immunofluorescence staining in the basal cortex at 24 h after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

nigericin administration (Figures 2A–H). No significant differences in the expressions of TAK1 (Figure 2C) and caspase1 (Figure 2E) were detected among all experimental groups.

3.3 Influence of takinib on M1/M2 microglial polarization after SAH

Microglial polarization plays a critical role in neuroinflammation after SAH. Numerous studies have demonstrated that TAK1 could induce microglial activation. However, whether TAK1 could modulate microglial polarization after SAH remains elusive. We then evaluated the effects of takinib on M1/M2 microglial polarization after SAH. The double immunofluorescence showed that takinib treatment significantly reduced the proportion of M1 microglial ($CD16/32^+$) and enhanced the quantity of M2 microglial ($CD206^+$) following SAH (Figures 3A–F). In contrast, NLRP3 activator nigericin abated the effects of takinib on M1/M2 microglial polarization (Figures 3A–F).

3.4 Influence of takinib on ROS production, and oxidative damage after SAH

ROS overproduction plays a key role in oxidative damage and contributes greatly to the development of EBI after SAH. Previous studies have demonstrated that TAK1 activation could aggravate ROS overproduction and oxidative damage. However, the influence of takinib on ROS production and oxidative damage following SAH remains unknown. We further evaluated the effects of takinib on ROS production and oxidative damage after SAH. 3-nitrotyrosine, a major product of tyrosine oxidation, is an important biomarker for ROS production. MDA is a biological marker for oxidative damage and lipid peroxidation. Our data revealed that SAH insults significantly induced ROS overproduction, lipid peroxidation, and oxidative damage, all of which were ameliorated by takinib treatment (Figures 4A–E). In contrast, the anti-oxidative effects of takinib could be partly reversed by nigericin treatment (Figures 4A–E).

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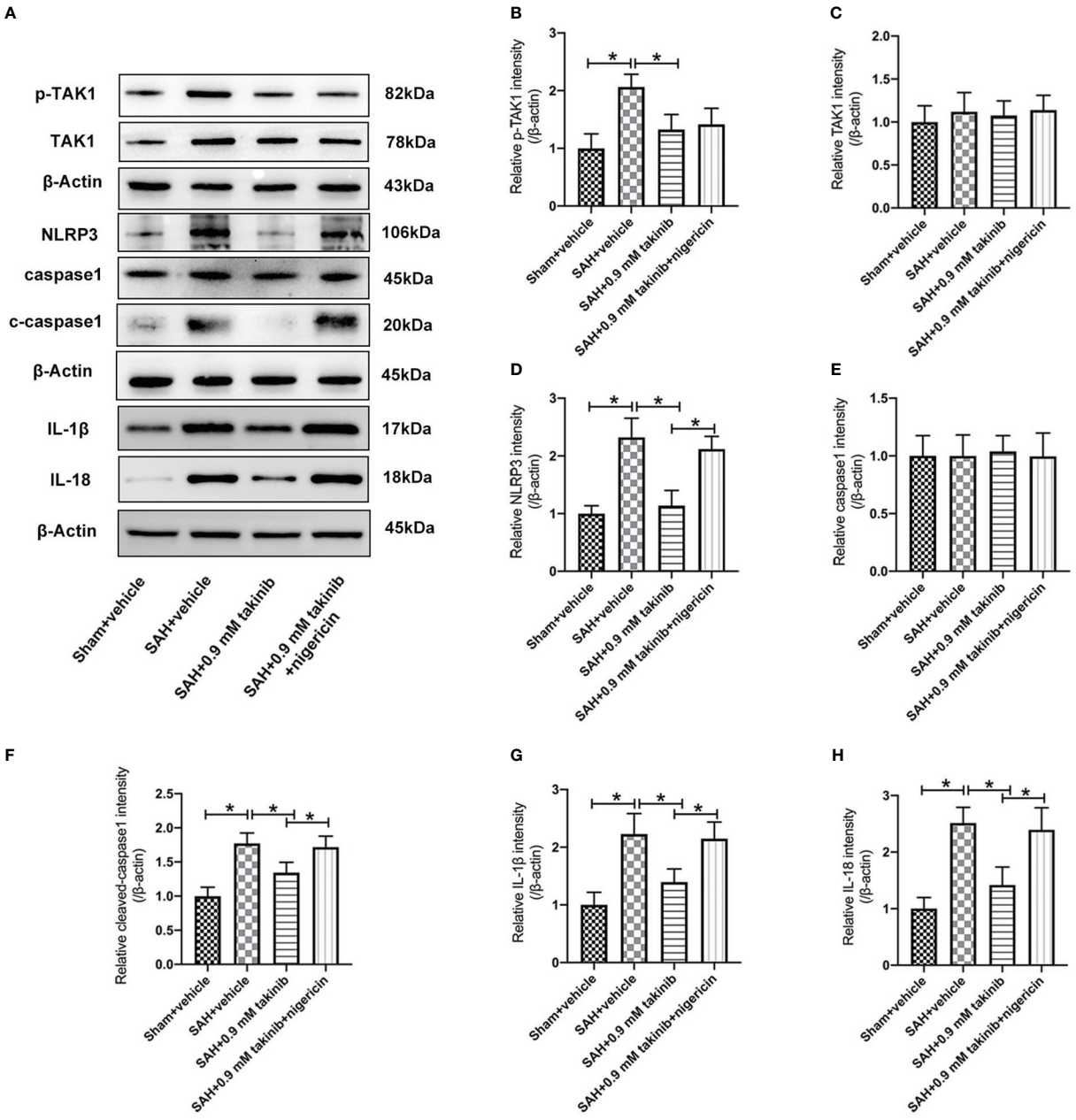

FIGURE 2
Effects of takinib on TAK1-NLRP3 inflammasome activation after SAH. **(A)** Representative western blot bands and quantitative analyses of p-TAK1 **(B)**, TAK1 **(C)**, NLRP3 **(D)**, caspase1 **(E)**, cleaved caspase1 **(F)**, IL-1β **(G)**, and IL-18 **(H)** after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

3.5 Influence of takinib on neurological function, brain edema, and neuronal apoptosis at day 1 after SAH

We then evaluated the effects of takinib on neurological outcomes, brain edema, and neuronal apoptosis after SAH. Consistent with the reduced neuroinflammation and oxidative damage, takinib treatment significantly improved neurological scores and motor functions, ameliorated brain edema, and reduced neuronal apoptosis after SAH (Figures 5A–E). In contrast, the pretreatment of nigericin statistically deteriorated the beneficial effects of takinib on neurological behavior, brain edema, and neuronal apoptosis after SAH (Figures 5A–E).

3.6 Influence of takinib on histopathological change and neurological behavior at day 3 after SAH

The first 72 h following SAH play a vital role in determining overall outcome. We then investigated the effects of takinib on histopathological change and neurological behavior at day 3 after

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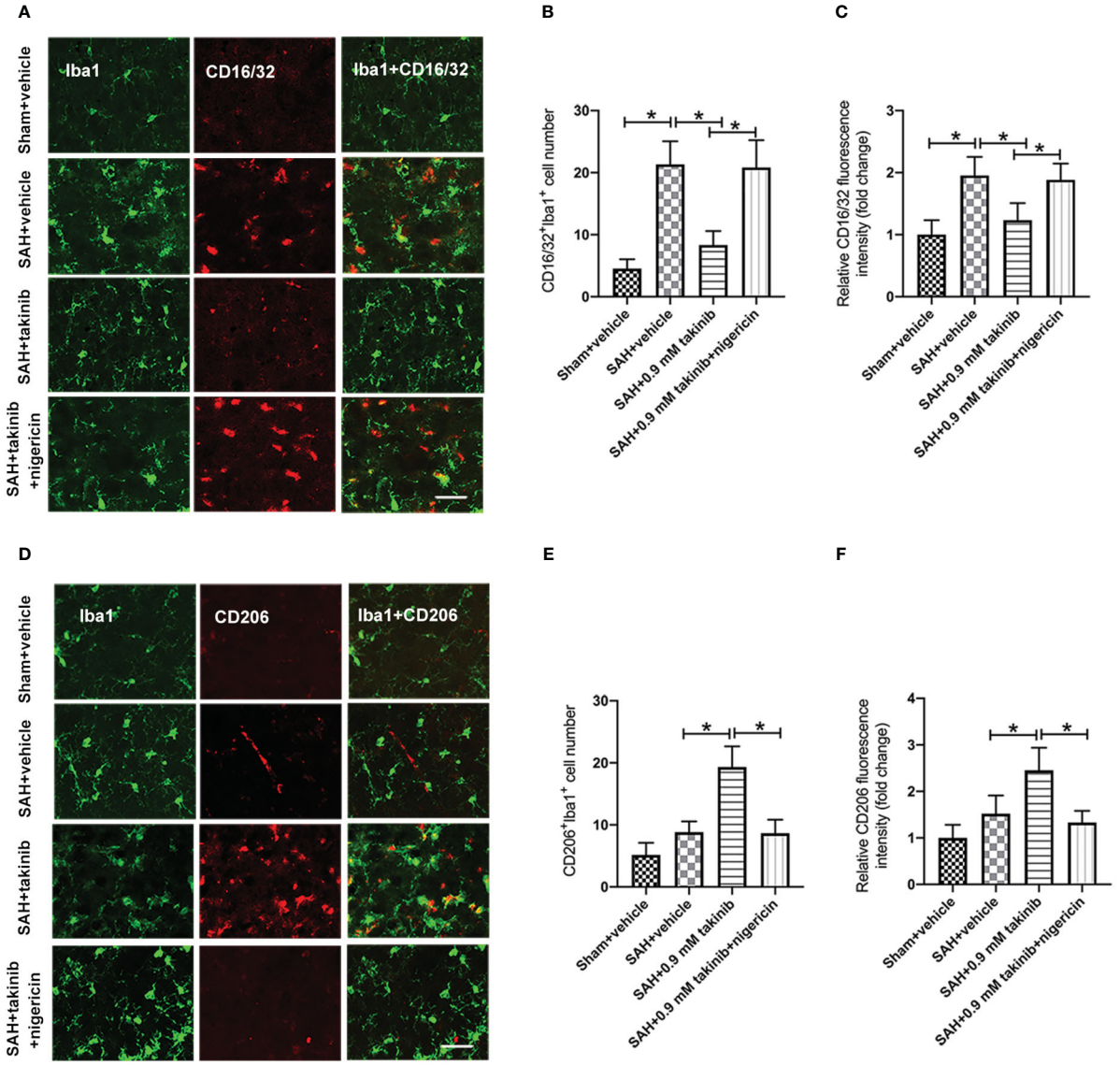

FIGURE 3
Effects of takinib on microglial phenotypic transformation after SAH. **(A)** Double immunofluorescence staining for CD16/32 in microglial in the basal cortex after SAH. **(B, C)** Quantification of CD16/32 immunofluorescence staining in the basal cortex at 24 h after SAH (n = 6 each group). **(D)** Double immunofluorescence staining for CD206 in microglial in the basal cortex after SAH. **(E, F)** Quantification of CD206 immunofluorescence staining in the basal cortex at 24 h after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

SAH. It indicated that SAH insults induced an evident histopathological damage in brain cortex as evidenced by sparse cell arrangements and integrity loss. In contrast, takinib significantly improved histopathological change and reduced neuronal degeneration. In addition, takinib also improved motor function in the early period after SAH. However, all these cerebroprotective effects were abated by nigericin (Figures 6A–D).

4 Discussion

In this study, we verified the beneficial effects of takinib on EBI after SAH. Our data indicated that takinib could ameliorate SAH-induced inflammatory response by inhibiting M1-microglial phenotype polarization and promoting microglial polarization to M2 phenotype. In addition, takinib reduced ROS generation and suppressed oxidative damage after SAH. Concomitant with the reduced neuroinflammation and oxidative stress, takinib improved functional outcome and neuronal survival after SAH. Mechanistically, takinib inhibited TAK1 activation and the subsequent ROS-NLRP3 inflammasome signaling pathway following SAH. In contrast, NLRP3 activation by nigericin abated the neuroprotective effects of takinib against EBI after SAH (Figure 7).

microglial polarization plays a critical role in the pathogenesis of neuroinflammation after SAH (29–31). Activated microglial exhibit different phenotypes under different stimulants and have distinct functions. M1-polarized microglial could increase proinflammatory

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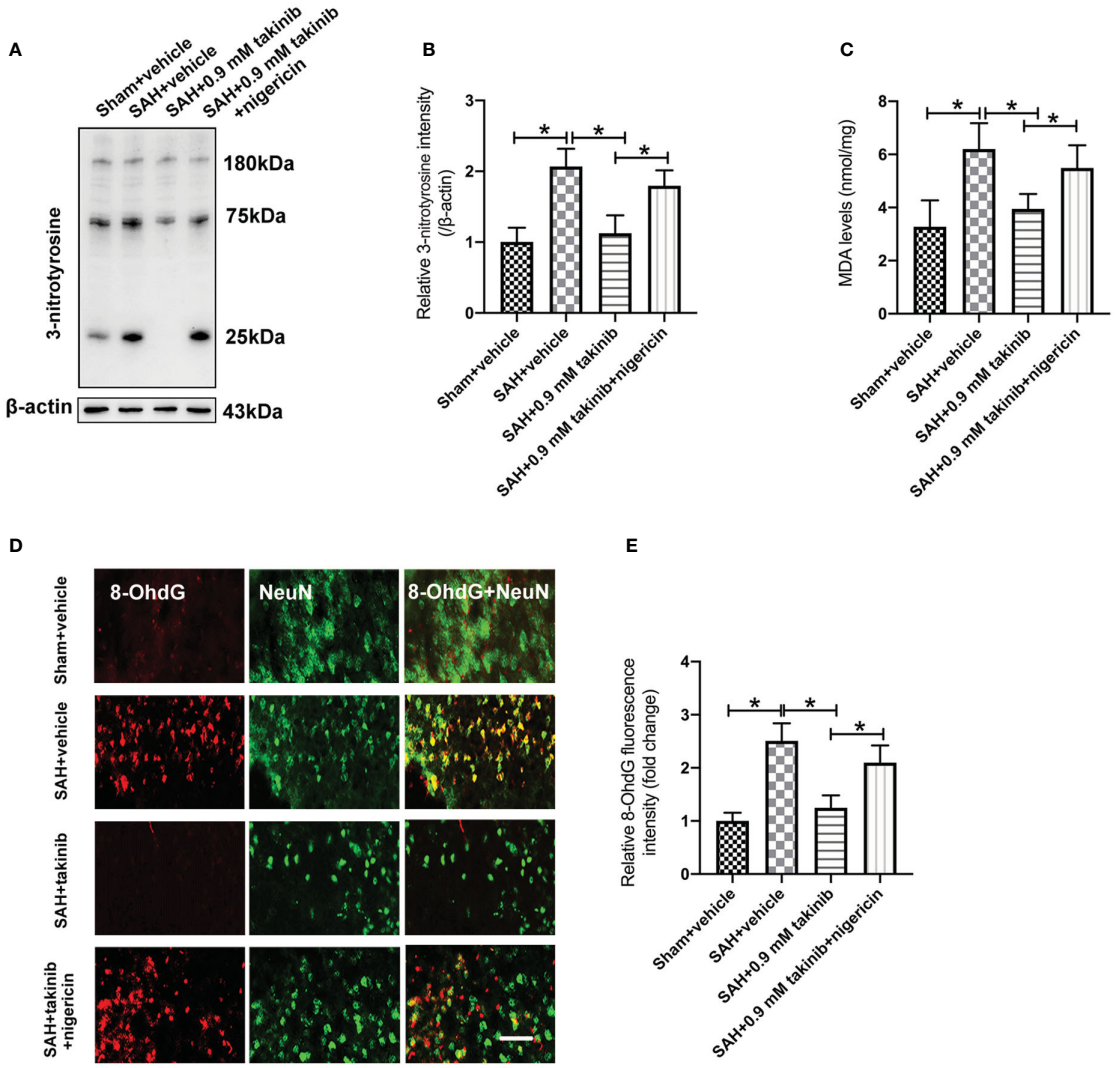

FIGURE 4
Effects of takinib on ROS and oxidative damage after SAH. Western blot assay **(A)** and quantification **(B)** for expression of 3-nitrotyrosine in different groups (n = 6 each group). **(C)** Quantification of MDA level in brain tissue at 24 h post-SAH (n = 6 each group). **(D)** Representative photomicrographs and quantification **(E)** of 8-OhdG immunofluorescence staining in the basal cortex after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

cytokines release and aggravate ROS production. In contrast, M2-polarized microglial produce anti-inflammatory cytokines to keep the immune balance (29, 32). It has demonstrated that inhibition of M1 microglial polarization and induction microglial polarization into M2 could alleviate neuroinflammation and improve neurological outcomes after SAH (29). In addition, oxidative stress also participates in the development of EBI after SAH. Numerous studies have reported that ROS overproduction could induce neuroinflammation to exacerbate brain damage after SAH (1, 24, 33). Therefore, inhibition of M1-like microglial polarization and oxidative damage might be a successful strategy to reduce EBI after SAH.

Multiple studies have proved that TAK1 inhibition is a promising therapeutic application for various CNS diseases including traumatic brain injury (TBI), ischemic stroke, and SAH (34–36). For example, Shen et al. demonstrated that microglial-selective deletion of Tak1 inhibited IL-18 production and ameliorated ischemic stroke injury in prolonged obesity (37). Shi et al. reported that pharmacological inhibition of TAK1 with 5Z-7-oxozeaenol provided long-lasting improvement of stroke outcomes (38). In SAH area, TAK1 inhibition also attenuated EBI by reducing neuroinflammation and neuronal death (34). These indicated that TAK1 might be a promising target for treating SAH. However, the effects of TAK1 inhibition on microglial polarization and oxidative damage after SAH remain unclear.

It has been reported that 5Z-7-oxozeaenol is a potent selective inhibitor of TAK1 and vascular endothelial growth factor receptor 2 (39). Different with 5Z-7-oxozeaenol, takinib is a novel and highly selective TAK1 inhibitor. Recent studies have showed that takinib could effectively inhibit TAK1 activation-mediated inflammatory

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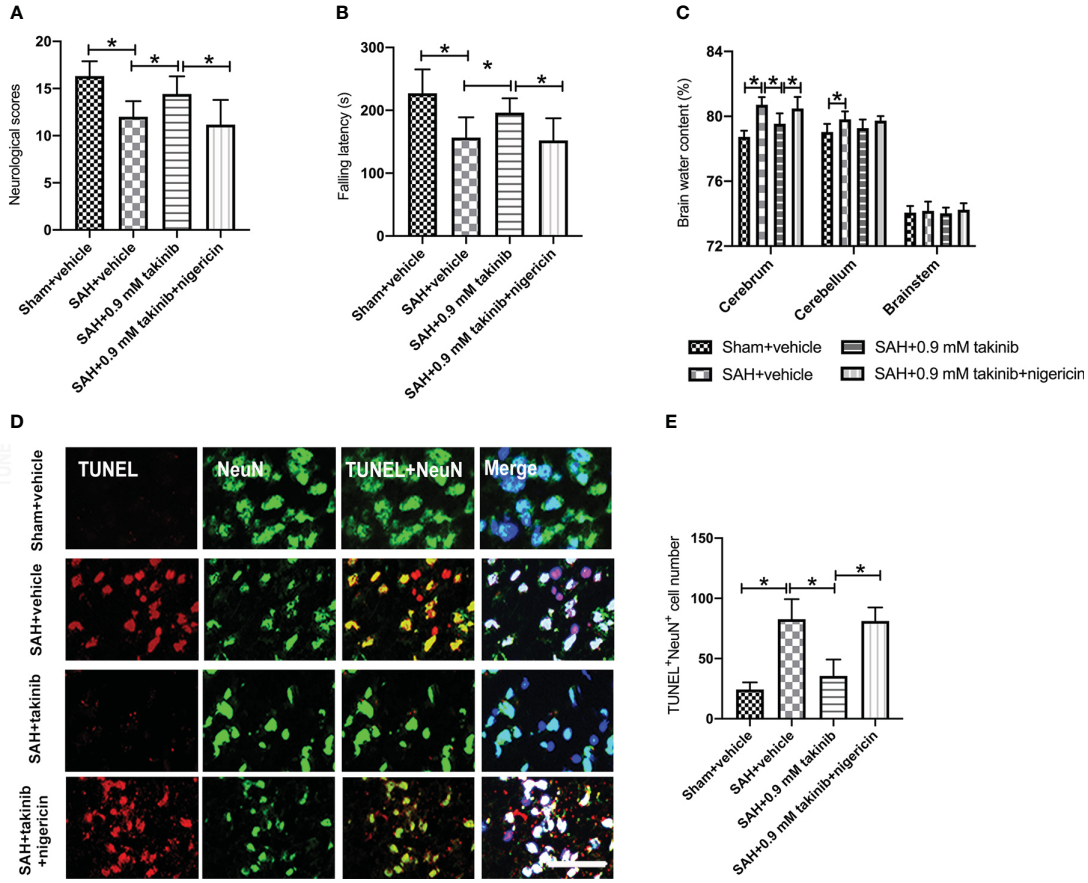

FIGURE 5
Effects of takinib on neurological behavior, brain water content, and neuronal survival after SAH. Quantification of **(A)** neurological deficits scores, **(B)** rotarod performance, and **(C)** brain water content in different groups after SAH (n = 10-12 each group). **(D)** Representative photomicrographs and quantification **(E)** of TUNEL staining in the basal cortex after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

and apoptotic signaling cascades in different research fields (20, 21). However, to date, no study investigated the potential role of takinib on EBI after SAH. In our study, we first evaluated the effects of takinib on TAK1 expression after SAH. In accordance with previous studies, our data revealed that p-TAK1 (Thr 187) was significantly increased in neurons after SAH. In contrast, takinib treatment significantly decreased p-TAK1 expression. We then evaluated the possible influence of takinib on microglial polarization. Intriguingly, it showed that SAH insults significantly induced M1 microglial polarization, which could be inhibited by takinib treatment. Moreover, TAK1 inhibition by takinib increased M2 phenotype microglial. These suggested that TAK1 inhibition by takinib might have the potential to inhibit M1 microglial polarization and promote the microglial phenotype toward M2.

ROS overproduction plays an important role in the development of EBI after SAH. It can disrupt cellular functions by damaging nucleic acids, proteins, and lipids (1). Moreover, ROS production can aggravate neuroinflammation by activation of NLRP3 inflammasome signaling (25, 40). NLRP3 inflammasome has been demonstrated to participate in microglial polarization after SAH. Previous studies have demonstrated that inhibition of ROS/NLRP3 inflammasome signaling could decrease microglial M1 polarization and promote microglial polarization to M2 phenotype (29, 41). Our experiments revealed that TAK1 inhibition by takinib regulated microglial M1-M2 phenotype transition after SAH. However, the possible mechanisms remain elusive. Mounting evidence has showed that TAK1 activation could induce the aggravation of oxidative stress by promoting ROS production (16, 42). Inhibition of TAK1 is able to attenuate ROS overproduction and might be a potential therapeutic target for oxidative stress-related injuries. Interestingly, TAK1 has been verified as a key regulator of NLRP3 inflammasome activation (11, 34, 43). In our experiments, our data showed that takinib successfully inhibited the ROS overproduction and the subsequent activation of NLRP3 inflammasome signaling after SAH. Moreover, nigericin, a NLRP3 activator, abated the protective effects of takinib against EBI after SAH, validating the interaction between TAK1 and NLRP3 inflammasome. Concomitant with the reduced oxidative damage and neuroinflammation, takinib treatment significantly reduced neuronal apoptosis and improved functional behavior after SAH. Together with our experimental results, we provided the evidence that TAK1-

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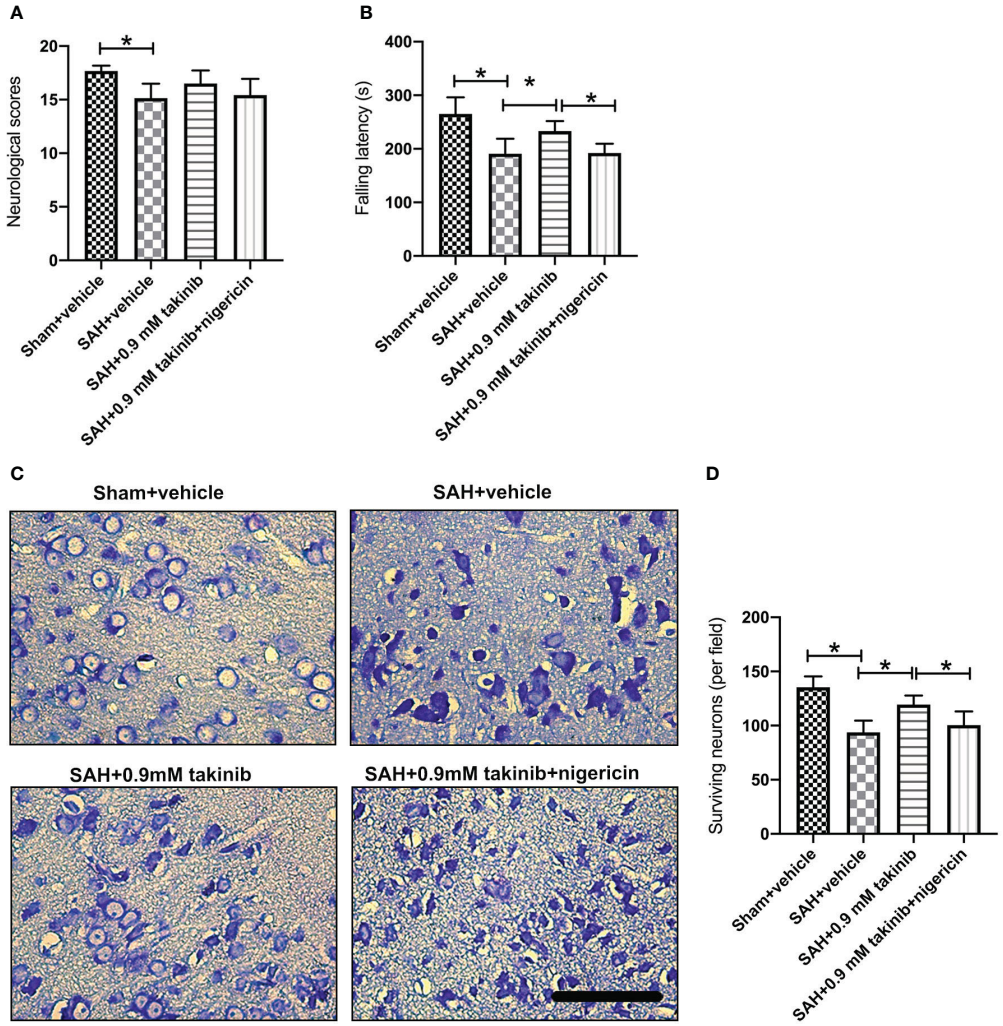

FIGURE 6
Effects of takinib on histopathological change and neurological behavior at day 3 after SAH. Quantification of **(A)** neurological deficits scores and **(B)** rotarod performance in different groups after SAH (n = 6 - 7 each group). **(C)** Representative photomicrographs and quantification **(D)** of survival neurons in the basal cortex after SAH (n = 6 each group). One-way ANOVA with Tukey, bars represent the mean ± SD. *$P < 0.05$.

ROS-NLRP3 inflammasome axis involved in the development of EBI after SAH. By intervening with TAK1-ROS-NLRP3 inflammasome axis, takinib modulated microglial polarization and inhibited oxidative damage.

It should be noted that NLRP3 inflammasome could modulate microglial polarization in a variety of disorders, including intracerebral hemorrhage, depression, ischemic stroke, white matter injury, as well as Alzheimer's disease (44–46). These suggested that targeting NLRP3 inflammasome might be a feasible method to relieve neuroinflammation. In SAH area, Xu et al. reported that TAK1 inhibition by siRNA could reduce NLRP3 inflammasome-mediated neuronal pyroptosis (34). However, the previous studies did not investigate whether TAK1 inhibition affected microglial polarization. In the present study, we demonstrated that TAK1 could affect microglial polarization by modulating NLRP3 inflammasome. Inhibition TAK1 by takinib suppressed NLRP3 inflammasome and decreased M1 microglial polarization. However, how TAK1 regulates NLRP3 inflammasome is not fully investigated. In addition to ROS, K^+ efflux, endosomal rupture, and mitochondrial dysfunction could trigger NLRP3 inflammasome activation (47). Hindi et al. indicated that TAK1 plays a critical role in regulating skeletal muscle mass and oxidative metabolism. TAK1 activation could induce an accumulation of dysfunctional mitochondria as well as oxidative damage in skeletal muscle (19). We suspected that TAK1 might also affect mitochondrial dysfunction to trigger NLRP3 inflammation. But further studies are still needed to decipher this question and whether other molecular targets are involved in this modulation.

Our study has several limitations. Firstly, the long-term effects of TAK1 inhibition in the delayed phase of SAH remains unclear. Meanwhile, no studies have performed to investigate the influence of TAK1 on cerebral vasospasm and delayed ischemic neurologic

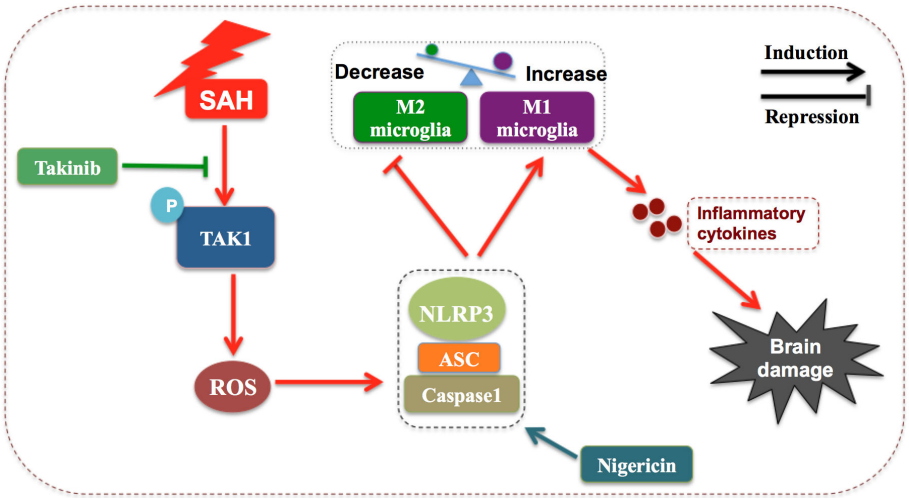

FIGURE 7
Schematic illustrating the possible mechanisms of takinib action after SAH. As illustrated, SAH significantly increases the expression level of p-TAK1 (Thr187) indicating that TAK1 is activated after SAH. TAK1 activation triggers reactive oxygen species (ROS) generation. In response to ROS accumulation after SAH, NLRP3 recruits the adaptor apoptosis-related speck-like protein (ASC) and pro-caspase-1 to form a large multiprotein complex. NLRP3 inflammasome activation promotes microglial phenotype toward M1 and inhibits M2 microglial polarization after SAH, subsequently aggravating neuroinflammation and brain damage. Takinib, a novel and highly selective TAK1 inhibitor, could suppress TAK1 activation and TAK1-mediaed ROS-NLRP3 inflammasome signaling after SAH. In contrast, NLRP3 inflammasome activator nigericin reverses the beneficial effects of takinib against SAH, eventually aggravating SAH-induced brain damage.

deficits after SAH. A recent study by Shen et al. indicated that prolonged high-fat diet (for 32 weeks) feeding-induced obesity has a significant cerebrovascular dysfunction, which is closely associated with microglial TAK1 activation. They showed that microglial TAK1 activation significantly aggravated basilar artery abnormalities. Both pharmacological suppression and genetic microglial-selective TAK1 deletion relieved basilar artery dysfunction and improved the outcome of ischemic stroke (37). On this background, we suspected that TAK1 inhibition might ameliorate cerebral vasospasm and relieve delayed ischemic neurologic deficits in the late phase of SAH. Secondly, the intracerebroventricular administration limits the clinical utility of takinib. Other administration routes instead of ventricle injections should be conducted to validate its clinical translatability. Thirdly, our data indicated that takinib could protect against M1 microglial polarization and oxidative damage after SAH by modulation of TAK1-ROS-NLRP3 inflammasome axis. However, whether these effects are mediated by NF-κB, AMPK, and p38 should be further determined (48, 49). Lastly, in addition to activate NLRP3, nigericin might modulate other signaling targets to aggravate neuroinflammation. Thus, more experiments are needed to solve these questions.

5 Conclusion

In conclusion, we provided the first evidence that takinib could modulate microglial polarization and inhibit oxidative damage after SAH primarily by targeting the TAK1-ROS-NLRP3 inflammasome axis. These findings implied that takinib might be a potential new therapeutic candidate for the treatment of SAH.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Animal Ethics Review Committee of Beijing Friendship Hospital. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WW: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. CP: Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. JZ: Investigation, Methodology, Validation, Writing – review & editing. LP: Methodology, Writing – review & editing. XZ: Methodology, Supervision, Writing – review & editing. LS: Conceptualization, Methodology, Project administration, Writing –

original draft, Writing – review & editing. HZ: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1266315/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Schematic illustration of experiment design.

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CD8$^+$ T cells in brain injury and neurodegeneration

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The interaction between the peripheral immune system and the brain is increasingly being recognized as an important layer of neuroimmune regulation and plays vital roles in brain homeostasis as well as neurological disorders. As an important population of T-cell lymphocytes, the roles of CD8$^+$ T cells in infectious diseases and tumor immunity have been well established. Recently, increasing number of complex functions of CD8$^+$ T cells in brain disorders have been revealed. However, an advanced summary and discussion of the functions and mechanisms of CD8$^+$ T cells in brain injury and neurodegeneration are still lacking. Here, we described the differentiation and function of CD8$^+$ T cells, reviewed the involvement of CD8$^+$ T cells in the regulation of brain injury including stroke and traumatic brain injury and neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), and discussed therapeutic prospects and future study goals. Understanding these processes will promote the investigation of T-cell immunity in brain disorders and provide new intervention strategies for the treatment of brain injury and neurodegeneration.

KEYWORDS

CD8$^+$ T cells, brain injury, neurodegeneration, ischemic stroke, traumatic brain injury, Alzheimer's disease, Parkinson's disease

1 Introduction

The incidence of brain injury and neurodegenerative diseases, which lead to the loss of specific neurons and dysfunction of neuronal networks resulting in impaired cognitive function, behavior, and motor functions, has increased worldwide in recent years (Carroll, 2019; Wareham et al., 2022). For many years, researchers have mainly focused on neurons to elucidate the mechanism of neuronal death, and it has only a few years since researchers have taken an interest in the roles of peripheral immune cells in brain injury. Upon brain injury, inflammation-mediated blood-brain barrier (BBB) damage leads to the recruitment of peripheral immune cells into the brain, which may directly interact with resident brain cells or indirectly release immune mediators to modulate the immune niche and ultimately affect the outcome of brain injury (Iadecola et al., 2020; Salvador and Kipnis, 2022). Similar to brain injury, immune cell-mediated inflammation is considered a hallmark of neurodegeneration. The neuroinflammatory responses mediated by innate and adaptive immunity indeed contribute to the progression of neurodegenerative diseases (NDDs) and regulate neuronal death (Ribeiro, 2023; Wilson et al., 2023). Thus, we summarized the role of CD8$^+$ T cells in both brain injury and neurodegeneration to reveal the similarities and differences underlying the regulation of neuronal death and function which will provide insights into new ways to treat neurological diseases.

At present, it is well established that T cells are involved in brain homeostasis as well as neurological diseases (Ellwardt et al., 2016; Evans et al., 2019). The roles of $CD4^+$ T cells in brain injury and neurodegenerative diseases have been summarized and discussed in many reviews (Iadecola and Anrather, 2011; Berriat et al., 2023). $CD8^+$ T cells are a subpopulation of T lymphocytes that can differentiate into cytotoxic effector T cells when exposed to antigens. In addition to secreting tumor necrosis factor alpha (TNFα) and interferon (IFN)-γ to exert immune regulatory functions, $CD8^+$ T cells can also directly release granzymes and perforins and upregulate the expression of FASL to trigger target cell death (Kaech and Cui, 2012; Reina-Campos et al., 2021). Considering their critical role and translational application in tumor therapies (Jiang et al., 2019; St Paul and Ohashi, 2020), understanding their functional properties and molecular mechanisms of the $CD8^+$ T-cell populations in brain injury and neurodegeneration may facilitate the design of therapies to alleviate diseases.

In this review, we described the properties of $CD8^+$ T-cell differentiation and function, summarized the roles of $CD8^+$ T cells in brain injury, including ischemic stroke and traumatic brain injury (TBI), and neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), and discussed future study goals.

2 Differentiation and function of $CD8^+$ T cells

$CD8^+$ T cells are generated in the thymus and act as important components of adaptive immunity, which play important roles in intracellular pathogen clearance and cancer (Kumar et al., 2018; Tabilas et al., 2023). Naïve $CD8^+$ T lymphocytes can be activated by recognizing peptides presented by major histocompatibility complex (MHC) class I molecules. Under the coordinated activation of signals mediated by antigens, costimulatory molecules, and various cytokines, naïve $CD8^+$ T cells undergo massive expansion and differentiation into various kinds of effector and memory subpopulations, which help to fight against pathogens and exert long-term protection (Mittrücker et al., 2014; Sun et al., 2023).

When $CD8^+$ T cells encounter antigens in an acute inflammatory context, such as bacterial or viral infection, they differentiate into cytolytic effector T cells, also known as $CD8^+$ cytotoxic T lymphocytes (CTLs). $CD8^+$ CTLs can directly secrete granzymes and perforins and enhance the expression of Fas ligand (FASL) to trigger target cell death (Golstein and Griffiths, 2018). In addition, effector $CD8^+$ T cells also secrete tumor necrosis factor α (TNFα) and interferon (IFN)-γ to exert immune functions (Kaech and Wherry, 2007). In tumor immunity, some $CD8^+$ T cells exhibit an exhausted state and become dysfunctional, during which $CD8^+$ T cells upregulate the expression of many inhibitory receptors, such as CTLA4, PD1, TIM3, and LAG3, and lose the ability to produce effector cytokines or cytotoxic molecules (Speiser et al., 2016; Philip and Schietinger, 2022). After pathogen or antigen clearance, most effector T cells die by apoptosis (Badovinac et al., 2004).

After exerting effector function, a small number of antigen-experienced $CD8^+$ T cells survive and remain as memory $CD8^+$ T cells, which can be rapidly reactivated and regain effector functions when re-exposed to antigens (Kaech and Cui, 2012). Currently, heterogeneous populations of $CD8^+$ memory T-cell types have been defined according to their expression of surface markers or functional properties, including central memory (T_{CM}) and effector memory $CD8^+$ T cells (T_{EM}), and tissue-resident memory (T_{RM}) cells (Jameson and Masopust, 2009; Gerlach et al., 2010). $CD8^+$ memory T cell types are identified mainly based on the expression of CD62L and CCR7; T_{CM} cells are mainly $CD62L^{hi}CCR7^{hi}$ and T_{EM} cells exhibit $CD62L^{low}CCR7^{low}$ phenotype. Unlike T_{CM} and T_{EM} cells, which continuously circulate in the peripheral blood (PB), T_{RM} cells mainly reside in the brain and mucosal tissues and have a characteristic of $CD103^{hi}CD69^{hi}$ $CD27^{low}$ phenotype (Kaech and Wherry, 2007; Sheridan and Lefrançois, 2011).

The differentiation and function of $CD8^+$ T cell subsets are orchestrated by transcription factors, epigenetic regulators, and metabolic programs at different tissues upon immune challenge (Kaech and Cui, 2012; Cao et al., 2023). After stimulation, the maintenance of memory $CD8^+$ T cell relies on cytokines including IL-7 and IL-15, which contribute to cell survival and self-renewal of memory $CD8^+$ T cell populations (Surh and Sprent, 2008). The roles of $CD8^+$ T cells in tumor immunity and infectious diseases are well established (St Paul and Ohashi, 2020; Reina-Campos et al., 2021). Recently, more specific $CD8^+$ T cell subpopulations in disease progression or tissue-specific regulation are under investigation with the development of single-cell RNA-sequencing (ScRNA-seq) technologies. Notably, the functions of $CD8^+$ T cells in brain injury and neurodegeneration are largely unknown, which is the focus of this review.

3 $CD8^+$ T cells in brain injury

Acute brain injuries such as ischemic stroke, hemorrhagic stroke and traumatic brain injury (TBI) and chronic autoimmune-induced brain injuries remain a major threat to human health (McKee and Daneshvar, 2015; Stampanoni Bassi et al., 2022). As many excellent reviews have summarized the well-established roles of $CD8^+$ T cells in autoimmune-related multiple sclerosis (Sinha et al., 2015; Brummer et al., 2022), we only review the role of $CD8^+$ T cells in acute brain injuries here. The common pathological aspect of these brain injuries is the occurrence of neuroinflammation (Jayaraj et al., 2019; Morganti-Kossmann et al., 2019). The released damage-associated molecular patterns (DAMPs) of dying cells trigger robust inflammatory responses within the brain that damage the BBB and lead to the infiltration of peripheral immune cells, such as neutrophils, monocytes/macrophages and T cells (Zhang Z. et al., 2022; Bersano et al., 2023). The role of $CD8^+$ T cells in brain injury is being discovered.

3.1 Ischemic stroke

Ischemic stroke caused by intracranial vascular occlusion is a devastating brain injury with considerable mortality and morbidity worldwide. Intravenous alteplase and thrombectomy are not always effective owing to reperfusion-induced injury.

Uncovering the underlying mechanism and alleviating brain damage remain the focus of research (Pan et al., 2007). Many reports have demonstrated the roles and mechanisms of different $CD4^+$ T-cell subsets in both the acute injury phase and long-term functional recovery phase (Zhang Z. et al., 2022; Wang et al., 2023). Similar to $CD4^+$ T cells, $CD8^+$ T cells are also profoundly activated after ischemic stroke, and the accumulation of $CD8^+$ T cells peaks approximately 3–4 days after stroke onset, with cell numbers decreasing overtime (Gelderblom et al., 2009), and of note many studies also showed its existence in the chronic phase (Xie et al., 2019; Ahnstedt et al., 2020). Therefore, $CD8^+$ T cells may participate in different stages of ischemic stroke.

$CD8^+$ T cells can both be detrimental and beneficial for acute ischemic brain injury (**Figure 1**). As cytotoxic lymphocytes, $CD8^+$ T cells can exert a direct cytotoxic effect on neurons. One study showed that depleting $CD8^+$ T cells by using a CD8α blocking antibody alleviates infarct volume and behavioral deficits in both transient middle cerebral artery occlusion (tMCAO) and permanent MCAO ischemia model mice. Correspondingly, adoptive transfer of $CD8^+$ T cells into RAG1-knockout mice increases infarct size. In addition, the transfer of perforin-deficient $CD8^+$ T cells reversed the detrimental effects, while IFNγ-knockout mice failed to alleviate the increased infarction, indicating perforin-mediated neurotoxicity of $CD8^+$ T cells (Mracsko et al., 2014b). Strategies that increase $CD8^+$ T-cell infiltration or cytotoxic function exacerbate ischemic brain injury (Li et al., 2017; Lee et al., 2018; Zhou et al., 2019; Fan et al., 2020). FASL enhances the cytotoxicity of $CD8^+$ T cells to neurons after ischemic stroke. Inactivation of FASL on $CD8^+$ T cells protects mice against neuronal death, which is mediated by compromised the expression of 3-phosphoinositide-dependent protein kinase-1 (PDPK1), a kinase responsible for the cytolytic effect of $CD8^+$ T cells by regulating the phosphorylation of mTOR (Finlay et al., 2012; Fan et al., 2020). IL-15 potently induces the proliferation of memory $CD8^+$ T cells in an antigen-independent manner and augments their effective function (Kim et al., 2008). Lee et al. (2018) showed that IL-15 blockade by using IL-15 knockout mice and an IL-15 blocking antibody reduces brain infarction. This neuroprotective effect is achieved by reducing the activation of $CD8^+$ T cells and NK cells (Lee et al., 2018). Consistently, GFAP-driven IL-15 transgenic mice exhibit increased brain infarction compared with non-transgenic mice owing to the augmented accumulation of $CD8^+$ T cells and NK cells (Li et al., 2017). Moreover, administration of an IL-2 neutralizing antibody alleviates brain infarction and promotes remyelination by limiting $CD8^+$ T-cell infiltration and activation (Zhou et al., 2019). Therefore, blocking the function of cytokines or molecules that are required for the maintenance and the cytotoxicity of $CD8^+$ T cells may be beneficial for the ischemic brain.

Risk factors for ischemic stroke may aggravate brain injury by regulating $CD8^+$ T-cell functions. Perioperative ischemic stroke is one of the most severe complications of surgery and has severe public health implications (Vlisides and Mashour, 2016). Compared to sham mice, mice subjected to ileocecal resection showed an obvious increase in the number of $CD44^{hi}CD62L^{lo}CD8^+$ T effector lymphocytes in peripheral and ischemic brain tissues at Day 7 after ischemic stroke, whereas the number of brain-infiltrating $CD4^+$ T lymphocytes and neutrophils was not significantly different. Further mechanistic study demonstrated that immunometabolite S-2HG accumulates in $CD8^+$ T cells in perioperative stroke mice, promotes proliferation and activation of $CD8^+$ T lymphocytes, and exerts direct neurotoxicity (Zhang F. et al., 2022). Aging is another important risk factor contributing to ischemic stroke (Chen et al., 2010). In the aging brain, a population of $CD8^+$ T cells are present in perivascular and parenchymal regions and exhibit a memory/effector phenotype with high T cell receptor (TCR) expression and show a positive correlation with anti-inflammatory phenotype of microglia which facilitates immune surveillance. However, when the aging mice are subjected to MCAO, these $CD8^+$ T cells contribute to age-related exacerbation of acute ischemic brain injury instead of alleviating it by promoting the production of proinflammatory cytokines as well as recruitment of peripheral leukocytes (Ritzel et al., 2016). Therefore, $CD8^+$ T cells may have opposing roles in homeostasis and ischemic stroke progression of the aging brain.

In addition to their detrimental effect, recent studies also discovered a subset of regulatory $CD8^+$ T cells that can exert an early protective effect. Bodhankar et al. (2015) discovered that transfer of IL-10-positive B cells into MCAO mice at an early time point leads to the generation of a dominant regulatory Treg population ($IL\text{-}10^+$ $CD8^+$ $CD122^+$) both in the ischemic brain and spleen. Coincidentally, Cai et al. (2022) showed that during the early stage of brain ischemia, the upregulated expression of CXCL10 interacts with CXCR3 on $CD8^+CD122^+CD49d^{lo}$ T regulatory-like cells ($CD8^+$ TRLs) to increase their infiltration in the brain. Interestingly, these recruited $CD8^+$ TRLs are reprogrammed to upregulate leukemia inhibitory factor (LIF) receptor and exert neuroprotection through direct neuronal protection via promoting the expression of epidermal growth factor-like transforming growth factor (ETGF), and indirect anti-inflammatory effect through increasing the production of interleukin 10 (IL-10) (Cai et al., 2022). Thus, transfer of this regulatory CD8 T-cell subset may offer new perspectives to protect the brain from acute injuries. In addition, with the rapid development of Sc-RNA-seq and cytometry by time-of-flight (CytOF) technologies (Zhang et al., 2020; Jovic et al., 2022), the characteristics and functions of various $CD8^+$ T-cell subsets in the acute phase and chronic phase will be clarified in the future.

In addition to the important function of $CD8^+$ T cells in the acute phase of ischemic stroke, the role of $CD8^+$ T cells in the chronic phase is also beginning to be exposed. One study found that $CD8^+$ T cells remain at a higher number in the chronic phase and worsen functional recovery by increasing the infiltration of other immune cells, such as B cells, neutrophils and monocytes, to promote neuroinflammation (Selvaraj et al., 2021). For many decades, multiple studies have shed light on the molecular mechanisms that regulate neurological recovery during the weeks after ischemic stroke (Carmichael, 2006; Cramer and Riley, 2008; Zhang Z. et al., 2022). Considering the existence of $CD8^+$ T cells during the chronic recovery phase of ischemic stroke, it will be interesting to reveal how these sustained $CD8^+$ T cells communicate with brain-resident cells to regulate functional aspects of recovery such as neurogenesis, oligodendrogenesis and neuronal regeneration in the long term. In addition, what signal determines whether $CD8^+$ T cells exert a protective effect or detrimental effect in homeostasis or during disease progression remains unknown.

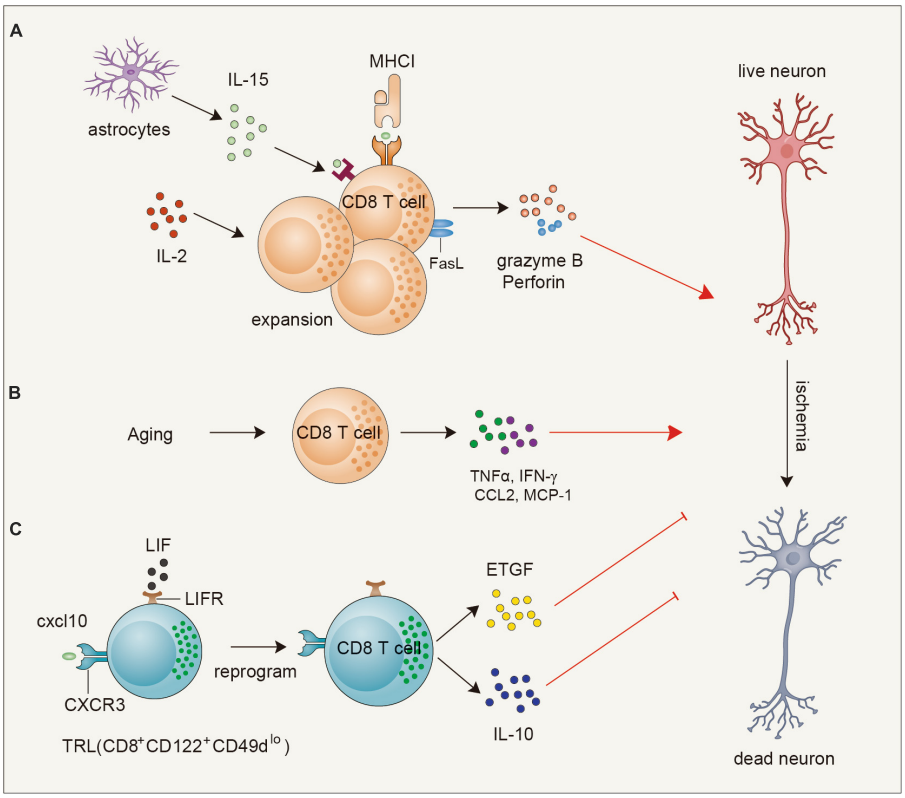

FIGURE 1
Functions and mechanisms of CD8$^+$T cells in ischemic brain injury. **(A)** Astrocyte-derived IL-15 or endogenous IL-2 can promote the expansion and activation of CD8 + T cells to secrete granzyme B and perforin or upregulate FASL to exacerbate ischemic neuronal death. **(B)** During the aging process, CD8$^+$ T cells are triggered to secrete TNFα, IFN-γ, CCL2, and MCP-1 to contribute to neuronal death. **(C)** A population of CD8$^+$ T cells, namely T regulatory-like cells (TRL, CD8$^+$CD122$^+$ CD49d^{lo}) infiltrate into the brain at early stages of ischemic stroke and are reprogrammed to upregulate leukemia inhibitory factor (LIF) receptor and induce epidermal growth factor-like transforming growth factor (ETGF), and interleukin 10 (IL-10) expression to exert neuroprotection through direct neuron protection or indirect anti-inflammatory effect. IL-2, interleukin 2; FASL, Fas ligand; TNFα, tumor necrosis factor alpha; IFN-γ, interferon-gamma; TRL, T regulatory-like cells; LIF, leukemia inhibitory factor; ETGF, epidermal growth factor-like transforming growth factor.

3.2 Hemorrhagic stroke

Hemorrhagic stroke which includes the subtypes of intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) has considerable morbidity and mortality rates. Similar to ischemic stroke, inflammation and the host immune response contribute to the pathophysiology of hemorrhagic stroke (Montaño et al., 2021; Nasa, 2022; Ohashi et al., 2023). After hemorrhagic stroke, the BBB is damaged and peripheral immune cells infiltrate into the brain. The function of peripheral infiltrated immune cells in hemorrhagic stroke has been studied, although less than ischemic stroke (Mracsko et al., 2014a; Li and Chen, 2023). Previous studies have observed the increase of CD8$^+$ T lymphocytes in the early phase of ischemic stroke and last for 21 or 28 days (Xue and Del Bigio, 2003; Mracsko et al., 2014a). Considering the regulatory role of CD8$^+$ T cells in ischemic stroke, the function of CD8$^+$ T cells in hemorrhagic stroke needs investigation in the future.

3.3 Traumatic brain injury

Traumatic brain injury (TBI) remains a substantial cause of morbidity and mortality in both adults and children. TBI involves complex neurological processes, including acute molecular changes and long-term neurocognitive sequelae (Bramlett and Dietrich, 2015; Taylor et al., 2017). Several studies have revealed the involvement of T cells in both the acute phase and chronic phase of TBI (Bao et al., 2021; Xu et al., 2021). Compared with the study of CD4$^+$ T cells in ischemic stroke, the study of CD8$^+$ T cells in TBI is still limited.

CD8$^+$ T cells can infiltrate the brain and accumulate in the TBI site (Ling et al., 2006). CD8$^+$ T cells exert a detrimental effect via their cytotoxic effect in the acute damage stage. By using a model of TBI, Ling et al. (2006) observed that astrocytes are activated and produce IL-15, which triggers CD8$^+$ T-cell activation and the release of granzyme B. The released granzyme B can in turn act on neurons and induce neuronal apoptosis by caspase-3-induced PARP cleavage (Wu et al., 2021). Pituitary adenylate cyclase activating polypeptide can protect mice from TBI-induced injury by balancing CD4$^+$ and CD8$^+$ T-cell ratios and functions (Hua et al., 2012). In addition to aggravating acute brain injury, traumatic brain injury (TBI) also results in myelin-related pathology and long-term disabilities in many survivors. At 8 weeks after TBI, the number of effector/memory CD8$^+$ T cells is increased in the injured brain and these CD8$^+$ T cells release granzyme B, which precedes Th17 cell infiltration, and is

associated with an elevated autoantibody response and progressive impairment of neurological functions. Genetic deletion of $CD8^+$ T cells by using β2-microglobulin-deficient mice or pharmacological depletion of $CD8^+$ T cells by using a $CD8^+$ blocking antibody improves neurological outcomes and produces a neuroprotective Th2/Th17 immunological shift. However, the deletion of $CD4^+$ T cells does not have this effect, and the depletion of B cells results in even more severe neurological dysfunction, demonstrating the specific effects of $CD8^+$ T cells in regulating long-term functional impairment after TBI (Daglas et al., 2019). Despite the above reports of $CD8^+$ T cells in the acute injury phase and chronic recovery phase of TBI (**Figure 2**), more questions still exist. For example, do $CD8^+$ T cell populations function differently during TBI progression? Can $CD8^+$ T cells directly interact with brain-resident cells to affect TBI progression? What is the difference in the cytokine profile of $CD8^+$ T cells during different stages of TBI? Which factors recruit $CD8^+$ T cells into the brain after TBI? Resolving these questions may provide important insights to increase the efficiency of TBI treatment using immunotherapy.

Like ischemic stroke, TBI also induces peripheral immunosuppression which is detrimental, as it increases the incidence of hospital-acquired infections (HAIs) (Ritzel et al., 2018; Sribnick et al., 2022). Mechanistically, this peripheral immunosuppression has been attributed to a disturbance in the well-balanced bidirectional communication between the brain and the immune system owing to injury (Hazeldine et al., 2015; Sribnick et al., 2022). One study showed that TBI induces the activation of the sympathetic nervous system, which triggers the expression of PD-1, a marker of T-cell exhaustion, on $CD4^+$ and $CD8^+$ T cells leading to immunosuppression. Inhibition of sympathetic nervous system by propranolol reverses this effect *in vivo* (Yang et al., 2019). More studies are needed to gain a better understanding of the mechanisms underlying TBI-induced changes in the immune system and how to manipulate these changes to treat hospital-acquired infections after TBI.

Notably, it is important to be aware of the specific staining of $CD8^+$ T cells when investigating $CD8^+$ T cells as one study found that a population of macrophage/microglia also expresses CD8 after TBI and localizes in the border of the pannecrosis, which may have a role in lesion development after TBI (Zhang et al., 2006). How to distinguish these cells to avoid false conclusions should be given immediate attention although why other immune cells also express CD8 remains unknown.

3.4 Other kinds of brain injuries

In addition to acute stroke-induced brain injury and TBI, other kinds of less frequent brain injuries also exist. Radiation-induced brain injury (RIBI) remains one of the most common medical complications of brain radiation therapy. In addition to the acute adverse effects of radiation exposure, cranial radiotherapy also results in severe and delayed onset of brain injury as well as cognitive impairment. Uncontrolled progressive development of brain lesions can eventually result in cerebral herniation and even death (Ali et al., 2019). However, the underlying mechanism of delayed RIBI is poorly understood. Notably, peripheral lymphocytes infiltration has been reported as a common feature in the necrotic brain area of patients with RIBI (Kureshi et al., 1994; Yoritsune et al., 2014), suggesting the involvement of adaptive immunity in the development of RIBI. Recently, one study found that $CD8^+$ T cells infiltrate and expand in the lesioned brain tissue of RIBI patients by using Sc-RNA-seq and T-cell receptor sequencing techniques. In addition, these infiltrated T cells are in an activation state as granzyme B and perforin are secreted from cytotoxic $CD8^+$ T cells in the CSF of RIBI patients. Further mechanistic studies showed that microglia-derived chemokines CCL2/CCL8 mediate the infiltration of $CCR2^+/CCR5^+$ $CD8^+$ T cells in the brain parenchyma. Inhibition of CCL2 and CCL8 function by conditional knockout mice and neutralization antibodies alleviate RIBI as well as ischemic brain injury (Kumari and Gensel, 2023; Shi et al., 2023). This study reveals the participation of microglial-mediated activation of $CD8^+$ T cells in brain damage and provides direct evidence of brain-immune communications in the regulation of RIBI.

Perinatal brain injury influences infants born at gestational ages and may lead to substantial long-term neurodevelopmental impairment, including cognitive, neurological, sensory and motor disabilities (Novak et al., 2018; Reiss et al., 2022). Perinatal brain injury can be triggered by hypoxia-induced ischemia, maternal infection or inflammation that elicits a cascade of events potentiating perinatal brain injury (Novak et al., 2018; Leavy and Jimenez Mateos, 2020). Several studies found an increase in $CD8^+$ T cell numbers in the placenta following maternal intrauterine inflammation (IUI). Maternal CD8 T-cell-depletion or maternal administration of bone marrow-derived mesenchymal stem cells (BMMSCs) results in a decreased number of placental $CD8^+$ T cells which reduces perinatal brain injury and improves neurological outcomes in the offspring (Lei et al., 2018; Zhao et al., 2020). Future studies are needed to reveal neuroinflammatory processes and design therapeutic strategies to alleviate perinatal brain injury.

4 $CD8^+$ T cells in neurodegeneration

Neurodegenerative diseases (NDDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are characterized by the processive accumulation of protein aggregates and the loss of neurons in specific brain regions. For example, in AD, the aggregates are intracellular Tau and extracellular Aβ and hippocampal as well as cortical neurons are impacted. In PD, the alpha-synuclein and TDP-43 are intracellular aggregates and the neurons in the midbrain substantia nigra are impacted (Dugger and Dickson, 2017; Berriat et al., 2023). For many years, researchers have focused on neurons to reveal the underlying mechanism of neuronal loss. Only in the past few years, has multiple pieces of evidence revealed the critical role of peripheral immune cells in the regulation of the pathogenesis of NDDs. Immune cell-mediated inflammation is now considered a hallmark of NDDs (Wilson et al., 2023). However, whether immune cell-mediated inflammation is a consequence or cause of PD remains unknown (Appel, 2012). It is widely accepted that the neuroinflammatory responses mediated by innate and adaptive immunity indeed contribute to the progression of NDDs and mediate neuronal death. As an important subpopulation of T cells, the role of $CD8^+$ T cells in NDDs is being recognized.

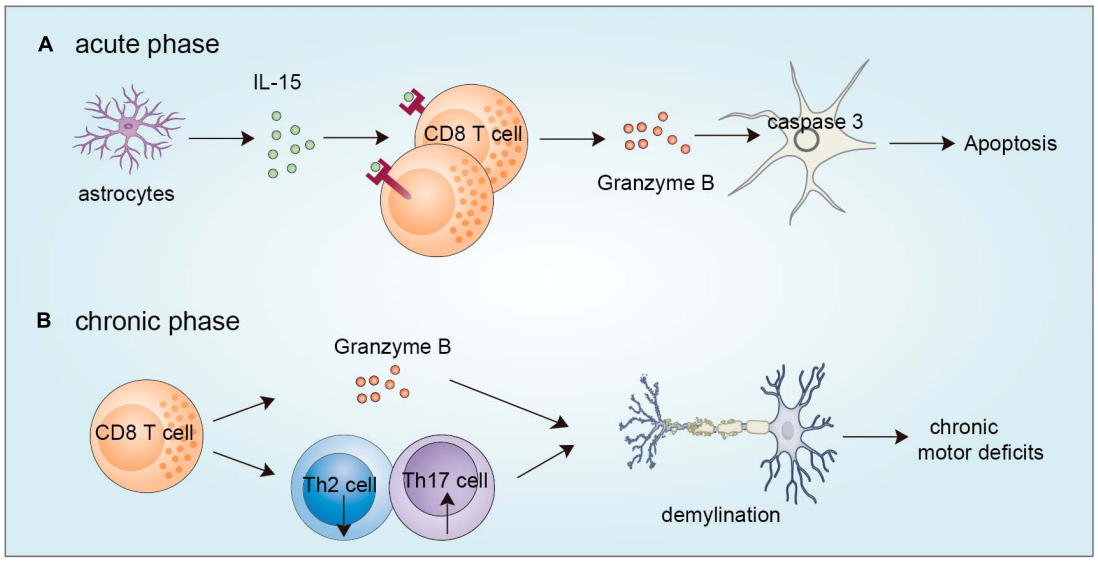

FIGURE 2
Functions of CD8$^+$ T cells in TBI. **(A)** During the acute phase, astrocytes are activated to produce IL-15, which evokes CD8$^+$ T-cell activation to release granzyme B, which in turn acts on neurons and leads to caspase 3-induced apoptosis. **(B)** During the chronic phase, brain-infiltrating CD8$^+$ T cells can secrete granzyme B and regulate Th2/Th17 shift to accelerate demyelination. TBI, traumatic brain injury.

4.1 Alzheimer's disease (AD)

In patients with AD, the immune cell diversity and activation states are altered, indicating their involvement in the progression of AD. Early studies suggested a decreased proportion of CD8$^+$ T cells in AD patients (Pirttilä et al., 1992; Shalit et al., 1995), and some observed low expression of the costimulatory molecules CD28 (Speciale et al., 2007). However, other studies have found no significant changes in CD8$^+$ T-cell frequency in the peripheral blood between mild AD patients and healthy controls (Larbi et al., 2009). These contrasting results may result from samples collected from patients in different stages of AD and testing methods used, as some macrophages and NK cells are also observed to express CD8 in some conditions (Popovich et al., 2003; McKinney et al., 2021). Thus, researchers should be cautious when testing the existence of CD8$^+$ T cells, and add other co-expression markers of T cells to draw a solid conclusion. With the development of mass cytometry and ScRNA-seq technologies, some studies have observed an increase in CD8$^+$ T cells numbers in the peripheral blood (PB) and CSF of AD patients and characterized the phenotype of CD8$^+$ T cells (Schindowski et al., 2007; Lueg et al., 2015; Gate et al., 2020). Gate et al. (2020) found an increased number of CD3$^+$CD8$^+$CD27$^-$ T effector memory CD45RA$^+$ (TEMRA) cells in the mononuclear cells of peripheral blood by using mass cytometry. These cells are negatively associated with cognitive function. In addition, the CD8$^+$ T cells in the cerebrospinal fluid of AD patients are clonally expanded and antigen-specific cells (Gate et al., 2020; Heneka, 2020; McManus and Heneka, 2020). This study raises the question of what antigen brain-infiltrating CD8$^+$ T cells recognize and mediate its clonal expansion. Interestingly, Gate et al. (2020) found some Epstein-Barr virus (EBV)-specific clones of CD8$^+$ T cells, although they are not highly expanded clones. EBV DNA is present in approximately six percent of AD brains (Carbone et al., 2014). In addition to EBV, cytomegalovirus (CMV) is a member of the herpesvirus family that is prevalent throughout the world. Approximately seventeen percent of patients with AD are positive for human herpes virus (Carbone et al., 2014). A reduced frequency of CMV-specific CD8$^+$ T cells is present in AD patients compared with other CMV-positive patients (Westman et al., 2013). Based on this evidence, we cannot define the direct relationship between viral infections and the progression of AD, as virus-induced immune response is commonly present in elderly people. Future studies are needed to investigate whether other antigen-specific T cell clones exist and participate in the pathogenesis of AD. Interestingly, in PD patients, peptides derived from α-synuclein can be presented to CD8$^+$ T cells and leads to its activation (Sulzer et al., 2017; Hobson and Sulzer, 2022). Whether Aβ can generate peptides to specifically activate CD8$^+$ T cells needs further investigation. In addition, if antigen specific CD8$^+$ T cells are identified, it will be interesting to further test whether they are derived in the brain or in the peripheral immune system. Comparing the antigen specificity of T cells may help identify some T cell specific subpopulations which may benefit both diagnosis and treatment.

In addition to the alteration of CD8$^+$ T cells in the PB and CSF, the presence of CD8$^+$ T cells is also observed in the brains of both AD patients (Itagaki et al., 1988; Merlini et al., 2018; Unger et al., 2020) and AD model mice (Laurent et al., 2017; Unger et al., 2020; Michael et al., 2021; Fernando et al., 2023). The substantial infiltration of CD8$^+$ T cells in the brain raises a question of what signal mediates CD8$^+$ T cell infiltration into the brain. One study indicated that pro-inflammatory cytokine IL-17 may mediate the enrichment of CD8$^+$ T cells. IL-17 was expressed in the cortex and hippocampus of APP/PS1 model mice. IL-17 enhances the production of chemokines CXCL1 and CXCL9/10 from glial cells to trigger the infiltration of myeloid cells and CD8$^+$ T lymphocytes (Ye et al., 2023). Then, comes another question. Which cells release IL-17? As Aβ immunization promotes

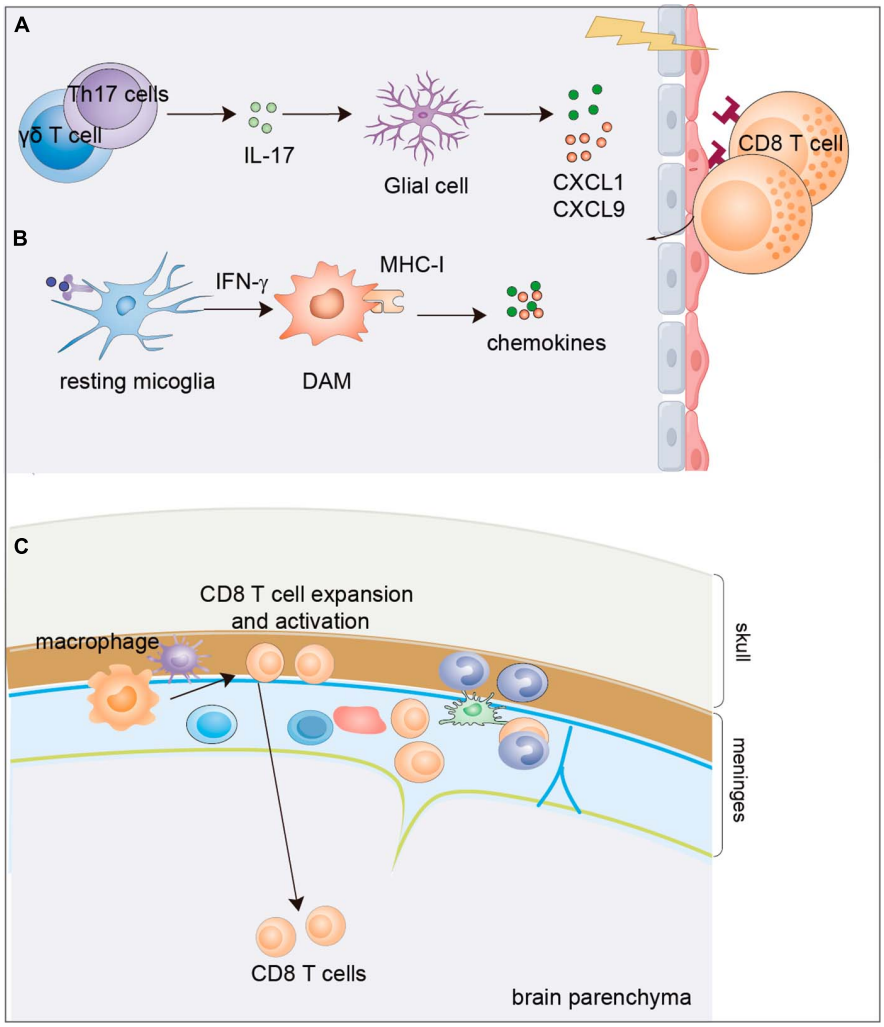

FIGURE 3
Mechanisms of $CD8^+$ T-cell infiltration into the brain parenchyma of AD. **(A)** IL-17 cytokine possibly produced by Th17 cells or γδ T cells was observed to express in the cortex and hippocampus of APP/PS1 mice which enhances the production of chemokines CXCL1 and CXCL9/10 from the glial cells to trigger the infiltration of $CD8^+$ T lymphocyte. **(B)** Disease associated microglia can be induced by cytokines such as IFN-γ to release chemokines and cytokines to recruit $CD8^+$ T cells. **(C)** Activation of meningeal $CD8^+$ T cells by meningeal macrophage facilitates their infiltration into the parenchyma. DAM, disease associated macroglia; AD, Alzheimer's disease.

IL-17 production by T helper 17 (Th17) cells (McQuillan et al., 2010; Zhang et al., 2013), one possibility is that the upregulated expression of IL-17 may result from elevated Th17 cell numbers. In addition, γδ T cells are regarded as one of the bridges connecting innate and adaptive immunity, and they can quickly secrete cytokines, especially IL-17 and IFNγ, after activation (Bonneville et al., 2010). More importantly, a subset of IL-17-producing γδ T cells populates the meninges in normal brains (Ribeiro et al., 2019). These evidence indicate that γδ T cells may also contribute to IL-17 production in the brain to incite neurodegeneration as an early response. In addition, brain-resident microglia also release chemokines and cytokines to recruit $CD8^+$ T cells. In human and mouse brains with tauopathy, parenchymal microglia exhibit a disease-associated phenotype with increased expression levels of MHC-I and inflammatory chemokines and cytokines, which promote the recruitment of peripheral T cells into the parenchyma and drive neurodegeneration (Chen et al., 2023). The important role of meningeal immunity is now being recognized. One study provides evidence that the activation of meningeal $CD8^+$ T cells facilitates their infiltration into the parenchyma. $CD8^+$ T cells are highly activated in the meninges and brains of patients with NDDs, and undergo clonal expansion which may further potentiate the infiltration of $CD8^+$ T cells into the parenchyma and exacerbate brain degeneration (Hobson et al., 2023; **Figure 3**).

$CD8^+$ T cells regulate the progression of AD (**Table 1**). One study used a CD8 blocking antibody to deplete $CD8^+$ T cells in APP-PS1 mouse models, and neither cognitive outcome nor plaque pathology was altered after depletion of $CD8^+$ T cells. However, the expression of several neuronal and synapse-related genes, including activity-regulated cytoskeleton-associated protein (Arc) and neuronal PAS domain protein 4 (Npas4) was altered in the hippocampus in the presence of a CD8 blocking antibody (Unger et al., 2020). In addition, $CD8^+$ T cells are present in the hippocampus of tau transgenic mice and the cortex of patient brains with P301L tau mutation.

TABLE 1 Evidence of CD8$^+$T cells in neurodegeneration.

Neurodegenerative diseases		Main evidence of CD8$^+$ T cell infiltration and effects on neurodegeneration
AD	Infiltration	PB or CSF of AD patients: decreased proportion (Shalit et al., 1995; Speciale et al., 2007; Larbi et al., 2009); no difference (McKinney et al., 2021); increased number (Schindowski et al., 2007; Gate et al., 2020; Heneka, 2020); increased number of effector/memory CD8$^+$ T cells (Heneka, 2020). Brain tissue of AD patients: present in the brain (Itagaki et al., 1988; Merlini et al., 2018; Hobson and Sulzer, 2022). Mice model: accumulated in the mouse brain (Laurent et al., 2017; Merlini et al., 2018; Unger et al., 2020; Michael et al., 2021)
AD	Effects	Blocking CD8 no alternation of cognitive outcome or plaque pathology (Merlini et al., 2018); Blocking CD3 improves spatial memory (Unger et al., 2020); β2m-deficiency alleviates AD (Fernando et al., 2023); β2m-deficiency exacerbates AD (Su et al., 2023).
PD	Infiltration	PB or CSF of PD patients: CD4 to CD8 ratio changes (He et al., 2022; Fu et al., 2023); decreased number (Baek et al., 2016); CD8$^+$ T cell terminal effector phenotype (Marsh et al., 2016), with high expression of TNFα (Wang P. et al., 2021; Heming et al., 2022) and show immunosenescence (Hisanaga et al., 2001; Chen et al., 2021). Brain tissue of PD patients: significant infiltration in the SN (Williams-Gray et al., 2018; Kouli et al., 2021; Jiang et al., 2023). Mice model of PD: present in the PD brains (Brochard et al., 2009; Galiano-Landeira et al., 2020).
PD	Effects	MHC-I knockdown suppresses dopaminergic neuronal loss (Thakur et al., 2017); RAG-KO alleviates 6-OHDA-induced PD (Williams et al., 2023); CD8$^+$ T cells depletion prevents colitis-induced suppression of dopaminergic markers (Ip et al., 2015).
ALS	Infiltration	PB or CSF of ALS patients: show a clonally expanded effector memory CD8$^+$ T cells (Shi et al., 2007; Fiala et al., 2010; Houser et al., 2021; Campisi et al., 2022; Yazdani et al., 2022), secrete increased granzyme B, IL-17, and IL-13 (Shi et al., 2007; Fiala et al., 2010; Yazdani et al., 2022). Mice model of ALS: present in the ALS brains (Campisi et al., 2022).
ALS	Effects	Depletion of CD8 T cell promotes the survival of motor neurons (Nardo et al., 2018; Coque et al., 2019).

CD3 blocking antibody inhibits T-cell infiltration in the tau transgenic mice and improves spatial memory impairment (Laurent et al., 2017). Administration of the leukotriene receptor antagonist montelukast (MTK) in 5XFAD mice improves cognitive function. Mechanistically, MTK treatment increases the number of Tmem119$^+$ microglia and decreases the expression level of AD-associated and lipid droplet accumulation-related genes, which reduces infiltration of CD8$^+$ T cells into the brain parenchyma (Michael et al., 2021). Therefore, factors or molecules that can specifically modulate CD8$^+$ T-cell expansion and function can be evaluated in AD models. Interestingly, a disease-associated CD8$^+$ T-cell population is present in the aged AD mouse brains on a 5XFAD background. These cells express high levels of immune-responsive genes (ISGs) at the transcriptional and epigenetic levels, which are normally induced by interferons (IFNs) (Fu et al., 2023). Depletion of CD8$^+$ T cells by using a β2m-deficient mouse leads to a reduction in amyloid-β plaque burden and improves spatial memory in AD model mice (Fernando et al., 2023).

Previous studies on the effects of T cells suggest that T cells may also have beneficial effects on the clearance of Aβ aggregates and improve cognitive behavior. Recently, one study showed that depletion of CD8$^+$ T cells using a β2m-deficient mouse or impairing the accumulation or clonal expansion of CD8$^+$ T cells by CCR6-deficiency increased Aβ deposition and cognitive impairment in 5XFAD mice by restraining the proinflammatory response of microglia (Su et al., 2023), which is in contrast with Fernando et al. (2023) study that also used β2m-deficient 5XFAD mice. In addition, the adoptive transfer of regulatory T cells into 3xTg transgenic AD mice reduces Aβ aggregate deposition and improves cognitive deficits by decreasing inflammatory cytokine production and microglial activation (Baek et al., 2016). Mice on a 5XFAD background with lymphocytes deficiency (RAG2-KO) exhibited greater Aβ deposition than that in control mice as a result of a reduction in microglia-mediated phagocytosis (Marsh et al., 2016). The studies indicate the complex and context-dependent roles of CD8$^+$ T cells in the pathogenesis of AD. Strategies aimed at modulating CD8$^+$ T-cell function to treat AD should distinguish the beneficial and detrimental roles of different CD8$^+$ T cell subpopulations.

4.2 Parkinson's disease (PD)

Similar to AD, many studies have reported an alteration in T lymphocytes in the peripheral blood (PBs) and CSF of PD patients (Baba et al., 2005; Wang P. et al., 2021; He et al., 2022; Heming et al., 2022). Early studies showed an increased or decreased ratio of CD4 to CD8$^+$ T cells in the PBs and CSF of PD patients (Hisanaga et al., 2001; Baba et al., 2005). Further mapping indicates that the number of naïve CD8$^+$ T cells is reduced (He et al., 2022) and that a large population of CD8$^+$ T cells continuously progress from central memory to terminal effector T cells in the blood and CFS of PD patients based on single-cell transcriptome expression combined with TCR-sequencing (Wang P. et al., 2021). Moreover, these CD8$^+$ T cells are activated and show high expression of TNFα (Chen et al., 2021; Jiang et al., 2023). During the early stages of PD, the population of terminally differentiated effector CD8$^+$ TEMRA T cells, the hallmarks of immunosenescence, in peripheral blood is reduced and PD patients exhibits downregulated expression of senescence-related markers such as p16^{INK4a} compared with age and sex-matched healthy controls (Williams-Gray et al., 2018; Kouli et al., 2021). Whether immunosenescence contributes to the development and progression of PD deserves further investigation.

Evidence of the presence of CD8$^+$ T cells in the brain is also increasing. CD8$^+$ T cells infiltrate the substantia nigra pars compacta (SNpc) in patients during the early stage of PD before α-synuclein aggregation, which leads to neuronal death and synucleinopathy (Brochard et al., 2009; Galiano-Landeira et al., 2020; Halliday, 2020). Moreover, CD4 and CD8$^+$ T cells are also present in the brains of PD model mice (Thakur et al., 2017).

In an age-dependent PD model mice with genetic A30P/A53T double-mutated α-synuclein, infiltrated $CD8^+$ and $CD4^+$ T cells are correlated with the loss of dopaminergic neurons in the late stage of PD progression. A high proportion of infiltrated $CD8^+$ T cells are tissue-resident memory T cells that express cytolytic granzymes and proinflammatory cytokines (Rauschenberger et al., 2022). This poses the question of how $CD8^+$ T cells are activated in the brain, and what antigens they recognize.

Increasing evidence indicates that neurons can express MHC-I to promote antigen presentation to $CD8^+$ T lymphocytes and leads to its activation. The neurotoxin MPTP induces oxidative stress in dopaminergic neurons which further upregulates the expression of MHC-I. The presentation of MHC-I is positively correlated with the number of $CD8^+$ T cells (Wang B. Y. et al., 2021). Another *in vitro* study showed that MHC-I molecules can be induced in neurons by IFNγ released from microglia, activated by neuromelanin or alpha-synuclein, which present antigens to $CD8^+$ T cells and mediate neurotoxicity (Cebrián et al., 2014). The antigens recognized by $CD8^+$ T cells have been extensively studied. Currently, it is accepted that peptides can be derived from α-synuclein in PD patients, which is then displayed by MHC-I and activates CD8 cytotoxic T cell responses (Sulzer et al., 2017; Hobson and Sulzer, 2022). In addition, one study found that the response of $CD4^+$ T cells and $CD8^+$ T cells in the PBMCs from PD patients or healthy controls to common antigens from human pathogens showed no obvious difference (Williams et al., 2023). Therefore, the altered T cell response observed in PD patients may be induced via the recognition of autoantigens like α-synuclein rather than common antigens from foreign pathogens.

Interfering with $CD8^+$ T-cell function affects the progression of PD (**Table 1**). Depletion of T cells by using RAG-KO mice exerts a protective effect on the 6-hydroxydopamine (6- OHDA)-induced PD model mice (Ip et al., 2015). Moreover, knockdown of MHC-I in neurons of the substantia nigra reduces the infiltration of $CD8^+$ T cells and suppresses the loss of dopaminergic neurons (Cebrián et al., 2014). With the understanding of gut-brain interactions in the pathogenesis of brain diseases, one study explored whether colitis promotes the neuropathology of PD. Colon biopsies from PD patients exhibit high expression of inflammatory indicators Cd8b and NF-κB p65 and low expression of regulator of G-protein signaling 10 (RGS10), an inhibitor of NF-κB. Moreover, an obvious reduction in tyrosine hydroxylase levels and increased $CD8^+$ T-cell infiltration and activation were observed in mice with experimental colitis. Experimental colitis-induced suppression of dopaminergic markers is recovered by depletion of $CD8^+$ T cells indicating the important regulation of $CD8^+$ T cells in mediating colitis-induced neuropathology (Houser et al., 2021). Investigation of the role of the gut-brain axis in other NDDs may provide common strategies for the treatment of NDDs.

4.3 Amyotrophic lateral sclerosis (ALS)

Similar to AD and PD patients, researchers discovered a clonally expanded effector memory $CD8^+$ T- cell population in the peripheral blood and CSF of ALS patients (Campisi et al., 2022; Yazdani et al., 2022) and ALS animal models with mutations in the senataxin gene (SETX) (Carroll, 2019; Campisi et al., 2022). In the peripheral blood of ALS patients, an increased number of $CD8^+$ T cell effector/memory cells was observed and these $CD8^+$ T cells secreted increased granzyme B, IL-17 or IL-13 (Shi et al., 2007; Fiala et al., 2010; Kaur et al., 2022). Moreover, depletion of $CD8^+$ T cells in MHC-I-deficient mice promotes the survival of motor neurons (Nardo et al., 2018; Coque et al., 2019; **Table 1**). The role of $CD8^+$ T cells in other neurodegenerative diseases needs further investigation.

5 Conclusion and perspectives

T lymphocytes contribute to the maintenance of brain homeostasis and are involved in neurological diseases. As a special population of cytotoxic T lymphocytes, the function of $CD8^+$ T cells in neurological disorders is being revealed. In this review, we mainly summarize evidence of the functions and molecular mechanisms of $CD8^+$ T cells in brain injury as well as neurodegenerative diseases which all involve neuroinflammation and neuronal death, and discuss their therapeutic potentials and future questions. It is well established that $CD8^+$ T cells infiltrate into the brain parenchyma after brain injury and neurodegeneration by responding to chemokines released by glial cells. It is interesting that some studies showed the significant infiltration prior to protein aggregates formation which indicates the involvement of $CD8^+$ T cells in early phase of NDDs. The role of $CD8^+$ T cells in brain injury and neurodegeneration seems to be more detrimental than beneficial, although a subset of regulatory $CD8^+$ T cells was found to be protective in ischemic stroke and several reports indicate the protective role of T cells in promoting the clearance of protein aggregates in NDDs. These studies indicate the complex and context-dependent roles of $CD8^+$ T cells in brain injury and neurodegeneration. Therefore, to avoid side effects, researchers should identify distinct $CD8^+$ T cell subsets in the brain during disease progression, elucidate their respective roles and then design specific therapeutic strategies. In addition, compared with neurons and glial cells, $CD8^+$ T cells are easy to detect and manipulate and are relatively accessible. However, their cytotoxic functions-mediated by granzymes and perforins and regulatory immune functions-mediated by IFNγ are not always exert the same effects on brain injury or neurodegeneration. To promote the application of $CD8^+$ T cells-mediated immunotherapy in brain injury and NDDs, more thorough investigations are needed.

Future outstanding questions:

(1) What type of $CD8^+$ T-cell subset is present and what are its functions during different stages of brain injury?
(2) Is there any specific feature of $CD8^+$ T-cell subset exist during brain injury and neurodegeneration?
(3) How do $CD8^+$ T-cell number and function change during the different stages of NDDs and affect the outcome of the disease, especially before severe protein aggregation?
(4) What antigens trigger $CD8^+$ T cell activation during brain injury and neurodegeneration?
(5) What is the impact of the alternations in peripheral $CD8^+$ T cells that do not infiltrate the brain on the progression of brain injury and NDDs?

(6) How do meningeal $CD8^+$ T cells contribute to brain injury and NDDs?

Author contributions

ZZ: Conceptualization, Funding acquisition, Software, Writing – original draft. ZD: Software, Writing – original draft. YC: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metabolic changes with the occurrence of atherosclerotic plaques and the effects of statins

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Atherosclerosis is a common cardiovascular disease caused by the abnormal expression of multiple factors and genes influenced by both environmental and genetic factors. The primary manifestation of atherosclerosis is plaque formation, which occurs when inflammatory cells consume excess lipids, affecting their retention and modification within the arterial intima. This triggers endothelial cell (EC) activation, immune cell infiltration, vascular smooth muscle cell (VSMC) proliferation and migration, foam cell formation, lipid streaks, and fibrous plaque development. These processes can lead to vascular wall sclerosis, lumen stenosis, and thrombosis. Immune cells, ECs, and VSMCs in atherosclerotic plaques undergo significant metabolic changes and inflammatory responses. The interaction of cytokines and chemokines secreted by these cells leads to the onset, progression, and regression of atherosclerosis. The regulation of cell- or cytokine-based immune responses is a novel therapeutic approach for atherosclerosis. Statins are currently the primary pharmacological agents utilised for managing unstable plaques owing to their ability to enhance endothelial function, regulate VSMC proliferation and apoptosis by reducing cholesterol levels, and mitigate the expression and activity of inflammatory cytokines. In this review, we provide an overview of the metabolic changes associated with atherosclerosis, describe the effects of inflammatory responses on atherosclerotic plaques, and discuss the mechanisms through which statins contribute to plaque stabilisation. Additionally, we examine the role of statins in combination with other drugs in the management of atherosclerosis.

KEYWORDS

atherosclerosis plaque, cell metabolism, inflammatory response, statins, anti-inflammatory drug, cardiovascular and cerebrovascular diseases

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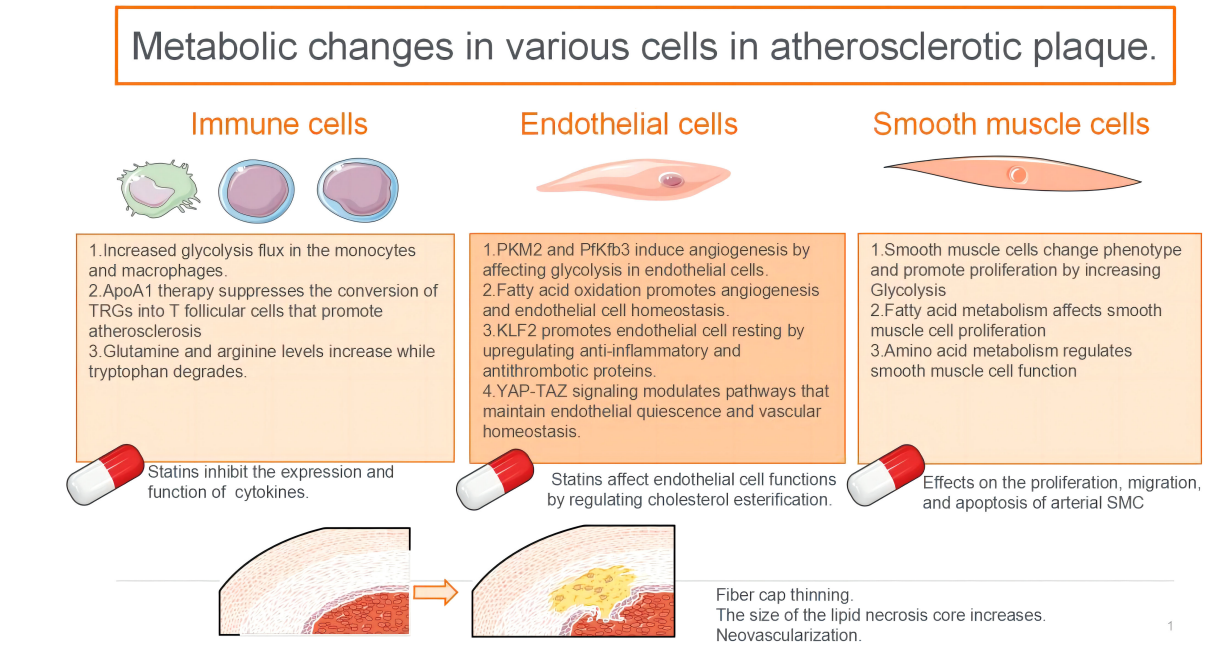

GRAPHICAL ABSTRACT

1 Introduction

Atherosclerosis is a common cardiovascular disease. It primarily manifests in the intima of affected arteries as lipid depositions, infiltrations of monocytes and lymphocytes, the migration and proliferation of vascular smooth muscle cells (VSMCs), and the formation of foam cells, lipid striations, and fibrous plaques, which further contributes to vascular wall sclerosis, stenosis, and thrombosis. Atherosclerosis can be asymptomatic for decades; however, when complications occur, such as the rupture of an atherosclerotic plaque, it can lead to myocardial infarction, stroke, peripheral vascular disease, and other high-fatality conditions. Treatment of these disease complications often relies on pharmaceutical interventions. In this review, we summarise the metabolic changes in some of the major cell types involved in atherosclerotic plaques and discuss the mechanisms, side effects, and progression of atherosclerotic plaques when treated with statins.

2 Metabolic changes with the occurrence of atherosclerosis

During the development of atherosclerotic lesions, the enhanced permeability of endothelial cells (ECs)facilitates the infiltration of peripheral inflammatory cells into the plaque (1). Macrophages, T cells, dendritic cells(DCs), and B cells are the most common types of immune cells found in growing arteriosclerotic plaques (2). Data from animal models show that selectively depleting or modulating the function of immune cells involved in atherosclerosis, inhibiting or blocking specific cytokines involved in inflammation and plaque development and regulating the immune cell bank and the secreted mediators in the arterial wall can impact atherosclerosis (2). Clinical trials, such as CANTOS (Canakinumab Anti-inflammatory thrombosis results study) and LoDoCo2 (low-dose colchicine for secondary prevention of stable coronary artery disease patients), also strongly suggest that immune regulation may be a relevant treatment option for human atherosclerosis (3, 4).

Metabolic regulation is closely related to the induction of immune responses. Metabolism in the microenvironment affects the proliferation and differentiation of immune cells and promotes the synthesis and secretion of immune mediators. In this section, we summarise the process of metabolic reprogramming in immune cells, particularly focusing on energy-related metabolic pathways. Moreover, we explore the potential of targeting the immune metabolism of macrophages and lymphocytes to control inflammatory responses in atherosclerotic lesions. The hypoxic environment within atherosclerotic plaques initially stimulates macrophage polarisation and enhances macrophage glycolysis.

2.1 Metabolic changes in macrophages, T and B cells that affect inflammation

2.1.1 Increased glycolysis flux in the monocytes and macrophages of patients with atherosclerosis

Adenosine triphosphate (ATP) is the universal energy currency within cells. The main sources of ATP for macrophages and lymphocytes involve glucose metabolism via glycolysis and the pentose phosphate pathway (PPP). Under normal oxygen conditions, cells generate 36 ATP molecules through the citric

acid cycle and oxidative phosphorylation (OXPHOS) pathway in the mitochondria. In an anaerobic environment, pyruvate is reduced as a hydrogen acceptor to lactate, resulting in a decrease in ATP production (two molecules) but an increase in ATP production rate. In the 1920s, the German scientist Warburg observed that, even in the presence of sufficient oxygen, tumour cells suppress aerobic respiration through a series of molecular mechanisms and promote efficient glycolysis reactions. This metabolic shift leads to the production of a large amount of ATP, creating a microenvironment suitable for the survival of tumour cells. This unique form of energy metabolism is known as the "Warburg effect". In recent years, several research groups have observed that the metabolic profile of activated macrophages, induced by phagocytosis or inflammatory stimulation, undergoes reprogramming. This reprogramming involves a transition from oxidative metabolism to "Warburg metabolism" (5), enabling the rapid supply of energy required for the inflammatory process and the essential metabolic intermediates needed for biosynthesis (Figure 1).

2.1.1.1 Macrophage metabolism

Nonpolarized macrophages, under steady-state conditions, primarily obtain energy via the OXPHOS pathway. However, the metabolic characteristics of polarised macrophages (M1 and M2) when obtaining energy are relatively complex (6), and they exhibit distinct metabolic profiles. According to the stimulation received from the microenvironment, M1 macrophages use glycolysis and the PPP to meet their ATP requirements. Simultaneously, OXPHOS and fatty acid oxidation (FAO) in the citric acid cycle are downregulated (7–10). In contrast, M2 macrophages exhibit a metabolic profile characterised by a complete citric acid cycle and enhanced FAO and OXPHOS (11, 12).

Glycolysis serves as a fundamental mechanism for energy production within cells. The glycolytic pathway occurring in the cytoplasm facilitates the conversion of glucose into pyruvate, yielding two ATP molecules per glucose unit. It also provides metabolic intermediates required for biosynthetic pathways, including ribose, amino acid, and fatty acid synthesis. The PPP operates in conjunction with the glycolysis pathway, harnessing the energy derived from glucose 6-phosphate conversion to ribulose 5-phosphate for NADP+ reduction to nicotinamide adenine dinucleotide phosphate (NADPH). This enzymatic process generates reactive oxygen species (ROS), which exhibit pathogenic activity. Additionally, high levels of NADPH are essential for maintaining the reduced form of the antioxidant glutathione, which protects cells from oxidative stress.

The adaptation of glycolysis metabolism depends on the activation of several transcription factors, including hypoxia-inducible factor 1 (HIF1α) (13). HIF1α regulates the expression of glycolytic enzymes, glucose transporter 1 (GLUT1), and genes encoding inflammatory mediators (13–15). The upregulation of GLUT1 promotes rapid glucose uptake by M1 macrophages (10).

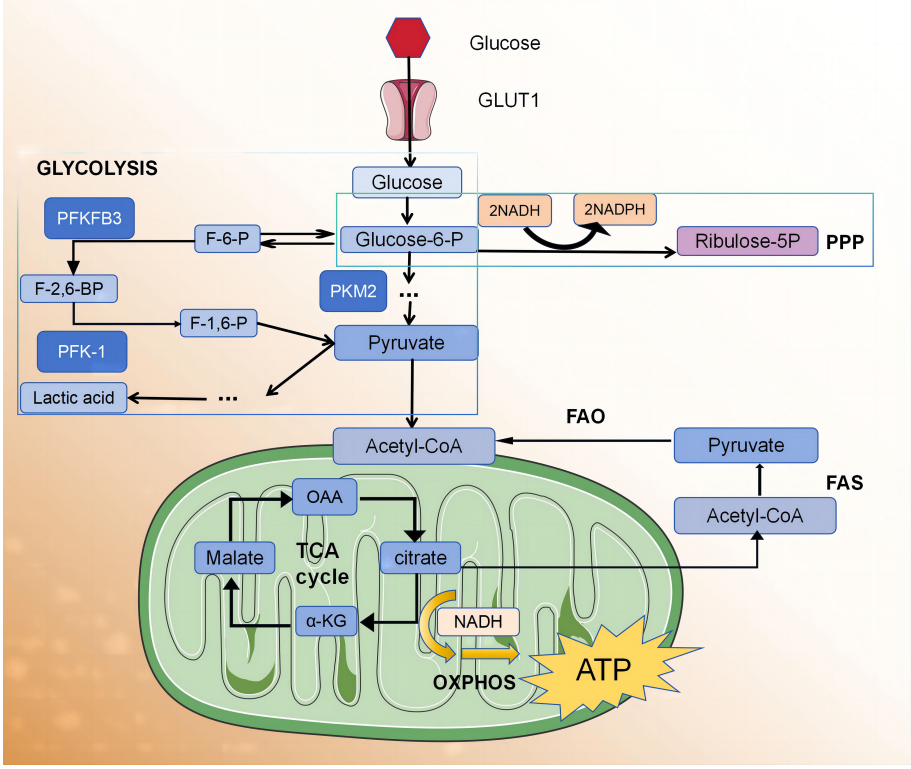

FIGURE 1
The glycolytic fluxes of immune cells, endothelial cells and smooth muscle cells in atherosclerotic plaque were increased.

Moreover, HIF1α supports the conversion of pyruvic acid to lactic acid by promoting the expression of lactate dehydrogenase (LDH) (16) and pyruvic acid dehydrogenase kinase (PDK) (17, 18), which are critical for restoring NAD+ levels and maintaining glycolytic flux. HIF1α regulation in macrophages predominantly occurs through two main signalling pathways, involving α expression of several genes, including toll-like receptor (TLR)/nuclear factor-κB (NF-κB) (15) and AKT/mammalian target of rapamycin (mTOR) (17–19) pathways. AKT kinase regulates macrophage polarisation in a subtype-specific manner, with AKT1 deletion promoting the M1 spectrum, while AKT2 deletion amplifies the M2 reaction (17). The other two factors that regulate glycolytic flux in M1 macrophages are 6-phosphofructose-2-kinase/fructose 2,6-diphosphatase 3 (Pfkfb3) and pyruvate kinase M2 (PKM2). Pfkfb3 catalyses the conversion of fructose-2,6-diphosphate to fructose-6-phosphate with low efficiency, thereby enhancing glycolytic flux. Moreover, M1 cells upregulate subtype PKM2, which, when overexpressed, exists in a balance between enzyme-inactive monomers or dimers and enzyme-active tetramers (20). The inactive enzymes translocate into the nucleus and bind to HIF1α, triggering the expression of HIF1α-regulated genes, whereas the enzymatically active tetramers promote glycolysis and M1 polarisation in the cytoplasm (20). In M1 macrophages, glycolysis significantly impacts macrophage functions, including phagocytosis, ROS production, and proinflammatory cytokine secretion (21). However, the role of glycolysis in M2 macrophages remains a subject of debate. Several studies have proposed that the presence of 2-deoxyglucose, an inhibitor of glycolysis, may impede M2 polarisation and impair its functionality (22, 23). There are also data indicating that M2 differentiation relies more on OXPHOS than glycolysis (24, 25). The tricarboxylic acid cycle (TCA cycle) is also complete in M2 macrophages.

Early studies using 18 fluorodeoxyglucose (18 FDG) positron emission tomography (PET) revealed elevated glucose uptake in atherosclerosis plaques of rabbits and humans compared to healthy blood vessels. Additionally, macrophage glycolysis levels, PPP activity, and TCA cycle metabolites were increased within plaques (12, 26). Notably, *in vitro* studies demonstrated that exposure to inflammatory factors, rather than a hypoxic environment, leads to a reduction in TCA cycle metabolism in polarized M1 macrophages, while the glycolytic metabolism level does not concurrently increase. These findings support the hypothesis that hypoxic stimulation enhances glycolysis in M1-polarized macrophages within atherosclerotic arteries (27). However, whether nonhypoxic stimulation enhances glycolysis in macrophages remains controversial (28).

2.1.1.2 T cell metabolism

Several studies have indicated that natural T cells are activated and differentiated into different subgroups after entering atherosclerotic plaques, and different T cell subgroups play distinct roles (29). For instance, a study has shown that the absence of $CD8^+$ T cells in mice did not affect plaque size, but immunotherapy with ApoB100 P210 peptide in $CD8^+$ $ApoE^{-/-}$ mice mitigated atherosclerotic lesions, suggesting that $CD8^+$ T cells may have a different function (30). The role of $CD4^+$ T cells in atherosclerosis is multifaceted. Static $CD4^+$ T cells differentiate into effector T cells (Teff cell) and regulatory T cells (Treg cell) upon activation. Different Teff cell lines proliferate and differentiate rapidly depending on their microenvironment (31).

Effector $CD4^+$ T cells in plaques primarily include Th1, Th2, and Th17 cells. Factors involved in the Th1 reaction, including tumour necrosis factor-α (TNF-α), recombinant human interferon-γ (IFN-γ), interleukin (IL)-12, and IL-18, have been proven to promote atherosclerosis through leukocyte recruitment, EC injury, and oxidative stress (32–36). However, the role of Th17 cells in atherosclerosis remains controversial. A study analysing atherosclerotic plaques in human coronary arteries showed that the cytokine IL-17 released by Th17 can synergistically increase the secretion of IL-6 with IFN-γ, promoting inflammation and atherosclerosis (37, 38). Conversely, in a study by Madhur MS et al., IL-17A did not affect atherosclerotic plaque burden in IL17A$ApoE^{-/-}$ mice fed a high-fat diet (39, 40). IL-10 secreted by Th17 cells may prevent the recruitment of T cells and macrophages and enhance protection against atherosclerosis by promoting the transformation of macrophages from an inflammatory to an anti-inflammatory phenotype (41, 42). The role of Th2 cells in the development of atherosclerosis remains uncertain. Th2 cells secrete IL-5, IL-10, and IL-13 and activate B cells to produce antibodies, which are believed to counteract the pro-atherosclerosis effects of Th1 cells. Furthermore, a study has shown that IL-4, an autocrine growth factor of Th2 cells, did not significantly affect the development of atherosclerotic lesions in $ApoE^{-/-}$ or female $ldlr^{-/-}$ mice (43). However, another study found that IL-4 deficiency reduced the formation of atherosclerotic lesions in female $ldlr^{-/-}$ mice (44). Therefore, the role of IL-4 in atherosclerotic lesions remains to be further clarified. Treg cells increase plaque stability mainly by secreting transforming growth factor-β (TGF-β) and IL-10. TGF-β can inhibit the recruitment and activation of T cells and macrophages, promote the proliferation of VSMCs, and maintain atherosclerotic lesion stability (31, 45). The balance between Teff and Treg cell subsets and their respective functions significantly influences the development of atherosclerotic lesions (46). Therefore, maintaining the equilibrium of the Treg/Teff ratio and function is essential to prevent the onset of atherosclerosis and slow its progression.

As T cells transition from a quiescent state to an activated state within atherosclerotic lesions, glycolysis becomes imperative for rapid energy generation. While OXPHOS is the primary pathway for energy generation in static T cells (31, 45), activated T cells require the GLUT1 receptor to increase glucose uptake and promote the upregulation of glycolytic enzymes to catalyse glycolysis, resulting in increased pyruvic acid production (47). Subsequently, pyruvic acid can be converted into lactic acid within the cytoplasm through LDH or into acetyl coenzyme A within the mitochondria through pyruvate dehydrogenase (PDH). Additionally, the NADH and flavin adenine dinucleotide (FAD) generated during this process are primarily produced within the mitochondria and ATP is produced through OXPHOS.

Teff cells (Th1, Th2, and Th17) predominantly rely on glycolytic metabolism and glutamine catabolism for energy, whereas Treg cells primarily depend on FAO (48). This metabolic

distinction between Teff and Treg subpopulations plays a pivotal role in governing the differentiation fate of $CD4^+$ T cells and maintaining optimal immune function (46, 49). Michalek et al. (48) differentiated Th1, Th2, and Th17 cells *in vitro* and found increased glycolysis and GLUT1 expression in these cells. Compared to wild-type mice, GLUT1 transgenic mice showed increased glucose uptake and selective enrichment of Teff cells. Conversely, inhibiting glycolytic metabolism through treatments like 2-deoxyglucose, a hexokinase inhibitor, reduces Teff cell production and impairs immune function *in vitro* and *in vivo* (13, 48, 50, 51). These findings indicate that the Teff subpopulation undergoes increased glycolytic metabolism, a necessity for differentiation and functional specialisation.

The high activity level of PDK1 in Th17 cells leads to higher extracellular acidification and glycolysis rates than in Th1 cells (46). Furthermore, PKM2 serves as the ultimate rate-limiting enzyme in $CD4^+$ T cell glycolysis. Hyperhomocysteinemia expedites the onset and progression of atherosclerosis by augmenting both PKM2 protein expression and activity in $ApoE^{-/-}$ mice (52). Therefore, targeted inhibition of the PKM2 metabolic pathway in $CD4^+$ T cells may represent a novel strategy for treating atherosclerotic lesions.

Glycolysis promotes the activation, proliferation, and migration of Treg cells to inflammatory tissues (53–55). The expression of the Treg transcription factor, forkhead box protein 3 (FOXP3), suppresses glycolytic metabolism and enhances OXPHOS by downregulating Myc protein expression. This metabolic adaptation allows Treg cells to function effectively in a low-glucose/high-lactate environment (56). However, the anoxic conditions within plaques may decrease FOXP3 expression and impair the protective effects of Treg cells on atherosclerosis (57, 58). In summary, the induction of the glycolytic pathway plays a crucial role in modulating the differentiation balance between Treg and Teff cells. However, further research is required to elucidate how the atherosclerotic plaque microenvironment affects the metabolic and functional properties of distinct T-cell subsets (Table 1).

2.1.1.3 DCs and B cells

In atherosclerotic lesions, vascular DCs are present within the plaque and outer membrane (59). Apart from their role in lipid absorption and clearance of apoptotic cells in plaques, DCs also facilitate T cell activation and proliferation by presenting antigens derived from autologous sources. DCs secrete a diverse array of cytokines that indirectly modulate the functionality of other immune cells, thereby contributing to immune-mediated vascular wall damage and the development of atherosclerosis, including their impact on B-cell activation (60).

B cells play a significant role in the development of atherosclerosis (61); however, there is ongoing debate regarding their specific involvement in plaque formation (62). B cells can be categorised into two subtypes: B1 and B2. The former produces natural antibodies (IgM) that are believed to exert protective effects against atherosclerosis by inhibiting necrotic nucleus formation on blood vessel walls (63, 64). Conversely, B2 cells participate in adaptive immune responses and secrete cytokines IL-10 and TNF-α, which influence Treg development and potentially promote the formation of atherosclerosis (65). Studies have investigated approaches such as anti-CD20 depletion or the absence of the BAFFR receptor to mitigate damage caused by B2 cell activation and protect hypercholesterolemic mice from developing atherosclerosis (66–68).

Glycolysis plays a crucial role in the activation of B cells within atherosclerotic lesions. The B-cell receptor (BCR), an immunoglobulin located on the surface of B cells, is responsible for specific antigen recognition and binding (69). BCR regulates glucose utilisation by promoting glucose uptake and the expression of the GLUT1 transporter in B cells, leading to a rapid and sustained increase in glucose metabolism that provides essential energy and the foundation for growth. After BCR activation, there is a significant increase in glycolysis. Previous studies have demonstrated that enhanced glucose utilisation primarily involves the PI-3K signalling pathway associated with BCR activity. This

TABLE 1 T cell typing and function.

T cell type	The main secretory factor	Effects in lesions		Contradiction
$CD8^+$ T cell	TNF-α, IFN-γ	Cytotoxic function	Plaque promotion (30)	
	IL-5, IL-10,IL-13	Modulating immune effects and assisting immune responses	Inhibition of plaque lesions (30)	
Th1	TNF-α, IFN-γ, IL-12, IL-18	Affecting leukocyte recruitment, EC damage and oxidative stress	Plaque promotion (33–36)	
Th2	IL-5, IL-10,IL-13	Activating B cells to produce antibodies	Inhibition of plaque lesions	
	IL-4	Did not affect the development of atherosclerotic lesions (43)		Plaque promotion (44)
Th17	IL-17	synergistically increase the secretion of IL-6 with IFN-γ	Plaque promotion (37, 38)	Did not affect the development of atherosclerotic lesions (40, 41)
Treg	TGF-β,IL-10	Inhibiting the recruitment and activation of T cells and macrophages, promote the proliferation of VSMCs	Inhibition of plaque lesions (31, 45)	

TNF, tumor necrosis factor; IFN, Human Interferon; IL, Interleukin; TGF, transforming growth factor; Th, helper T; Treg, regulatory T.

pathway facilitates precise regulation of glucose utilisation within B lymphocytes through the PI-3K/AKT signalling cascade, ensuring their capacity to meet the energy demands essential for growth-related processes (70) (Table 2).

2.1.2 Lipid metabolism: ApoA1 therapy suppresses Treg-to-T follicular cell conversion

During atherosclerosis, apolipoprotein A1 (ApoA1) indirectly affects T cell responses during inflammation. ApoA1 is the major protein component of high-density lipoprotein (HDL) and is produced in hepatocytes. Before being released into the plasma, it interacts with pre-HDL particles and ATP-binding cassette transporter A1 (ABCA1), acquiring phospholipids and cholesterol to form new HDL or ABCA1 (73). The formation of pre-HDL promotes the efflux of cholesterol from cells, resulting in a reduction in plaque volume. Research has shown that treating $ApoE^{-/-}$ mice with ApoA1 increased ABCA1 expression in Treg cells and restored cholesterol levels in these cells to normal (74). The anti-inflammatory properties of ApoA1 are also associated with changes in the lipid raft composition. Lipid rafts are microdomains on the cell membrane that are rich in sphingolipids and cholesterol, serving as enriched sites for IL-2 receptors. Lipid raft components regulate immune cell signalling and proliferation. Several studies have demonstrated that ApoA1 exerts regulatory effects on cholesterol levels, IL-2 receptor expression, and IL-6 expression in Treg cells during the progression of atherosclerosis, thereby impeding the transition from exTregs to Tfh cells and ultimately reducing atherosclerosis (73, 74).

Notably, diet-induced disruption of intracellular cholesterol metabolism is a significant factor affecting the differentiation and function of Tregs in atherosclerotic lesions. Maganto-García et al. (75) found that diet-induced hypercholesterolemia promoted the differentiation and migration of Treg cells in mouse splenic and prevented atherosclerosis. The classification of fatty acids into saturated, monounsaturated, and polyunsaturated forms is determined by the saturation level of their hydrocarbon chains. They can also be categorised as short-chain (SCFAs), medium-chain, or long-chain fatty acids (LCFAs) based on their carbon chain length. Fatty acids play a crucial role in regulating specific aspects of the body's innate immunity and cholesterol metabolism within atherosclerotic plaques. The innate immune system relies on a diverse group of pattern-recognition receptors called toll-like receptors (TLRs) (76, 77). TLR4 is highly expressed in atherosclerosis and has multiple functions. It activates cell adhesion, enhancing the uptake of oxidized lipids by macrophages and foam cell formation (78, 79). It also influences cholesterol metabolism and its impact on atherosclerosis development (80). In contrast to saturated fatty acids, polyunsaturated acids do not activate the TLR4 signalling pathway (81–83) and instead inhibit NACHT-, leucine-rich repeat (LRR)-, and pyrin domain (PYD)-containing protein 3 (NLRP3) activity (84, 85). This is significant because NLRP3 may contribute to the pathogenesis of atherosclerosis through a signalling pathway that triggers PCSK9 secretion via IL-1β stimulation (86). LCFAs and SCFAs also play different roles in arteriosclerotic lesions. LCFAs can enhance the proliferation and differentiation of Teff cells into Th1 and Th17 cells, aggravating the progression of atherosclerosis (87). Conversely, dietary SCFAs affect Treg differentiation, which in turn improves and treats autoimmune-related diseases (87, 88).

These findings suggest the potential of using fat-free ApoA1 therapy and dietary adjustments to regulate the conversion between Treg and Tfh cells, offering potential benefits to patients with atherosclerosis and other inflammatory diseases.

2.1.3 Amino acid metabolism: Alterations in glutamine, leucine, arginine, and tryptophan

Although glucose is generally considered the most important nutrient for inflammatory cells, amino acid metabolism also plays a crucial role in inflammatory cell proliferation and activation. Abnormal amino acid metabolism contributes to the occurrence

TABLE 2 Main metabolic patterns of inflammatory cells and their effects on disease.

Main metabolic patterns of inflammatory cells and their effects on disease				
cell type	Metabolic pattern	Effects in lesions		
		sample	Intervention method	result
M1 macrophages	glycolysis PPP	macrophages from mice and patients with atherosclerotic lesions within plaque	IFN-γ stimulation	M1 polarisation; Increased secretion of inflammatory cytokines, chemokines (18)
M2 macrophages	TCA FAO	anti-inflammatory alternatively activated macrophages	Etomoxir inhibits FAO; Oligomycin inhibits OXPHOS; FCCP inhibits uncoupled mitochondrial respiration	Mitochondrial oxidative metabolism is directly involved in M2 macrophage polarisation (11, 71, 72)
$CD4^+$ T cell	glycolysis	$CD4^+$ T cells and Tregs from Glut1 transgenic mice	Etomoxir stimulation	Th1, Th2, and Th17 cells primarily use glycolysis Tregs primarily use lipid metabolism (48)
Treg cell	FAO			
B2 cell	glycolysis	B cells from the mouse spleen	Incubated quiescent B cells with 1 mM 2-DOG along with anti-Ig	glycolytic flux is necessary for BCR-induced B-cell growth (70)

PPP, pentose phosphate pathway; IFN-γ, Human Interferon-γ; TCA, tricarboxylic acid cycle; FAO, fatty acid oxidation; OXPHOS, oxidative phosphorylation; Treg cell, regulatory T cell; BCR, B-cell receptor.

and development of atherosclerotic lesions (89). Relevant studies mainly focus on monocytes and macrophages, with relatively fewer studies on lymphocytes.

Glutamine is one of the most widely studied amino acids involved in inflammation. It enters cell mitochondria through amino acid transporters and is converted to glutamic acid by the action of glutaminase. It also provides nutrients for the synthesis of other amino acids and NADPH and is an important energy source (90, 91). Glutamine, a non-essential amino acid in plasma, exerts intracellular effects on macrophage polarisation. Glutamine undergoes conversion to alpha-ketoglutaric acid (α-KG) through the enzymatic actions of glutaminase (GLS) and glutamate dehydrogenase (GDH/GLUD1). α-KG plays a crucial role in the tricarboxylic acid cycle, and its deficiency disrupts normal metabolic processes, thereby promoting the polarisation of macrophages towards the M1 phenotype and enhancing pro-inflammatory cytokine secretion. Conversely, active glutamine metabolism can stimulate macrophages to polarise towards the M2 type and secrete anti-inflammatory factors (9). Activated T cells exhibit increased glutamine uptake and metabolism, similar to cancer cells (90, 92). Depletion or deficiency of glutamine can disrupt T cell activation and proliferation (92). Rapid extracellular glutamine uptake depends on the amino acid transport weight group solute carrier family 1, member 5 (Slc1a5, also known as ASCT2). In mouse immune and autoimmune models, Slc1a5 defects impair Th1 and Th17 cell differentiation and thus affect inflammatory T cell responses (93). Under these circumstances, targeted therapy with glutamine hydrolase holds promise in preclinical models of cardio-cerebral vascular disease (94).

Leucine is an essential amino acid that affects the differentiation of Teff cells. Leucine entry into activated T cells requires the involvement of the L-leucine transporter (LAT1) (95). T cells lacking LAT1 cannot differentiate into Th1 or Th17 cells, even with appropriate polarising cytokines (96). Genetic defects in the leucine sensor sestrin 2 also limit T-cell activation and differentiation (97).

There is growing evidence that tryptophan metabolism is also involved in inflammatory responses. Overexpression of aminophenamide 2,3 dioxygenase 1 (IDO1) during inflammation can drive tryptophan consumption, produce bioactive metabolites, and control the interaction between general control non-derepressible 2 and specific receptors, thereby shifting cytokine production toward an anti-inflammatory phenotype. Conversely, the ablation of IDO1 promotes the pro-inflammatory effect of immune cells. The induction of IDO1 is associated with protection against atherosclerosis and increased plaque stability (98, 99). Other studies suggest that in addition to IDO1, other enzymes involved in tryptophan degradation, such as kynurenine 3-hydroxylase, are also involved in inflammation regulation, potentially increasing the instability of atherosclerotic plaques (100).

Arginine metabolism and its byproduct, nitric oxide (NO), play crucial roles in the early stages of atherosclerosis (101), such as involvement in immunity and affect the phenotypic polarisation of macrophages (102). Under inflammatory conditions, macrophages exhibit overexpression of inducible nitric oxide synthase (iNOS), thereby promoting arginine metabolism and subsequent NO production. Elevated NO levels can impede the repolarisation process from M1 to M2 by interfering with the electron transport chain (103). Additionally, arginine inhibits T-cell proliferation and impairs their ability to migrate to related chemokines, possibly contributing to its protective effect against atherosclerosis (104) (Table 3).

2.2 Changes in the immune system affect EC metabolism

Vascular ECs play a crucial role in vascular homeostasis and disease. One of the earliest events of atherosclerosis development is the activation and dysfunction of ECs in vulnerable arterial regions. As atherosclerosis progresses, it exhibits characteristics such as the formation of a fibrous cap covering a lipid-rich necrotic core and the accumulation of leukocytes at the plaque's periphery. These immune cells influence the phenotype of ECs and promote plaque instability. Although the endothelium was initially considered an inert and semi-permeable barrier between blood components and underlying endothelial tissues, it is now recognised as an organ with active metabolic functions that profoundly impact vascular homeostasis and atherosclerosis throughout life (107).

Metabolic pathways regulate angiogenesis, inflammation, and barrier function of ECs in atherosclerotic lesions. The following section outlines the changes in glycolysis and lipid metabolism observed in ECs within atherosclerotic plaques, as well as the regulation of EC homeostasis and function through Krüppel-like transcription factor 2 (KLF2) and yes-associated protein/the transcriptional coactivator with PDZ-binding motif (YAP/TAZ) signalling.

2.2.1 Pfkfb3 and PKM2 induce angiogenesis by enhancing glycolysis in ECs

The formation of functional blood vessels and vascular plexuses, including processes like EC junction reorganisation, tip cell migration, stem cell proliferation, and phagocytosis (108–112), requires a substantial amount of energy in the form of ATP (108). Vascular sprouting relies on the differentiation of ECs into specialised subtypes, each with a specific function. Once the blood vessels are perfused, ECs transition into a quiescent state while being firmly anchored in the extracellular matrix. Blood vessels in which ECs germinate are regulated by genetic signalling and metabolic factors. ECs prefer to rely on glycolytic metabolism to minimise the production of ROS and rapidly produce ATP (95). Glycolytic enzymes and ATP are concentrated within the lamellar and filamentous pseudopods of ECs, facilitating their rapid motility (111).

Pfkfb3, the most prominent member of the Pfkfb family, plays a pivotal role in glycolysis by serving as the key enzyme (111). Conversely, the isozyme Pfkfb4 exhibits diminished kinase activity and can either stimulate or inhibit glycolysis (113). Signalling molecules known to induce germination in tip and stem cells, such as VEGF and FGF2 have been found to upregulate Pfkfb3 expression, subsequently promoting glycolysis.

TABLE 3 Amino acid metabolism in inflammatory cells.

Amino acid metabolism	Cell type	Intervention method	Result
Glutamine	Macrophages were extracted from C57BL6/J mice	Cells were cultured in glutamine medium and inhibited by membranomycin (1 μM or 2 μM) with n-glycosylation	Promoted the polarisation of macrophages towards M2, and then secrete anti-inflammatory factors (9)
	Activated T cells	Cells were cultured in glutamine-deficient media	Decreased Th1 production of IFNγ and Th17 production of IL-17 (93)
Leucine	Activated T cells	Stimulated with the leucine antagonist NALA	Th17 differentiation was inhibited, but Th1 and Th2 polarisation were not affected (105, 106)
tryptophan	Arterial samples were obtained from 30 patients undergoing vascular surgery and the T cells in them were analysed	IDO-induced tryptophan degradation-dependent pathways	Inhibiting T cell activation and may prevent atherosclerosis (98)
Arginine	Macrophages	Arginine was increased *in vivo* models of mouse peritoneal inflammation and *in vitro* RAW 264.7 macrophages	Arginine is also involved in immunity and affects the phenotypic polarisation of macrophages (102)
	T cells	Female APOE-deficient mice were supplemented with high arginine (2mg/L) for 14 weeks	inhibiting T cell proliferation and impairs their ability to migrate to related chemokines (104)

Furthermore, Pfkb3 also governs EC proliferation and influences their motility. *In vitro* and *in vivo* experiments have demonstrated that deactivating Pfkfb3 leads to reduced EC proliferation, impaired formation of filopodia/lamellipodia, and compromised directional migration, ultimately resulting in impaired vascular growth and branching in mice deficient in endothelial Pfkfb3 (114).

Alternatively, the Pfkfb3 blockade may reduce angiogenesis through other mechanisms. For example, in the context of tumour angiogenesis, lactic acid can activate HIF1α, upregulating vascular endothelial growth factor receptor 2 (VEGFR2) and promoting angiogenesis, or activate proangiogenic nuclear factors by inhibiting oxygen sensor PHD2 κB/IL-8 (115, 116). Reduced lactate levels after the Pfkfb3 blockade may inhibit angiogenesis through these mechanisms. Additionally, it has been proposed that Pfkfb3 may regulate cell proliferation through nuclear activity independent of glycolysis (117). However, further research is required to ascertain whether targeting Pfkfb3 can effectively reduce pathological angiogenesis.

Pyruvate kinases, which are involved in the production of pyruvate and ATP, are crucial for regulating glycolytic flux (109). PKM2, one of the PK isoenzymes located at the junctions of ECs expressing VE-cadherin, provides the material and energy required for promoting EC binding dynamics, migration, and proliferation through hyperactive glycolysis (118). Silencing PKM2 reduces ATP levels near EC junctions, affects the dynamics and internalisation of VE-cadherin at EC junctions, reduces the number of filopodia in endothelial tip cells, and ultimately disrupts EC junction remodelling, collective migration, and angiogenic germination.

2.2.2 FAO promotes angiogenesis and EC homeostasis

ECs serve as gatekeepers for fatty acid transport. The fatty acids at atherosclerotic sites are associated with alterations in biophysical properties and membrane protein function within EC membranes. Polyunsaturated fatty acids not only serve as carbon sources for cultured ECs but are also regulated by ECs for transport to metabolically active tissues (119). Circulating fatty acids can be locally released from triglyceride-rich lipoproteins in the lumen of ECs via lipoprotein lipase-mediated lipolysis. Subsequently, they can enter ECs via passive diffusion or fatty acid transporters (120). The silencing or deletion of fatty acid-related genes can affect various EC functions, including migration capacity, vascular sprouting ability, and permeability regulation. Additionally, it can impact the activity of endothelial nitric oxide synthase (eNOS), leading to the production of excessive NO, an important vasodilator. Therefore, dysregulation of eNOS and excessive NO production can lead to pathological dysfunction and contribute to the progression of atherosclerosis (121).

2.2.3 KLF2 and YAP-TAZ regulate EC homeostasis and function

Vascular ECs undergo different flow patterns depending on their location. For instance, the aortic arch, near the branching of the large ductal artery, and the tip of the coronary artery are exposed to oscillating shear stress known as disturbed flow (d-flow). In contrast, the larger curvature of the aorta and thoracic aorta experience high shear stress known as steady flow (s-flow). Exposure to d-flow increases the susceptibility of ECs to intima-media thickening and atherosclerosis (122). Among the numerous mechanosensitive transcription factors that differentially regulate vascular pathophysiology, our focus lies on KLF2 and YAP/TAZ.

2.2.3.1 KLF2 affects EC function by regulating the expression of LOX-1 and HRD1

KLF2 expression was down-regulated in ECs exposed to d-flow. KLF2 is the primary activator of eNOS expression and other

regulatory genes in the ECs, Its ability to maintain optimal expression and activity is indispensable for preventing pathological alterations in blood vessels, including thrombosis, oxidative stress, and inflammation (123). While early atherosclerosis is associated with Lectin-type oxidized low-density lipoprotein receptor 1 (LOX-1) expression. The imbalance between the excessive production of ROS and inadequate antioxidant defences in atherosclerosis leads to profound oxidative stress and the transformation of low-density lipoproteins (LDL) into highly atherogenic oxidized LDL (ox-LDL). These ox-LDL particles are subsequently deposited subcutaneously and bind to the clearance receptor LOX-1. This leads to an increased expression of cell adhesion molecules in ECs, promoting increased adhesion and migration of inflammatory cells into the intima. Concurrently, endothelial dysfunction worsens owing to increased vasoconstrictor production, increased ROS, and depletion of endothelial nitrogen oxide production (124). The activation of KLF2 is crucial for LOX-1 expression under shear stress. Downregulating KLF2 increases LOX-1 expression while overexpressing it inhibits LOX-1 upregulation. KLF2 regulates the degradation of 3-hydroxy-3-methylglutaryl reductase (HRD1), an E3 ubiquitin ligase, by binding to its promoter. HRD1 expression in atherosclerotic ECs is significantly reduced due to ox-LDL. Conversely, overexpression of HRD1 prevents ox-LDL-induced apoptosis in ECs (125).

In summary, KLF2 affects EC function by regulating the expression of various proteins and is a promising target for the treatment of atherosclerosis and related diseases (Figure 2).

2.2.3.2 YAP/TAZ signalling modulates pathways that maintain EC quiescence and vascular homeostasis

On the contrary, YAP/TAZ was activated and translocated into the nuclei of ECs that were exposed to atherosclerotic interference in blood flow. Relevant findings were substantiated by Wang et al. through *in vivo* and *in vitro* experiments. they also found target genes such as angiogenesis inducer 61 (CYR61), connective tissue growth factor (CTGF), and ANKRD1 were upregulated. Mouse arterial surface analysis also revealed increased nuclear localisation of YAP/TAZ and elevated levels of endothelial target genes in atherosclerotic regions. In contrast, protective laminar flow inhibited YAP/TAZ activity. Knockdown of YAP/TAZ significantly reduced EC proliferation and the induction of pro-inflammatory phenotypes. Conversely, overexpression of YAP promoted EC proliferation and inflammation. Thus, inhibiting YAP/TAZ activation may be a promising therapeutic strategy for atherosclerotic protection. Notably, statins inhibit YAP/TAZ activity, thereby reducing disturbed flow-induced proliferation and inflammation (126).

In summary, the regulation of various EC functions is influenced by metabolic pathways, including those related to

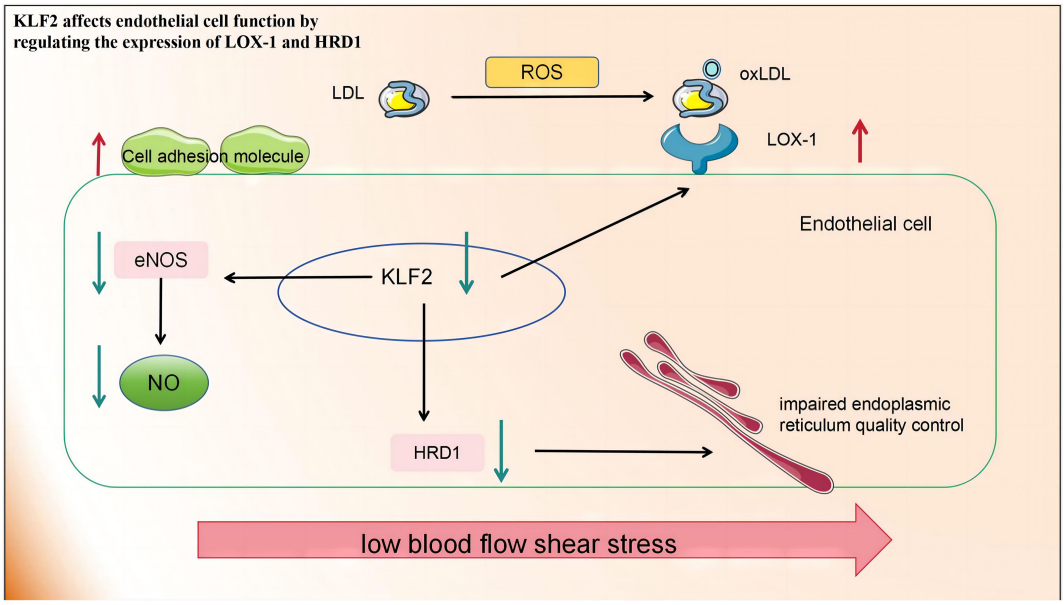

FIGURE 2
Low blood flow shear stress in atherosclerotic lesions leads to the downregulation of KLF2 in endothelial cells, thereby impacting endothelial cell function in the following aspects: 1) The production of eNOS and NO is diminished, consequently impairing their roles in promoting vascular relaxation and inhibiting inflammation. 2) The upregulation of LOX-1 expression on the cell membrane facilitates increased ox-LDL entry into cells, resulting in cellular damage. 3) The downregulation of HRD-1 expression involved in endoplasmic reticulum-related protein degradation pathway compromises endoplasmic reticulum quality control. KLF2, Recombinant Human Krueppel-like factor 2; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; LOX-1, lectin-like oxidised LDL receptor 1; HRD-1, hydroxy-3-methylglutaryl reductase degradation.

angiogenesis, inflammation, and barrier function. Despite advancements in understanding EC metabolism, many questions remain unanswered. A deeper comprehension of metabolic disturbances in ECs could lead to the development of novel therapeutic strategies for treating atherosclerosis (Figure 3).

2.3 Metabolic changes affect VSMCs function

The stability of atherosclerotic plaques depends on the thickness of the fibrous cap and the level of inflammation within it. Thinning of the vascular smooth muscle cell cap, which increases plaque rupture risk, is caused by cellular death and degradation of collagen and extracellular matrix (ECM). Vascular smooth muscle cells need to proliferate and synthesise new matrix components for effective repair. However, this process is hindered by cell death and senescence. The balance between cell proliferation, migration, death, and senescence plays a crucial role in determining the population size of vascular smooth muscle cells in atherosclerotic plaques. Understanding and regulating these processes are essential for maintaining stability during atherosclerosis development and plaque formation. Vascular smooth muscle cells are mainly found in the tunica media of arteries, exhibiting a mature "contractile" phenotype characterized by limited proliferation rates and expression of specific contractile proteins crucial for optimal vascular function (127). However, in response to vascular injury, these cells undergo a phenotypic transition from their static "contractile" state to a highly migratory and proliferative "synthetic" state. This shift significantly contributes to the development of Atherosclerosis, hypertension, and intimal hyperplasia formation (128–130). Recent research has revealed that metabolic reprogramming drives this transformation in VSMCs, involving key metabolic pathways such as glycolysis, fatty acid oxidation, and amino acid metabolism in both physiological and pathological vascular systems (130).

2.3.1 Glycolysis and phenotypic changes in SMCs

During the development of atherosclerotic plaques, VSMCs transition from their contractile state to a more synthetic state, involving proliferation and migration from the tunica media to the intima. This phenotypic alteration is closely linked to alterations in glucose metabolism, particularly glycolysis (131). One key driver of VSMC proliferation in atherosclerosis is the upregulation of GLUT1, a glucose transporter. This upregulation results in a substantial increase (44%) in the intracellular glucose concentration within these cells (132–134). The higher glucose levels provide ample energy and lactic acid for further VSMC proliferation. Therefore, modulating glycolysis presents a promising therapeutic avenue for treating atherosclerosis.

Inhibition of the glycosylation pathway rate-limiting enzyme PKM2 leads to a decrease in extracellular and intracellular lactate production (135). Lactate dehydrogenase A (LDHA) converts pyruvate to lactic acid, which affects the survival, proliferation, migration, and invasion of human and rat aortic SMCs (133, 134). Additionally, LDHA down-regulates adenosine monophosphate-

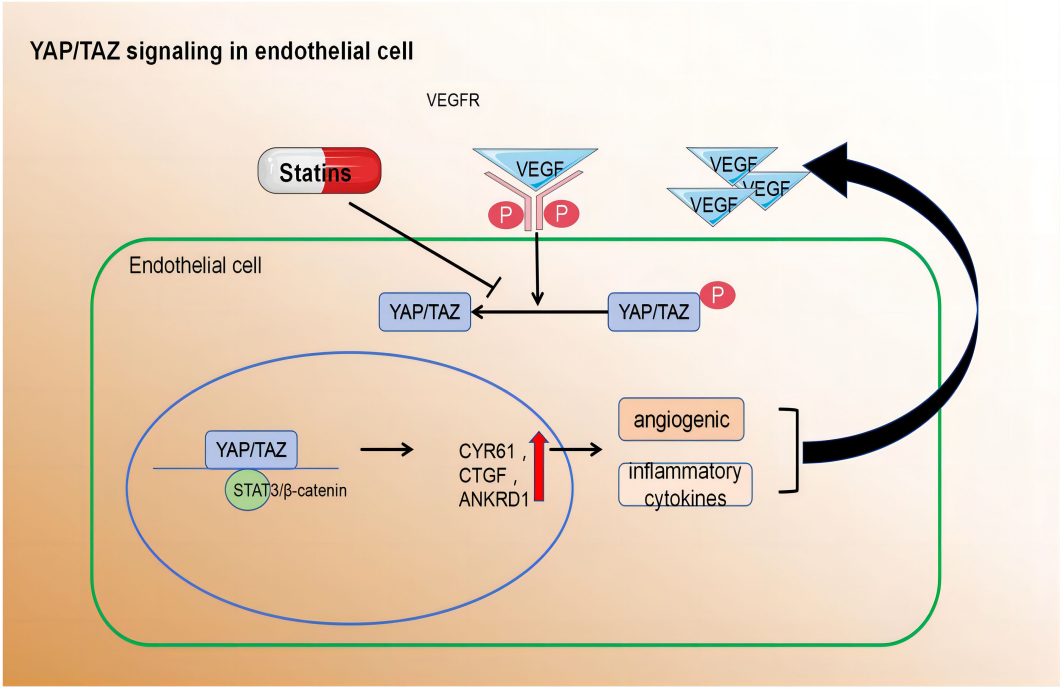

FIGURE 3
Pro-angiogenic growth factors, inflammatory cytokines, hypoxia, and disturbed blood flow activate YAP/TAZ leading to their translocation to the nucleus.Within the nucleus, YAP/TAZ interacts with STAT3 and β-catenin to induce transcription of downstream target genes. cysteine-rich angiogenic inducer 61 (CYR61), connective tissue growth factor (CTGF), and ankyrin repeat domain 1 (ANKRD1) Activated YAP/TAZ induce the expression of angiogenic and inflammatory cytokines.

activated protein kinase (AMPK), which is involved in the vascular injury pathway. Furthermore, pyruvate can be oxidized to form acetyl-CoA by the PDH enzyme complex in the mitochondria of SMCs, entering the TCA cycle (136). Therefore, inhibition of PDK1 weakens the TCA cycle and shifts glucose metabolism from OXPHOS to glycolytic pathways (137).

In summary, the regulation of glycolysis is a key factor influencing VSMC proliferation. Additionally, VSMCs possess functional mitochondria, which not only produce energy but also provide metabolites necessary for biomass synthesis. Targeting mitochondrial complex I activity may reduce neointimal hyperplasia by inhibiting VSMC proliferation and migration (138). Furthermore, the presence of ox-LDL induces oxidative stress and ROS production in human VSMCs. High levels of ox-LDL exacerbate VSMC apoptosis, resulting in matrix and collagen loss and the thinning of the fibrous cap (139). These intricate metabolic processes play critical roles in the development of atherosclerotic plaques and offer potential therapeutic targets for intervention.

2.3.2 Fatty acid metabolism affects SMC proliferation

In addition to glucose, VSMCs can also derive energy from fatty acids. FAO yields a higher amount of energy compared to glucose metabolism but requires higher oxygen levels. The presence of fatty acids can impact the utilisation of glucose and glycogen in both resting and contracting VSMCs (140). During the phenotypic transformation of VSMCs, there is a decrease in glucose oxidation and an increase in FAO. This elevation in FAO may provide VSMCs with additional energy for rapid proliferation, migration, synthesis, and secretion of the extracellular matrix (141, 142). The Randall cycle plays a pivotal role in altering the preference for utilising either glucose or fatty acids as fuel sources. This shift towards increased FAO impedes the oxidation of glucose (143). The Randall cycle has been implicated in regulating oxidative metabolism in muscle tissue, adipose tissue (143, 144), and brain energy balance (145). However, its influence on VSMC metabolism remains unclear. Exploring this aspect could present an intriguing avenue for future research.

Notably, dysfunctional FAO has been observed in plaques within the carotid artery of humans (146). This impaired FAO capacity may limit the functions of VSMCs, such as proliferation and migration, which are essential for plaque stability and vascular health.

2.3.3 Amino acid metabolism regulates SMC function

Currently, there is a growing body of research focusing on the role of amino acid metabolism in VSMCs. Glutamine, the primary non-essential amino acid in plasma, plays a pivotal role in this process. Recent findings indicate that Slc1a5 plays a pivotal role in the efficient transportation of L-glutamine, and this transport mechanism has the potential to enhance VSMC proliferation (147). Moreover, glutamine is utilised to produce glutathione (γ-glutamyl-L-cysteinylglycine, GSH), an essential component involved in combating free radicals. Within VSMCs, both reduced GSH and its oxidized form (glutathione disulfide, GSSG) play critical roles in maintaining cellular redox balance (148). Depletion of GSH and increased DNA damage have been shown to inhibit growth and induce cell death in human VSMCs (149–151). Nitric oxide, a free radical in VSMCs, induces p53 expression and triggers programmed cell death by consuming intracellular GSH, making it a potent initiator of apoptosis (152). Additionally, it impedes mitochondrial respiration by suppressing the functions of complexes I and II; thereby affecting the relaxation of vascular smooth muscle (107). Furthermore, L-arginine effectively suppresses the proliferation and migration of VSMCs, even in the absence of NOS (153–155). Tryptophan, an essential amino acid serving as a substrate for serotonin synthesis, significantly enhances both proliferative and migratory tendencies of rat VSMCs under laboratory conditions (156). Elevated cysteine levels cause inflammation, oxidative stress, and increased proliferation and migration of VSMCs (157, 158). Notably, endogenous synthesis of hydrogen sulphide (H_2S) from cysteine has protective effects on blood vessels. It inhibits NADPH oxidase, ROS production, glutathione disulfide formation, glutathione synthesis, and cysteine uptake (159, 160).

In summary, amino acid metabolism in atherosclerotic lesions affects smooth muscle proliferation and migration. These processes have profound implications for vascular health and the development of conditions such as atherosclerosis (Figure 4) (Table 4).

3 Effects of inflammatory reactions on arteriosclerosis plaques

3.1 Inflammatory cells interact with ECs during the initial stages of lesion development

In the initial stage of plaque development, there is an accumulation and aggregation of LDL and Very Low-Density Lipoprotein (VLDL) particles beneath the endothelium. These particles undergo oxidation and enzymatic modifications, resulting in the formation of oxidized phospholipids (oxPLS). The presence of oxPLS promotes inflammation and activates ECs. Simultaneously, various adhesion molecules are expressed, leading to the recruitment of white blood cells and platelets into the endothelial lining of blood vessels (161).

Monocytes firmly adhere to ECs through the interaction between monocyte integrins and ligands on ECs. Immunohistochemical analysis of human lesions and genetic studies in mice have demonstrated the significance of monocyte integrins VLA-4 and LFA-1, as well as their respective EC ligands VCAM-1 and ICAM-1, during the early stages of atherosclerosis. Moreover, platelet aggregation on the endothelium covering atherosclerotic lesions may enhance monocyte-EC interactions by inducing NF-κB signalling, promoting the expression of adhesion molecules, and depositing platelet-derived chemical factors on activated endothelium (162).

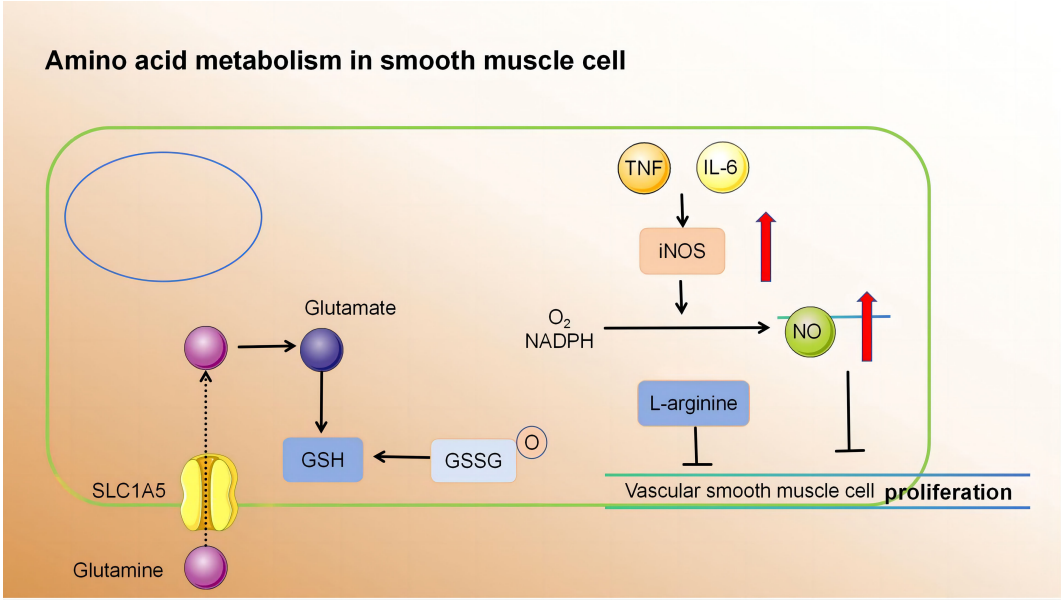

FIGURE 4
(1) Slc1a5 is a high-affinity L-glutamine transporter.glutamine absorption mediated by Slc1a5 can enhance the proliferation of VSMC. (2) In VSMCs, GSH and GSSG play crucial roles in cellular redox maintenance. (3) NO is a free radical. In VSMCs, NO activates programmed cell death through the consumption of intracellular GSH, thereby acting as a powerful apoptosis trigger. (4) L-arginine inhibits the proliferation and migration of VSMCs in the absence of NOS. Slc1a5, Solute Carrier Family 1, Member 5; VSMC, vascular smooth muscle cell; GSH, γ-glutamyl-L-cysteinylglycine; GSSG, Oxidized glutathione.

3.2 Inflammatory factors regulate the internal environment of atherosclerosis plaques

In addition to inflammatory cells, cytokines synthesised and expressed within atherosclerotic plaques play a significant role in shaping the internal environment for these plaques. Various stimuli trigger the release of inflammatory factors, including IL-1, IL-6, IL-8, IL-12, IL-18, soluble CD40 (SCD40), and TNF. These factors exert diverse effects that contribute to increased vascular permeability caused by inflammation (SCD40), and TNF. These factors exert diverse effects that contribute to increased vascular permeability caused by inflammation (163).

Clinical research on anti-thrombotic therapy involving canakinumab has investigated the role of IL-1 in the induction of atherosclerosis. Monoclonal antibodies targeting IL-1 effectively inhibit the formation and progression of atherosclerotic plaques (164). Studies like MIRACL have demonstrated an association between stroke risk and levels of high-sensitivity C-reactive protein (hs-CRP), serum amyloid A protein (SAA), and the inflammatory marker IL-6 (165). A phase II clinical trial demonstrated the potential of ziltivekimab, an all-human

TABLE 4 Smooth muscle cell metabolism.

Metabolic pattern		Sample processing	Result or conclusion
Glycolysis		PDGF stimulates VSMCs in primary rat aorta	Promoting VSMC proliferation and migration (131)
Fatty acid metabolism		VSMCs were exposed to PDGF	Synthetic VSMCs demonstrated a 20% decrease in glucose oxidation, which was accompanied by an increase in fatty acid oxidation (141)
Amino acid metabolism	Glutamine	Slc1a5 is consumed by silencing RNA or blocking Slc1a5-mediated glutamine uptake	Inhibition of VSMC proliferation (147)
	Glutathione	Exogenous H_2O_2 depletion of GSH	VSMC growth inhibition and cell death were induced (149)
	L-arginine	L-arginine treatment of carotid artery injury rat model	L-arginine effectively suppresses the proliferation and migration of VSMC (153)
	Tryptophan	The 5-HT2BR antagonist acts on smooth muscle cells	Inhibition of VSMC migration (156)
	Elevated cysteine	High homocysteine stimulated carotid artery injury in rats	Promoting VSMC proliferation (157)

PDGF, platelet-derived growth factor-BB; 5-HT2BR, 5-HT receptor 2B; VSMC, vascular smooth muscle cell.

monoclonal antibody targeting IL-6 ligand, to significantly reduce multiple biomarkers associated with systemic inflammation and thrombosis (166). Furthermore, elevated levels of IL-8 within atherosclerotic plaques promote the recruitment and migration of monocytes toward vascular ECs, resulting in firm adhesion (167, 168). CD40 is predominantly expressed in the pro-inflammatory M1 phenotype of macrophages (169), and plasma CD40 levels are correlated with carotid artery severity. TNF-α stimulates interstitial cells to induce the expression of various adhesion molecules and triggers the secretion of inflammatory cytokines and chemical factors, thereby enhancing the recruitment of activated white blood cells to affected areas (170). TNF-α is a pleiotropic cytokine that acts through two primary receptors, TNF-1 and TNF-2. TNF-1 mediates pro-inflammatory signals, apoptosis, and degeneration, while activation of TNF-2 by TNF-α induces anti-inflammatory and cytoprotective responses, leading to cellular proliferation, differentiation, angiogenesis, and tissue repair (171). Within plaques, there are also anti-inflammatory cytokines such as IL-10 and TGF. TGF is a multifaceted late-stage cytokine with both protective and atherogenic properties. Vascular endothelial TGF generates positive signalling cascades that inhibit inflammation, reduce vascular permeability, and slow disease progression in hyperlipidaemic mice (172). The absence of TGF-β1 results in reduced VSMC differentiation within the body, accelerated lesion formation, and increased inflammation.

3.3 Characteristics of unstable plaques in advanced atherosclerosis

In advanced atherosclerotic plaques, the fibrous cap ruptures, exposing the necrotic core to thrombotic material, which initiates platelet aggregation and subsequent clot formation. The active release of plaque-derived cytokines, proteases, and coagulation/thrombosis-related factors further promotes the progression of vulnerable plaques (173).

In unstable atherosclerotic plaques, the thickness of the fibrous cap is reduced owing to decreased collagen synthesis in SMCs and/or enhanced collagen degradation in fibroblasts. This thinning promotes the development of vulnerable plaques. Reduced abundance of VSMCs in vulnerable plaques can contribute to decreased collagen synthesis. In areas of vulnerable plaque where apoptotic cells are present, macrophages can attenuate collagen production in VSMCs without inducing cell death by secreting lower levels of TGF-β, a key stimulator of collagen synthesis in SMCs. Additionally, macrophage-derived matrix metalloproteinases (MMPs), which refer to a group of enzymes responsible for activating proteins and breaking down different types of extracellular matrix proteins, can also contribute to weakening the fibrous cap (174).

Unstable plaques are characterised by the presence of a necrotic core, which results from programmed cell death of mature macrophages and impaired phagocytic ability to engulf dying macrophages in advanced plaques (175). Early atherosclerotic lesions efficiently clear apoptotic macrophages through phagocytosis, leading to minimal compromise in cellular integrity, and limited plaque progression. Apoptotic macrophages play a crucial protective role through three essential mechanisms (1): eliminating cells before they release harmful substances into the surrounding environment; (2) inducing an anti-inflammatory response mediated by IL-10 and TGF-β; (3) enhancing cell survival by counteracting internal toxic factors. The benefits of apoptotic macrophages encompass efficient cholesterol esterification and removal, elimination of proapoptotic oxidized lipids, and activation of AKT and NF-κB signalling pathways. However, in the advanced stages of atherosclerotic lesions characterised by oxidative stress and increased inflammation, macrophage endocytosis signalling pathways are impaired. This impairment leads to secondary necrotic cell death, and elevated levels of inflammation lead to cytotoxicity (176).

The low-oxygen, inflammatory, and oxidative stress environment in atherosclerotic plaques can induce both conventional and unconventional angiogenic factors, promoting the formation of neovascularisation (177). The presence of neovascularisation in the shoulder region is typically characterised by an incomplete and immature structure, rendering it prone to leakage, which contributes to intra-plaque haemorrhage (IPH). In 1936, it was postulated that repetitive IPH is implicated in the progression of atherosclerosis and thrombosis (178). Magnetic resonance imaging (MRI) studies on human carotid atherosclerosis over the past two decades have confirmed histological observations and indicated a significant influence of IPH on plaque evolution (179). Moreover, the potential of 18F-FDG as a functional imaging technique for identifying vulnerability by analysing signals associated with plaque histology has been suggested. However, concerns regarding the accuracy and precision of PET in detecting fragile plaques remain significant. Combining 18F-FDG-PET imaging with dynamically enhanced MRI is expected to enhance the diagnosis of plaque fragility in the future (Figure 5).

4 Mechanism of action, side effects, and drug combinations of statins

4.1 Statins stabilise plaques by acting on the inflammatory system

HMG-CoA reductase plays a crucial role in the production of cholesterol and was identified as a target enzyme for statins in the 1980s. Statins exert a wide range of effects beyond their primary function of lowering LDL cholesterol levels. Statin therapy has been associated with inflammatory responses triggered by stimulants such as oxidized LDL.

These drugs affect the function of various cells within atherosclerotic plaques through different signalling pathways. Statins enhance macrophage-mediated cholesterol esterification, increase the uptake and degradation of LDL, and promote NO synthesis in ECs. Additionally, they reduce inflammation, improve EC function, and inhibit SMC proliferation and apoptosis. These

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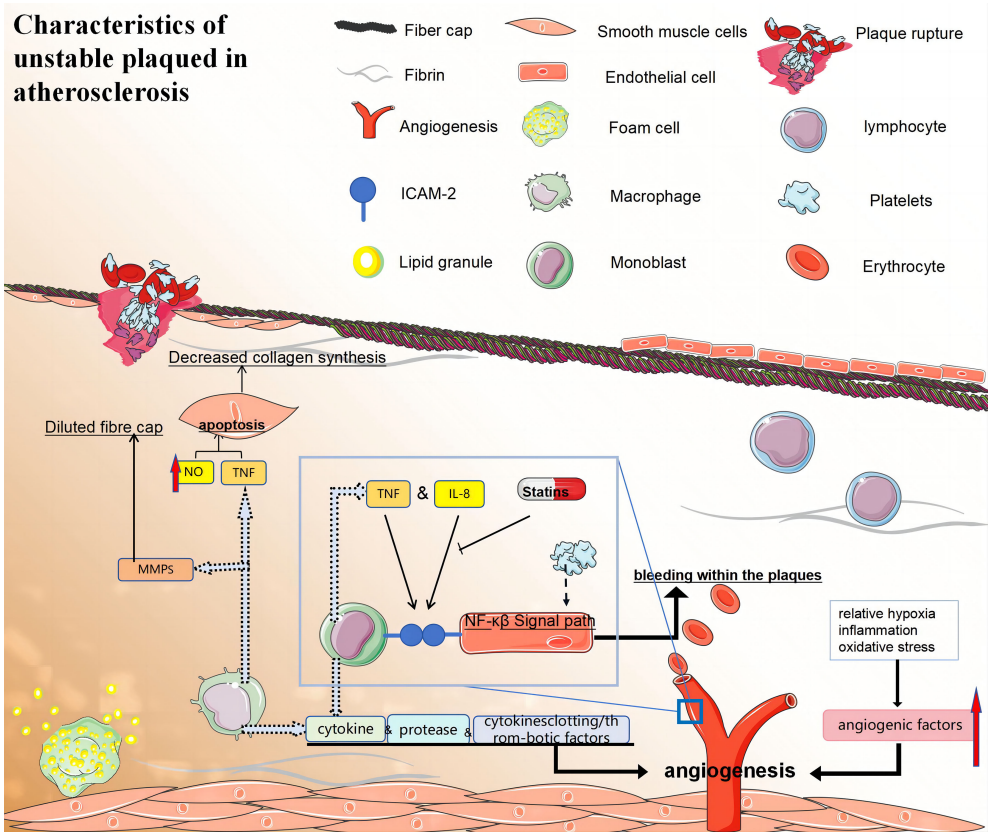

FIGURE 5
(1) Macrophages promote the formation of vulnerable plaques by secreting cytokines, proteases, and clotting/thrombotic factors. (2) i:The fibrous caps of late unstable atherosclerotic plaques became thinner. VSMCs may promote the formation of vulnerable plaques by reducing collagen synthesis and/or leading to collagen degradation. ii:Macrophages activate their apoptotic pathways and secrete TNF that is preapoptotic α, and that nitric oxide can trigger the apoptosis of SMC cells, which leading to a decrease in the number of VSMCs. iii:Macrophage-derived MMPS may also be involved in the dilution of fibre caps. (3) Specific local conditions (relative hypoxia, inflammation, and oxidative stress) in atherosclerotic plaques can also induce classical and non-classical angiogenic factors and promote neovascularisation. Neovascularisation increases bleeding within the plaques, which may lead to their instability and rupture. Monocytes firmly attach to endothelial cells through ICAM-1 interactions, while atherosclerotic lesions can enhance monocyte-endothelial interactions by activating NF-κB signaling. (4) Statins also exhibit anti-inflammatory properties by decreasing the expression of intercellular adhesion molecule-1 and inhibiting IL-6 secretion in monocytes and macrophages stimulated by LPS. (5) TNF:tumor necrosis factor; NF-κB:nuclear factor-κB; ICAM-1, intercellular cell adhesion molecule-1; MMPS, matrix metalloproteinase.

effects contribute to enhanced stability within atherosclerotic plaques, which, in turn, leads to the restoration of platelet activity and the clotting cascade. Therefore, statins are the most effective drugs for reducing lipid levels and mortality rates in patients with coronary issues. They significantly decrease the incidence of atherosclerosis in both primary and secondary prevention settings. Commonly prescribed statins include lovastatin, pravastatin, fluvastatin, simvastatin, pitavastatin, rosuvastatin, and atorvastatin.

4.2 Pharmacological mechanisms of statins

4.2.1 Inhibition of HMG-CoA reductase

Statins exert their pharmacological effects through a multi-faceted approach. Primarily, they act on hepatocytes and inhibit the activity of HMG-CoA reductase, a critical enzyme responsible for the synthesis of mevalonate, a precursor to cholesterol. This leads to a decrease in cellular cholesterol levels. Moreover, statins promote the removal of sterol regulatory element-binding proteins (SREBPs) from the endoplasmic reticulum by inducing protease activity. SREBPs are responsible for increasing LDL receptor expression when they translocate to the cell nucleus. The decreased cholesterol levels in liver cells lead to an increase in the number of LDL receptors on their membranes, which facilitates the efficient clearance of LDL cholesterol particles from the bloodstream. Recent research suggests that statins may also enhance the expression of PCSK9, an enzyme involved in LDL receptor breakdown, potentially affecting their effectiveness in reducing LDL-C levels and preventing coronary heart disease risk (180).

4.2.2 Statins reduce the sensitivity of LDL to oxidation and inhibit NLRP3 the activation of inflammasomes and the TLR signalling pathway

Several mechanisms underlie the antioxidant properties of statins (1): Statins reduce cholesterol levels, leading to decreased lipoprotein cholesterol and oxidative substrate levels (181). (2) They

inhibit superoxide production in macrophages, thereby reducing cellular oxygen production. Additionally, statins inhibit the isopentenylation process of the p21 Rac protein in ECs, thereby impeding the formation of superoxide anions (182). This preserves the functionality of the endogenous antioxidant system, counteracting LDL oxidation (183). (3) By binding with phospholipids on lipoprotein surfaces, statins obstruct the penetration of free radicals generated during oxidative stress into the core region of lipoproteins. (4) Metabolites resulting from statin usage exhibit potent antioxidant capabilities that effectively protect against lipoprotein oxidation.

Ox-LDL promotes the activation of the NLRP3 inflammasome and its receptor pathway in plaques. The first signal is triggered by pattern recognition receptors such as TLR, which activates the NF-κB pathway, leading to the transcription of NLRP3 and other pro-inflammatory cytokines. The second signal involves the oligomerisation of activated NLRP3, ultimately forming the inflammasome, which activates pro-inflammatory cytokines. Statins exert a pleiotropic effect on the NLRP3 complex. They cause a reduction in TLR agonists and inhibit the TLR4/MyD88/NF-κB pathway. Additionally, statins inhibit the NLRP3 inflammatory response via the LOX-1/NF-κB pathway (184).

4.3 Effects of statins on intracellular cell functions in plaques

4.3.1 Statins affect EC functions by regulating cholesterol esterification

The development of endothelial dysfunction due to elevated cholesterol levels is an early event in the progression of atherosclerosis. Hindered by hypercholesterolemia, the capacity of ECs to generate NO, a pivotal regulator of anti-atherosclerotic functions, is compromised. Research has demonstrated that statins effectively enhance endothelial function by reducing cholesterol levels. In 2002, Simionescu et al. (185) discovered that simvastatin alleviated the intracellular effects of LDL and restored endothelium-dependent relaxation, possibly due to increased NO synthesis. In 2018, Geng et al. (186) demonstrated that rosuvastatin protects endothelial cells in an *in vitro* model of human umbilical vein endothelial cells induced by ox-LDL, through its antioxidant function and up-regulation of the expression of eNOS, an endothelial protective factor. These findings confirm the advantageous impact of statins on promoting eNOS expression and preventing LDL-induced suppression of eNOS expression (187).

Furthermore, ox-LDL induces ECs to generate adhesion molecules and selective proteins, thereby facilitating immune cell infiltration into the intima. Statin therapy inhibits EC adhesion and permeability while reducing white blood cell migration, ultimately mitigating the inflammatory response within plaques (188).

4.3.2 Effects on inflammatory cells

Cytokines released by macrophages and lymphocytes influence endothelial function and promote SMC proliferation, collagen degradation, and thrombosis. Statins act by inhibiting the expression and activity of these cytokines in the pathogenesis of atherosclerosis. In hypercholesterolemic rabbits, atorvastatin has been demonstrated to reduce the presence of macrophages, monocyte chemoattractant protein-1 (MCP-1), and nuclear factor NF-κB activation within the intima layer (189). Moreover, statins exhibit anti-inflammatory effects on monocytes and macrophages by downregulating intercellular adhesion molecule-1 expression induced by LPS and suppressing IL-6 secretion. The presence of crystalline cholesterol is widely acknowledged in the scientific community to play a significant role in arterial inflammation. This inflammatory response occurs due to the activation of NLRP3 (or cryopyrin) inflammasomes by cholesterol, subsequently triggering caspase-1 and leading to the release of IL-1 family cytokines (190). Additionally, studies have demonstrated that statins can effectively reduce hs-CRP levels and potentially mitigate adverse events in patients, even in the absence of evident hypercholesterolemia (191).

4.3.3 Effects on the proliferation, migration, and apoptosis of arterial SMCs

In atherosclerotic lesions, the proliferation, migration, and invasion of SMCs into the subendothelial layer can induce intimal hyperplasia, while the secretion of collagen fibres by SMCs can influence the thickness of fibrous caps within plaques. In 2000, Bellosta et al. (192) conducted a study using both *in vitro* and *in vivo* models. The findings suggest that fluvastatin, simvastatin, lovastatin, and atorvastatin exhibit dose-dependent inhibition of SMC migration and proliferation. Chandrasekar et al. (193) demonstrated that the pro-atherosclerotic cytokine IL-18 stimulates SMC migration in an MMP9-dependent manner, and atorvastatin inhibits this process. Zhou et al. (194) established a diabetic mouse model by utilising *ApoE*$^{-/-}$ mice and administered atorvastatin treatment. Subsequently, they quantified SMCs and collagen composition, revealing that atorvastatin effectively reduced the number of SMCs while promoting collagen fibre synthesis. Moreover, it resulted in diminished atherosclerotic plaque area and enhanced arterial plaque stability through modulation of the RAGE pathway. However, Palomino-Morales et al. (195) demonstrated that statins effectively attenuated the activity of RhoA in SMCs, consequently leading to a reduction in collagen expression. The differences among these findings may be attributed to the different animal models used. By 2021, Jo et al. (196) conducted experiments to elucidate the mechanism underlying the inhibitory effect of statins on SMC apoptosis. Upon stimulation by ox-LDL in atherosclerotic lesions, platelet-derived growth factors induce the proliferation and migration of VSMCs. Prolonged stimulation ultimately leads to VSMC apoptosis. Statins exert their inhibitory effects by suppressing p38 activation through autophagy, thereby attenuating intracellular ROS levels and preventing apoptosis.

In summary, statins can promote the stability of atherosclerotic plaques by inhibiting the proliferation, migration, and apoptosis of SMCs and by affecting the collagen secreted by SMCs.

4.3.4 Effects on platelet activation

Hyperlipidaemia and atherosclerotic plaque formation are associated with increased platelet activation and blood hypercoagulability. Elevated LDL levels promote an increase in

thromboxane A2 production, which augments platelet responsiveness. Statin treatment leads to a reduction in collagen- and fibrinogen-induced platelet aggregation and thromboxane production. In a clinical trial, Barale et al. (197) assessed the impact of simvastatin treatment on platelet aggregation response and inflammatory cytokine expression in patients with hypercholesterolemia for a duration of 2 months. The findings demonstrated that in addition to ameliorating lipid distribution, simvastatin treatment also attenuated platelet aggregation rate and reduced circulating levels of pro-inflammatory factors, endothelial markers, and platelet markers. These results indicate that statins effectively reduce lipids, inhibit platelet activation, and improve inflammation levels and EC dysfunction within atherosclerotic plaques associated with primary hypercholesterolemia (Table 5).

4.4 Side effects of statin therapy

Statins demonstrate favourable drug tolerance but are commonly associated with adverse reactions such as hepatotoxicity and myopathy. Hanai et al. (199) have proposed a direct explanation for statin-related muscle toxicity, suggesting that these drugs can induce the expression of the Atrogin1 gene in skeletal muscles, leading to cytotoxic effects combined with interference in muscle differentiation processes like insulin induction. Moreover, some studies, including the final assessment of the JUPITER trial, have raised concerns about an elevated incidence of diabetes as a potential risk associated with the use of statins (200). Additionally, reports of potential toxic effects such as proteinuria and haematuria have also emerged (201).

TABLE 5 Effects of statins on intracellular cell functions in plaques.

	Experimental operation	Result or conclusion
Effects on ECs	A single injection of LDL (4mg/kg, 48 h) induced endothelial injury in rats Endothelial injury was also induced by incubation with LDL (300 mg/L) or ox-LDL (100 mg/L) in ECV304 cells	Simvastatin protects the vascular endothelium against the damages induced by LDL or ox-LDL in rats or cultured ECV304 cells (187)
	Thirty adult male hamsters were divided in three groups (1): hyperlipemic hamsters (HH) fed with 3% cholesterol and 15% butter, (2) hyperlipemic animals (HS) treated daily for 16 weeks by gavage with 0.3-mg/kg simvastatin and (3) normal hamsters. The blood and tissues were collected for biochemical assays and structural analysis.	Simvastatin reduces transcytosis of LDL and is able to restore the endothelial-dependent relaxation by an increase in NO synthesis (185)
	Human pulmonary artery EC was treated with simvastatin (5μM, 24 h)	Statin inhibits EC adhesion and permeability while reducing white blood cell migration, ultimately mitigating the inflammatory response within plaques (198)
Effects on inflammatory cells	Atherosclerotic lesion in rabbit was treated with Atorvastatin (5 mg/kg/d)	Atorvastatin reduces the presence of macrophages, MCP-1, and nuclear factor NF-κB activation within the intima layer (189)
	Normal human PBMCs and THP-1 cells were cultured with inhibitors of HMGR (simvastatin), geranylgeranyltransferase (GGTI-298), farnesyltransferase (FTI-277), and/or caspase-1 (Z-VAD (Ome)-FMK)	Statin activates pro-IL-1 processing and IL-1 release by human monocytes (190)
	1095 patients with non-cardiogenic ischemic stroke were assigned to the pravastatin (n=545) or control groups (n=550), and the endpoints were serum hs-CRP reduction and stroke recurrence	Pravastatin treatment may reduce hs-CRP, and higher hs-CRP levels increase the risk of vascular events (191)
Effects on the arterial SMCs	Studied the ability of statins to arterial myocyte migration and proliferation using *in vitro* and ex vivo models	Fluvastatin, simvastatin, lovastatin, and atorvastatin exhibit dose-dependent inhibition of SMC migration and proliferation (192)
	SMC was pretreated with atorvastatin prior to the determination of IL-18-induced migration	IL-18 stimulates SMC migration in an MMP9-dependent manner, and atorvastatin inhibits this process (193)
	Atorvastatin was used to treat $ApoE^{-/-}$ DM models	Atorvastatin reduces the number of SMCs while promoting collagen fiber synthesis (194)
	Lovastatin treated primary SMC	Statin affects the production of extracellular matrix in SMCs, especially for type I collagen (195)
	Sustained high concentrations of rosuvastatin (100 ng/ml) stimulated VSMCs	Rosuvastatin reduces intracellular ROS levels through autophagy, leading to its vascular protective activity (196)
Effects on platelet activation	In hypercholesterolemic patients allocated to diet (n=20) or a 2-month treatment with diet plus 40 mg simvastatin (n=25)	Simvastatin treatment reduced platelet activation and subclinical inflammation and improved endothelial dysfunction (197)

MCP-1, monocyte chemoattractant protein-1; LDL, low-density lipoprotein; ROS, reactive oxygen species.

Statins are powerful lipid-lowering drugs that can reduce the incidence and mortality of atherosclerosis, and they have been widely used to prevent primary and secondary cardiovascular diseases for more than 25 years. Interestingly, while statins treat atherosclerotic lesions by regulating lipid metabolism, they also regulate the function of various cells in the plaque and the secretion and expression of certain cytokines, thereby influencing the inflammatory response and plaque stability. However, since there may be other factors contributing to atherosclerotic lesions, such as hypertension and diabetes, and statins have some side effects including myopathy, the effect of statin monotherapy may be limited, and patient compliance may be low. Therefore, in clinical practice, statins are often used in combination with other lipid-lowering, antihypertensive, hypoglycaemic, and anti-inflammatory drugs.

4.5 A combination of statins and other drugs

The future of reducing atherosclerotic disease lies in combining statins with other medications. For example, inhibition of the regulatory protein PSCK9 effectively reduces plasma LDL levels, making it an ideal complement to statins (202). Experts propose that the combination of rosuvastatin and ezetimibe is safe and effective for treating hypercholesterolemia or hyperlipidaemia, regardless of diabetes or cardiovascular disease status. The fixed combination of 40 mg rosuvastatin/10 mg ezetimibe has been approved and evaluated (203). Statins can also be combined with fibrates, niacin, and omega-3 fatty acids to lower triglycerides in atherosclerosis development. However, extensive clinical studies are needed to assess the impact on cardiovascular outcomes and risk reduction for patients with hypertriglyceridemia. Hypertension is the primary risk factor for intravascular atherosclerosis, wherein the combination therapy of antihypertensive and statin treatment exhibits superior efficacy compared to monotherapy with antihypertensive agents alone in hypertensive patients without complications (204). The synergistic effect of lipid-lowering and inflammation-suppressing therapies is evident. Dicarboxylic acid, commonly prescribed as an agent for lowering blood sugar levels, not only regulates macrophage function in atherosclerosis but also suppresses inflammatory responses. By combining dimethylformic acid with statins, inflammation can effectively be inhibited while simultaneously reducing blood sugar levels and lipids, thereby enhancing the therapeutic potential for treating atherosclerosis (204, 205). Furthermore, ongoing clinical trials are investigating alternative anti-inflammatory agents targeting the CRP/IL-6/IL-1 axis such as low-dose methylidene and colchicine (206). When combined with aggressive LDL-C therapy, this approach may become the standard treatment for most patients with atherosclerosis. Aspirin and statins are well-established treatments for both atherosclerosis and coronary heart disease due to their cohesive properties and effective reduction of inflammation.

5 Conclusions

This review provides a comprehensive overview of the metabolic regulation in immune, endothelial, and smooth muscle cells and their potential contributions to the pathogenesis of atherosclerosis. Cells exposed to hypoxic conditions undergo distinct alterations in energy metabolism, including augmented glycolysis, impaired fatty acid synthesis, and abnormal amino acid metabolism. Inflammatory processes and lipid accumulation within atherosclerotic plaques are intricately linked to cellular proliferation, migration, senescence, and apoptosis. Different inflammatory responses within plaques affect plaque stability.

Statins and their combinations play an important role in the treatment of atherosclerotic lesions. In this review, the main pharmacological mechanisms of statins and their effects on the function of various cells in atherosclerotic plaques were briefly discussed. Considering the limitations and potential adverse reactions of statins in the treatment of atherosclerotic lesions, a comprehensive regimen is required, which combines statins with other lipid-lowering, antihypertensive, hypoglycaemic, antiplatelet aggregating, and anti-inflammatory drugs.

Although previous animal experiments, *in vitro* experiments, and clinical data analyses have provided extensive research on specific metabolic pathways, products, and the mechanisms and applications of lipid-lowering drugs, our understanding of drugs regulating inflammatory responses and influencing the pathological and physiological processes of atherosclerotic plaques remains limited. The continuous accumulation of clinical research data, utilisation of proteomics analysis, and the integration of advanced technologies will aid in the development of a more robust theoretical framework. This will enhance our understanding of angiogenesis, inflammatory changes, lipid stability, barrier function, atherosclerosis metabolic mechanisms, and treatment-related knowledge. These efforts may lead to the identification of new drug targets for treating atherosclerosis and related diseases, the development of more promising treatment strategies, and the reduction of unnecessary side effects.

Author contributions

LMZ: Writing – original draft, Writing – review & editing. DM: Conceptualization, Supervision, Validation, Writing – review & editing. LW: Conceptualization, Writing – original draft, Writing – review & editing. XS: Writing – original draft. LF: Writing – original draft. LCZ: Writing – original draft. YC: Writing – original draft. YH: Writing – original draft. XW: Writing – original draft. JF: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

EC	endothelial cell
VSMC	vascular smooth muscle cell
LDL	low-density lipoprotein
OXPHOS	oxidative phosphorylation
ATP	Adenosine triphosphate
PPP	pentose phosphate pathway
FAO	fatty acid oxidation
NADPH	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
PFKFB	phosphofructose-2 kinase B
PKM2	pyruvate kinase M2
TCA cycle	tricarboxylic acid cycle
Teff cell	effector T cell
Treg cell	regulatory T cell
TNF	tumor necrosis factor
IFN	Human Interferon
IL	Interleukin
TGF-b	transforming growth factor-b
PDK1	Pyruvic acid dehydrogenase kinase 1
LDH	lactate dehydrogenase
PDH	pyruvate dehydrogenase
HDL	high density lipoprotein
ABCA1	ATP binding cassette transport A1
HIF1a	hypoxia inducible factor 1
Slc1a5	Solute Carrier Family 1, Member 5
IDO1	indoleamine 2,3dioxygenase 1
iNOS	inducible nitric oxide synthase
Pfkfb3	phosphofructose-2-kinase/fructose 2,6-diphosphatase 3
eNOS	endothelial nitric oxide synthase
KLF2	Recombinant Human Krueppel-like factor 2
LOX-1	lectin-like oxidised LDL receptor 1
HRD1	hydroxy-3-methylglutaryl reductase degradation
NRF2	Nuclear Factor erythroid 2-Related Factor 2
HO-1	heme oxygenase-1
NF-kB	nuclear factor kB
d-flow	disturbed blood flow
s-flow	stable flow
ECM	extracellular matrix
LDHA	lactate dehydrogenase A
AMPK	Adenosine monophosphate-activated protein kinases
SAA	Serum Amyloid A protein
SR-BI	scavenger receptor class B type I
IPH	intra plaque hemorrhage
SREBPs	strip sterol regulatory element-binding proteins
GLUT1	glucose transporter 1
FOXP3	forkheadbox Protein 3
DCs	dendritic cells
BCR	B-cell receptor
ApoA1	Apolipoprotein A1
pre-HDL	pre-high density lipoprotein
NLRP3, NACHT-	leucine-rich repeat (LRR)-, and pyrin domain (PYD)-containing protein 3
NO	nitric oxide
CPT1A	Carnitine palmitoyltransferase- 1A
ECM	extracellular matrix

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Peripherally derived myeloid cells induce disease-dependent phenotypic changes in microglia

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In central nervous system (CNS) injury and disease, peripherally derived myeloid cells infiltrate the CNS parenchyma and interact with resident cells, propagating the neuroinflammatory response. Because peripheral myeloid populations differ profoundly depending on the type and phase of injury, their crosstalk with CNS resident cells, particularly microglia, will lead to different functional outcomes. Thus, understanding how peripheral myeloid cells affect the phenotype and function of microglia in different disease conditions and phases may lead to a better understanding of disease-specific targetable pathways for neuroprotection and neurorepair. To this end, we set out to develop an *in vitro* system to investigate the communication between peripheral myeloid cells and microglia, with the goal of uncovering potential differences due to disease type and timing. We isolated peripheral myeloid cells from mice undergoing experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, or acute cerebral ischemia by permanent middle cerebral artery occlusion (pMCAO) at different times after disease and probed their ability to change the phenotype of primary microglia isolated from the brain of adult mice. We identified changes not only dependent on the disease model, but also on the timepoint after disease onset from which the myeloid cells were isolated. Peripheral myeloid cells from acute EAE induced morphological changes in microglia, followed by increases in expression of genes involved in inflammatory signaling. Conversely, it was the peripheral myeloid cells from the chronic phase of pMCAO that induced gene expression changes in genes involved in inflammatory signaling and phagocytosis, which was not followed by a change in morphology. This underscores the importance of understanding the role of infiltrating myeloid cells in different disease contexts and phases. Furthermore, we showed that our assay is a valuable tool for investigating myeloid cell interactions in a range of CNS neuroinflammatory conditions.

KEYWORDS

myeloid cells, microglia, multiple sclerosis, ischemic stroke, neuroinflammation

1 Introduction

Diseases of the central nervous system (CNS) are etiopathologically diverse, ranging from acute disorders such as traumatic brain injury and stroke, to chronic neurodegenerative diseases like multiple sclerosis (MS) and Alzheimer's. Because an immune-inflammatory response is common to virtually all of them (Lucas et al., 2006), targeting specific immune processes has been pursued for the treatment of neurological diseases (Correale et al., 2017; Lambertsen et al., 2019; Ruiz et al., 2019; Iadecola et al., 2020).

Cells of the myeloid lineage play an important role in the response to CNS injury and disease (Brendecke and Prinz, 2015). Microglia are the CNS-resident myeloid cells and are key regulators of neural homeostasis (Nayak et al., 2014). They are also a crucial component of the response to injury/disease and, in disease states, they undergo profound phenotypic changes signaling their activation. These include morphological changes, such as the transition to an amoeboid shape, and transcriptional changes that lead to increased expression and release of cytokines and chemokines (DiSabato et al., 2016; Becher et al., 2017). The advent of single cell omics has made it clear that microglial responses are highly heterogeneous and vary in time and space depending on the type, severity, and localization of injury/disease within the CNS. This has led to the identification of multiple coexisting microglial phenotypes with distinct and often opposite functions (Zheng et al., 2022; Vainchtein et al., 2023).

In MS, microglia can have both detrimental and protective effects (Rawji and Yong, 2013; Guerrero and Sicotte, 2020; Plastini et al., 2020). They are involved in demyelination (Zrzavy et al., 2017; Savarin et al., 2018), and in the experimental autoimmune encephalomyelitis (EAE) model, the early depletion of microglia or reduction of their activation improves the outcome (Goldmann et al., 2013; Nissen et al., 2018). Microglia can also be protective in EAE through production of anti-inflammatory cytokines and clearance of myelin debris (Kocur et al., 2015; Wlodarczyk et al., 2015). Depletion of microglia at late timepoints after EAE exacerbates inflammation and demyelination (Tanabe et al., 2019), indicating a finely tuned microglial function that differs according to the disease phase.

In acute neurological disease, such as ischemic stroke, microglia are the first cells to be activated and, as in MS, they have dual functions (Ma et al., 2017; Qin et al., 2019). Microglia release pro-inflammatory cytokines after experimental stroke, leading to disruption of the blood-brain-barrier (Clausen et al., 2008; Jolivel et al., 2015), synapse elimination, and neuronal death (Neher et al., 2013; Shi et al., 2021). Reducing the number of activated microglia increases the number of new neurons and improves functional outcome (Liu et al., 2007). Microglia can also limit the ischemic damage by reducing the size of the ischemic lesion, thus improving the outcome (Lalancette-Hébert et al., 2007; Lambertsen et al., 2009; Szalay et al., 2016), and the phagocytic properties of microglia are important in the recovery post-stroke (Kawabori et al., 2015).

Peripheral immune cells of the myeloid lineage are the first to infiltrate the CNS in neurological disease. Neutrophils are present in the inflamed areas shortly after the onset of ischemic stroke and EAE (Lewis et al., 2014; Beuker et al., 2021) and have been proposed as a biomarker for MS (Bisgaard et al., 2017). Macrophages also infiltrate the ischemic brain in large numbers early after stroke (Beuker et al., 2021), and in EAE, where T-cells are required for the induction of disease, monocytes were shown to be necessary for disease progression (Ajami et al., 2011). When participating in the neuroinflammatory response, peripheral myeloid cells carry out many of the same functions as their CNS-resident counterpart, microglia. However, their disease-induced phenotypic changes may occur with different timing and localization, leading to distinct and sometimes opposing functions (Ajami et al., 2011; Giles et al., 2018; Werner et al., 2020; Gao et al., 2017, 2023). Peripherally derived myeloid cells interact with CNS cells driving the acute neuroinflammatory response but can also persist long-term in the CNS parenchyma participating in the chronic neuroinflammatory response (Garcia-Bonilla et al., 2016), where they sustain various processes (Brendecke and Prinz, 2015; Giles et al., 2018; Lambertsen et al., 2019).

Because of the co-existence of microglia and peripherally derived myeloid cells in CNS disease, it is important to understand how their cross-talk might affect the outcome in different CNS pathologies (degenerative vs. traumatic) and disease stages (acute vs. chronic). The complexity and plurality of CNS cellular networks make it challenging to study discrete cell-cell interactions *in vivo*, and even more so under inflammatory conditions. A further obstacle is the functional and transcriptional similarities of the two populations, making it difficult to distinguish them *in vivo*. To overcome these challenges, we developed an *in vitro* system to investigate the communication between peripheral myeloid cells and microglia, with the goal of uncovering potential differences due to disease type and timing.

Abbreviations: Ccr, CC chemokine receptor; CD, cluster of differentiation; cDNA, copy deoxyribonucleic acid; CFSE, carboxyfluorescein succinimidyl ester; Cmklr, chemerin chemokine-like receptor; CNS, central nervous system; Cxcr, C-X-C motif chemokine receptor; DAPI, 4′,6-Diamidino-2-Phenylindole; Dpi, days post injury/induction; EAE, experimental autoimmune encephalomyelitis; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; Gapdh, Glyceraldehyde-3-Phosphate Dehydrogenase; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; Il1b, interleukin-1 beta; Lrp, low density lipoprotein receptor-related protein; MACS, magnetic-activated cell sorting; MCA, middle cerebral artery; Mertk, MER proto-oncogene tyrosine kinase; MS, multiple sclerosis; Olig2, oligodendrocyte transcription factor 2; PBS, phosphate buffered saline; PFA, paraformaldehyde; pMCAO, permanent middle cerebral artery occlusion; PCR, polymerase chain reaction; PPR, pathogen recognition receptor; RNA, ribonucleic acid; SEM, standard error of the mean; Tnf, tumor necrosis factor; Tnfrsf, tumor necrosis factor receptor; Trem2, triggering receptor expressed on myeloid cells 2.

2 Materials and methods

2.1 Animals

Adult female C57Bl/6 mice (2 months old) were used for this study. The animals were kept in group housing under controlled temperature and humidity, a 12/12-h light/dark cycle, and with food and water *ad libitum*. The experiments carried out at the

Animal Core Facility of The Miami Project to Cure Paralysis were performed according to protocols and guidelines approved by the Institutional Animal Care and Use Committee of the University of Miami. The experiments conducted at the Animal Facility of the University of Southern Denmark were performed in accordance with approved permits (J. no. 2019-15-0201-01620).

2.2 Permanent middle cerebral artery occlusion

The distal part of the left middle cerebral artery (MCA) was permanently coagulated to induce an experimental stroke, as previously described (Lambertsen et al., 2009; Yli-Karjanmaa et al., 2019). The surgery was performed under anesthesia with a mix of Hypnorm [fentanyl citrate (0.135 mg/mL, VetaPharma) and fluanisone (10 mg/mL, VetaPharma)], Midazolam (5 mg/mL, Hameln), and sterile water in the ratio 1:1:2. When the mouse was fully sedated, the skin was incised from the eye to the ear on the left side, the muscles were dissected to expose the skull, and a small hole was drilled above the MCA. The MCA was coagulated by pinching it with bipolar forceps and administering an electric current, hereby inducing a permanent middle cerebral artery occlusion (pMCAO). Mice were given subcutaneous injections of analgesics [Temgesic (0.001/20 mg body weight buprenorphine; Indivior)] every 8 h for 24 h, starting at the time of surgery, while being kept in a temperature-controlled heating cabinet. Eyes were coated in Viscotears ointment (2 mg/g, Bausch and Lomb) to prevent dehydration. The mice were euthanized at acute disease (1 day post-injury, dpi) or chronic disease (14 dpi) for isolation of peripheral myeloid cells, using an intraperitoneal injection of 0.2 mL pentobarbital (200 mg/mL) containing lidocaine (10 mg/mL) followed by transcardial perfusion with 20 ml phosphate-buffered saline (PBS).

2.3 Experimental autoimmune encephalomyelitis

Mice were induced with experimental autoimmune encephalomyelitis (EAE) following a protocol well established in the laboratory (Brambilla et al., 2011; Madsen et al., 2020). Briefly, on day 0 and 2, pertussis toxin (PTX, List Biological Laboratories, #181) diluted in PBS was injected intraperitoneally (500 μg/mouse). On day 1, myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}, Bio-Synthesis, #A9349-1) emulsified in complete Freund's adjuvant was injected subcutaneously (0.33 mg/mouse). Disease development was tracked daily, and locomotor dysfunction was scored on a standard 0–6 scale where 0 = no symptoms; 1 = loss of tail tone; 2 = fully flaccid tail; 3 = complete hind limb paralysis; 4 = complete forelimb paralysis; 5 = moribund; 6 = dead. Mice were euthanized at acute disease (20 days post-induction, dpi) or chronic disease (30 dpi) for isolation of peripheral myeloid cells. Inclusion criterion for the acute timepoint was presence of symptoms for a maximum of 7 days. Inclusion criterion for the chronic timepoint was presence of symptoms for a minimum of 17 days.

2.4 Primary microglia cultures

Primary microglia cultures were generated from whole brains of naïve adult (2 months old) C57Bl/6 mice. Following transcardial perfusion with PBS, brains were dissected out and enzymatically dissociated into a single-cell suspension using Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, #130-092-628) followed by myelin removal with debris removal solution (Miltenyi Biotec, #130-109-398). Microglia were then isolated by magnetic-activated cell sorting (MACS) with LS columns (Miltenyi Biotec, #130-042-401) after incubation with anti-CD11b conjugated magnetic microbeads (Miltenyi Biotec, #130-093-634) according to the manufacturer's protocol. Cells were seeded on poly-D-lysine coated plates (Sigma-Aldrich, #P7280) and maintained in complete medium consisting of RPMI 1640 Medium (Gibco, A1049101) supplemented with 20% L929 fibroblast conditioned media, 10% fetal bovine serum (FBS) (GEMINI, #900-108), 50 μM beta-mercaptoethanol, and 1% antibiotic-antimycotic (Gibco, #15240-062). Cells were plated in 24-well plates at a density of 37,000 cells/cm^2 for RNA isolation, and in 24-well plates (indirect co-cultures) or 96-well plates (direct co-cultures) at a density of 16,000 cells/cm^2 for morphological analysis. Microglia were cultured for 5 days with a partial media change (50%) every other day.

The primary microglia culture was assessed for purity by immunohistochemical staining and cell counting. Paraformaldehyde (PFA) fixed cells were incubated with goat anti-Iba1 (1:200, Novus Biologicals, #NB100-1028), rat anti-glial fibrillary acidic protein (GFAP) (1:500, Invitrogen, #13-0300) and rabbit anti-oligodendrocyte transcription factor 2 (Olig2) (1:200, Sigma-Aldrich, #AB9610) overnight at 4°C, followed by washing and subsequent 1-h incubation with secondary antibodies (1:750, Thermo Fisher, Donkey anti-goat 594 #A-11058; Donkey anti-rat 488 #A-21208; Donkey anti-rabbit 647 #A-31573). The cells were washed, and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:6000, Invitrogen, #D1306). Seventeen (17) randomly selected fields of view from 6 individual cell culture wells were imaged at 20X magnification, and the percentage of $Iba1^+$ microglia, $GFAP^+$ astrocytes, $Olig2^+$ oligodendrocytes and $Iba1^-GFAP^-Olig2^-$ cells out of the total number of $DAPI^+$ cells were estimated using ImageJ software.

2.5 Peripheral myeloid cell isolation

Since secondary lymphoid organs, such as the spleen, coordinate the immune response in immune-mediated pathologies such as MS and EAE, (Zhu et al., 2007), while in ischemic stroke the direct output of myeloid cells from the bone marrow drives the peripheral innate immune response (Courties et al., 2015), we isolated myeloid cells from the spleen of EAE induced mice and from the bone marrow of pMCAO injured mice at acute and chronic disease phases. After perfusion with PBS, spleen or bone marrow was isolated and manually dissociated into single cell suspensions followed by red blood cell lysis. Myeloid cells

were isolated by magnetic bead cell separation with anti-F4/80 MicroBeads (Miltenyi Biotec, #130-110-443) and LS columns (Miltenyi Biotec, #130-042-401) according to the manufacturer's protocol. For indirect co-cultures, 100,000 myeloid cells in 1 ml media (density 100 cells/μl) were plated in cell culture inserts (cellQART 24-well Cell Culture Insert, Sterlitech, #9320402), which were then placed on top of naïve microglia that were maintained in culture for 5 days. For direct co-cultures, myeloid cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (2 μM dilution in PBS, BioLegend, #423801) for 10 min at 37°C. Then 50,000 myeloid cells in 500 μl media were plated in the 24-well plates and 10,000 myeloid cells in 100 μl media were plated in the 96-well plates (density 100 cells/μl) directly on top of naïve microglia maintained in culture for 5 days. Naïve unstimulated microglia without myeloid cells were used as controls. Three-five (3–5) biological replicates/condition with 2–6 technical replicates for gene expression analysis and 1–8 technical replicates for morphological analysis were plated.

2.6 High content analysis of cell morphology

After 24 h in co-culture, microglia (either alone or mixed with peripheral myeloid cells) were fixed with 4% PFA, washed, and stained with CellMask Deep Red Plasma Membrane Stain (1:5000, Invitrogen, #C10046) and DAPI (1:2000, Invitrogen, #D1306). Cells were imaged with the Opera Phenix Plus High Content Screening System (Perkin Elmer), and micrographs were analyzed with Harmony® software package. Multiple filters were applied to eliminate artifacts using empirically determined cutoffs. For the purpose of this study, we extracted the following parameters: area, perimeter, roundness, length/width ratio, and percentage of elongated cells (with length/width ratio above 3). Within each well, multiple fields of view were automatedly sampled, and the average value per well was calculated for each parameter. For direct co-cultures, the algorithm was instructed not to include CFSE-labeled cells (peripheral myeloid cells) in the analysis. Morphological data were calculated as the mean of 1–8 technical replicates per mouse. To account for variability between cultures, the results were normalized to internal unstimulated microglia controls, before being calculated as fold change of the acute timepoint with direct exposure.

2.7 Collection of microglia for RNA extraction

Direct co-cultures underwent fluorescence-activated cell sorting (FACS) to separate microglia from peripheral myeloid cells after 24 h in co-culture. Briefly, cells were lifted using a cell scraper, spun down, and resuspended in DMEM-F12 (Gibco, #11320-033) supplemented with 5% FBS (GEMINI, #900-108). Cell suspensions were passed through a strainer to ensure single cell separation, and DAPI was added to label dead cells (NucBlue Fixed Cell Stain ReadyProbes reagent, Invitrogen, #R37606). Live $DAPI^{-}CFSE^{-}$ microglia were sorted directly into RNA extraction buffer from the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, #12204-01). Microglia from indirect co-cultures after 24 h in co-culture and unstimulated microglia controls were collected as described above and immediately resuspended in RNA extraction buffer from the Arcturus PicoPure RNA Isolation Kit. In all experiments, 2–6 technical replicates were pooled to obtain 3–5 samples/condition.

TABLE 1 Primers used for RT-PCR.

Gene	Forward primer	Reverse primer
Ccr6	5′GGT GCA GGC CCA GAA CTC CA	5′TGC AGC TCC GGC CCA CTT TG
Cd40	5′CTA TGG GGC TGC TTG TTG AC	5′CCA TCG TGG AGG TAC TGT TT
Cmklr1	5′CTG GTG GTG ATC TAC AGC TT	5′ACA GTG TTC ACG GTC TTC TT
Cxcr3	5′CTC CTC TTC TTG CTG GGG CTG CTA	5′GAA GGT GTC CGT GCT GCT CA
Gapdh	5′GAG GCC GGT GCT GAG TAT GTC GTG	5′TCG GCA GAA GGG GCG GAG ATG A
Il1b	5′CTT CAA ATC TCA CAG CAG CAC ATC	5′CCA CGG GAA AGA CAC AGG TAG
Lrp1	5′GCG GTG TGA CAA CGA CAA T	5′GCA CTT GAA CTG GGT ACT GG
Mertk	5′AGA CTC CCA GTC AAC CAC AG	5′CAG GAG GTA GGA GCT TTG AT
Tnf	5′AGG CAC TCC CCC AAA AGA TG	5′TCA CCC CGA AGT TCA GTA GAC AGA
Tnfrsf1a	5′GCC CGA AGT CTA CTC CAT CAT TTG	5′GGC TGG GGA GGG GGC TGG AGT TAG
Tnfrsf1b	5′GCC CAG CCA AAC TCC AAG CAT C	5′TCC TAA CAT CAG CAG ACC CAG TG
Trem2	5′CAG CCC TGT CCC AAG CCC TCA AC	5′CTC CTC ACC CAG CTG CCG ACA CC

2.8 Gene expression analysis

Microglial RNA was isolated with the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, #12204-01). RNA concentration and purity were measured using a 2100 Agilent Bioanalyzer. RNA was reverse-transcribed to cDNA with the Sensiscript RT Kit (Qiagen, #205211) together with random primers (Promega, #C1181). Semiquantitative RT-PCR was performed with PowerUp SYBR Green Master Mix (Applied Biosystems, #A25742) and specific primers for the genes of interest (Table 1), according to the manufacturer's instructions. cDNA samples from the pMCAO co-cultures underwent pre-amplification (14 cycles at 57°C) due to low concentrations. The PCR data were normalized to *Gapdh* expression before being calculated as fold change of unstimulated naïve microglia using the deltadeltaCt method.

2.9 Statistical analysis

All data are expressed as mean ± SEM of 3–5 biological replicates. Data were analyzed with unpaired two-tailed *t*-test, and

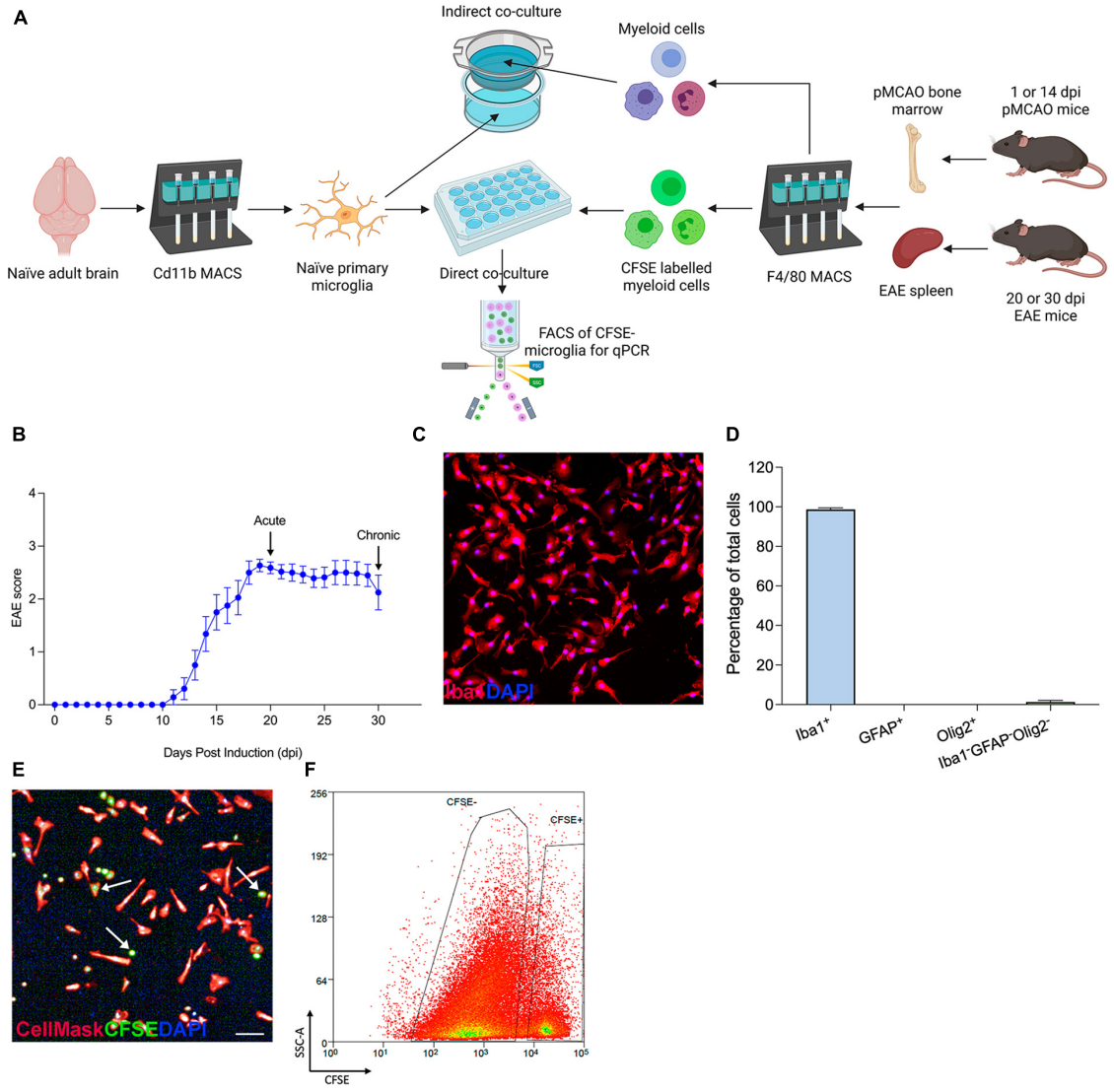

FIGURE 1
In vitro assay setup and validation. **(A)** Schematics of co-culture experiments. **(B)** Daily clinical scores of the EAE mice used in the present study. Data are shown as mean ± SEM, $n = 14$. **(C)** Representative 10X image of Iba1$^+$DAPI$^+$ naïve microglia. **(D)** Assessment of microglial purity by quantification of Iba1$^+$ microglia, GFAP$^+$ astrocytes, Olig2$^+$ oligodendrocytes, and Iba1$^-$GFAP$^-$Olig2$^-$ cells out of total DAPI$^+$ cells. Data are expressed as mean ± SEM of 17 randomly selected areas imaged from 6 cell culture wells. **(E)** Representative image of a direct co-culture, with microglia stained with CellMask (red) and peripherally derived myeloid cells labeled with CFSE (green); scale bar = 100 μm. Arrows point at examples of CFSE-stained myeloid cells. **(F)** CSFE$^-$ microglia isolated by FACS to exclude peripherally derived CSFE$^+$ myeloid cells. Figure created with BioRender.com.

differences between groups were considered statistically significant at p-values ≤ 0.05.

3 Results

3.1 *In vitro* assay to study the interaction between peripherally derived myeloid cells and microglia

To investigate the cell-cell communication between myeloid cell populations in different disease conditions, we devised an *in vitro* assay in which peripherally derived myeloid cells isolated from mice undergoing EAE or cerebral ischemia (pMCAO) were co-cultured with microglia. This enabled us to study both direct communication requiring cell-to-cell contact and indirect communication driven by soluble factors between peripherally derived and CNS-resident myeloid cells. Adult microglia were cultured from naïve mice for 5 days and then exposed to peripherally derived myeloid cells (either from pMCAO or EAE mice) that were plated directly over the microglia monolayer or seeded in cell culture inserts (Figure 1A). In EAE, myeloid cells were obtained from the spleen at 20 dpi (the most acute phase of disease) and 30 dpi (during chronic disease) (Figure 1B). In pMCAO, myeloid cells were obtained from the bone marrow at 1 dpi (acute disease) and 14 dpi (chronic disease). The purity of adult primary microglia was confirmed by immunohistochemistry, with 99% of cells being positive for Iba1, and no contamination

by GFAP$^+$ astrocytes or Olig2$^+$ oligodendrocytes (Figures 1C, D). In direct co-cultures (Figure 1E), to allow for discrimination of peripherally derived myeloid cells from microglia, myeloid cells were pre-labeled with CFSE and CFSE$^-$ microglia were isolated by FACS (Figure 1F). Taken together, these validation experiments confirm that our *in vitro* assay works as intended and can be used to study different types of interactions between myeloid cell populations.

3.2 Peripherally derived myeloid cells induce disease-specific changes in microglial gene expression

Myeloid cells respond to injury/disease by transitioning to an activated phenotype that is often characterized by upregulation of inflammatory genes such as cytokines and chemokines (Brendecke and Prinz, 2015; Becher et al., 2017). To investigate how peripheral myeloid cells in acute traumatic and chronic neurological disease may differ in their ability to drive microglial responses, we analyzed the expression of key inflammatory genes in microglia from our co-culture systems. Myeloid cells from both pMCAO and EAE mice increased microglial expression of *Tnf* compared to naïve unstimulated microglia, but no differences were observed between acute and chronic myeloid cell exposure (Figure 2A). Expression of TNF receptors *Tnfrsf1a* and *Tnfrsf1b* was also increased compared to baseline levels (Figures 2B, C). Direct contact with pMCAO myeloid cells induced higher expression of *Tnfrsf1a* and *Tnfrsf1b* when cells were obtained at the chronic timepoint, whereas direct contact with EAE myeloid cells induced lower expression of *Tnfrsf1a* and *Tnfrsf1b* when cells were obtained at the chronic timepoint. The highest expression of *Tnfrsf1a* was observed in pMCAO (chronic 14 dpi), and the highest expression of *Tnfrsf1b* was in EAE (acute 20 dpi) (Figures 2B, C). Interestingly, indirect interaction had minimal effect on microglial expression of these *Tnf* signaling genes, except for *Tnfrsf1b* which was elevated in both acute and chronic EAE (Figure 2C). For *Il1b*, direct contact with myeloid cells from acute disease (both pMCAO and EAE) increased microglial expression levels (Figure 2D). Finally, expression of *Cd40*, a member of the TNF superfamily whose ligand, CD40L, is important for microglial activation in inflammatory conditions (Ponomarev et al., 2006), was elevated by direct contact with chronic EAE myeloid cells compared to acute (Figure 2E). A similar trend ($p = 0.065$) was observed with pMCAO myeloid cells (Figure 2E).

To look further into microglial inflammatory changes induced by myeloid cells, we examined genes involved in chemokine signaling, which are important for microglial migration toward sites of injury/disease (Becher et al., 2017; Ifergan and Miller, 2020). *Cmklr1*, the receptor for chemerin, was mildly elevated over baseline levels with EAE-derived cells at both timepoints, and with pMCAO-derived cells, but only with direct cell-cell interaction (Figure 3A). *Cxcr3* expression changed primarily with pMCAO myeloid cells in direct contact with microglia, showing a significant increase in chronic cells over acute (Figure 3B). With EAE-derived myeloid cells, *Cxcr3* changed minimally, showing a significant increase in acute over chronic cells after indirect cell-cell interaction (Figure 3B). No notable differences were found in *Ccr6* in any of the conditions (Figure 3C).

Next, we tested whether myeloid cells might affect the microglial phagocytic function. Phagocytosis is key in maintaining CNS homeostasis, and in disease states it is important to clear toxic cell debris (Fu et al., 2014). We examined the expression of *Trem2*, *Mertk*, and *Lrp1*, which have all been shown to be important for microglia phagocytic properties (Fu et al., 2014; Chuang et al., 2016; Shi et al., 2021). Myeloid cells from the chronic phase of pMCAO induced a higher expression of microglial *Trem2* compared to acute pMCAO cells, both with direct and indirect interaction (Figure 3D). Microglial *Trem2* expression with direct exposure to chronic pMCAO myeloid cells was the highest compared to naïve microglia (Figure 3D). Interestingly, *Trem2* changes with EAE myeloid cells were minimal. *Trem2* was mildly elevated over baseline levels in all conditions, and acute EAE myeloid cells induced higher expression than chronic EAE myeloid cells with indirect interaction (Figure 3D). A similar pattern with EAE myeloid cells was observed for *Mertk*, where expression was higher with acute than chronic cells after indirect interaction (Figure 3E). pMCAO myeloid cells did not induce any changes in *Mertk* expression (Figure 3E). Similar to *Trem2*, *Lrp1* was higher with chronic pMCAO myeloid cells compared to acute. EAE myeloid cells increased *Lrp1* expression after indirect contact, with no difference between acute and chronic (Figure 3F).

Collectively, these data illustrate that peripherally derived myeloid cells can induce transcriptional changes in naïve microglia, dependent on disease type and stage and mode of interaction (direct or indirect).

3.3 Peripheral myeloid cells from EAE mice induce changes in microglial morphology

Microglial activation driven by injury and disease is denoted by a series of phenotypic changes that include morphological adaptations whereby microglia transition from a ramified, branched phenotype to an ameboid phenotype with larger cell body and shorter processes (Woodburn et al., 2021). To further investigate whether myeloid cells changed microglial activation state, we evaluated microglial morphology using high content analysis, focusing on area, roundness, perimeter, length/width ratio, and quantifying the percentage of elongated cells. Overall, the morphological changes were relatively mild in all experimental conditions (Figures 4A–E). When exposed to myeloid cells from pMCAO mice, the most notable change was observed in cell size, where indirect cell interaction led to a reduced cell area (Figure 4A) and perimeter (Figure 4C) with acute myeloid cells, showing a significant difference compared to chronic. However, the microglia exposed to pMCAO myeloid cells appeared to have a similar morphology across conditions (Figure 4F). When exposed to myeloid cells from EAE mice, direct contact most notably affected microglial length/width ratio (Figure 4D) and % of elongated cells (Figure 4E), which decreased with acute interaction compared to chronic. These changes were evident as microglia became visibly more rounded (Figures 4B, G).

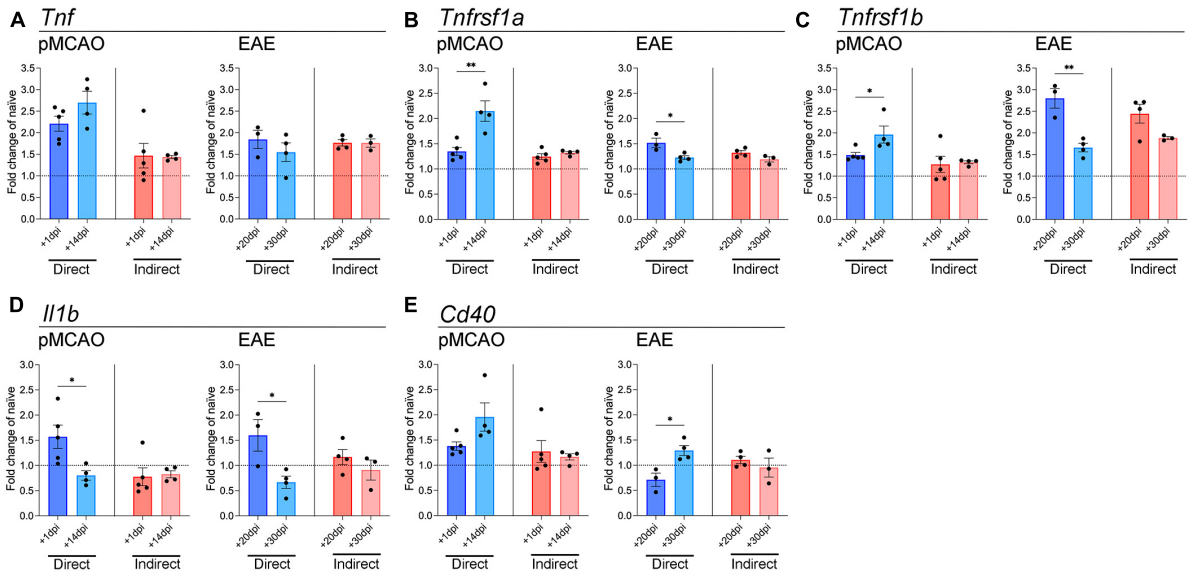

FIGURE 2
Gene expression analysis of pro-inflammatory cytokines and TNF receptor superfamily members in microglia. qPCR analysis of *Tnf* **(A)**, *Tnfrsf1a* **(B)**, *Tnfrsf1b* **(C)**, *Il1b* **(D)**, and *Cd40* **(E)** in adult microglia exposed to pMCAO or EAE myeloid cells. Direct: Direct contact and interaction between myeloid cells and microglia. Indirect: Indirect interaction between myeloid cells and microglia, only via soluble factors. Data are expressed as fold change of naïve microglia after normalization to *Gapdh* gene expression. The horizontal line at $y = 1$ indicates baseline expression in naïve microglia. Data are presented as mean ± SEM of 3–5 biological replicates. Each biological replicate is derived from 2–6 technical replicates. $*p \leq 0.05$, $**p \leq 0.01$, unpaired two-tailed *t*-test.

4 Discussion

Although microglia and infiltrating myeloid cells have previously been thought to act in similar ways in neurological disease, RNA sequencing studies revealed distinct microglia and myeloid cell populations with key differences in disease conditions (Lewis et al., 2014; Yamasaki et al., 2014; Guo et al., 2021; Zheng et al., 2022). Despite the considerable involvement of peripheral myeloid cells in neurological disease, we still lack a full understanding of how they drive different disease processes and affect CNS-resident cells. In the current study, we developed an *in vitro* assay to identify potential differences between acute traumatic and chronic neurodegenerative disease by investigating how peripheral myeloid cells from pMCAO or EAE mice affect naïve microglia. As the spleen coordinates the immune response in MS and EAE (Zhu et al., 2007) and cells from the bone marrow drive the immune response in ischemic stroke (Courties et al., 2015), we isolated myeloid cells from the spleen of EAE induced mice and from the bone marrow of pMCAO injured mice at acute and chronic disease phases. We found key differences in their ability to modulate gene expression and morphology of microglia, not only between the two disease models, but also through the course of disease. These changes were dependent on whether the cells were in direct contact or had interactions via soluble factors only. Peripheral myeloid cells increased microglial expression of most of the investigated genes compared to the baseline expression. This validates our *in vitro* system, as an increase in microglial gene expression is expected with exposure to myeloid cells derived from disease conditions.

We tested *Il1b* expression as it is released from microglia and infiltrating myeloid cells acutely in neuroinflammatory conditions and is involved in EAE progression and ischemic damage (Clausen et al., 2008; Yang et al., 2014; Lévesque et al., 2016). The finding that peripheral myeloid cells from the acute phase of both EAE and pMCAO mice induced higher *Il1b* expression in microglia compared to chronic phase myeloid cells suggests that activated peripherally derived myeloid cells might activate pathogen recognition receptors (PRRs) and inflammasome assembly acutely (Lee et al., 2013; Yang et al., 2014). Interestingly, even though microglial *Tnf* expression was increased upon interaction with peripheral myeloid cells from both disease models, no changes were seen between acute and chronic timepoints, indicating that differences between disease phases are not triggered by peripheral myeloid cells. Expression of microglial *Tnfrsf1a* and *Tnfrsf1b* was increased with direct exposure to peripheral myeloid cells from both pMCAO and EAE mice. In the acute phase of EAE, peripheral myeloid cells highly express TNF and other cytokines (Renno et al., 1995; Ifergan and Miller, 2020), which may cause the increased expression of microglial *Tnfrsf1a* and *Tnfrsf1b* after interaction with acute EAE peripheral myeloid cells in our experimental setting. In stroke, increased TNF receptor expression peak days after pMCAO induction (Lambertsen et al., 2007), supporting our finding that peripherally derived chronic pMCAO myeloid cells increase microglial TNF receptor expression to a larger extent than acute myeloid cells. It is noteworthy that peripherally derived myeloid cells in both disease models induced changes in TNFR1 and TNFR2 simultaneously, as these two receptors have distinct and opposing functions in neurological disease (Dong et al., 2016; Papazian et al., 2021). This suggests that peripheral myeloid cells can induce both detrimental and protective effects in the microglia.

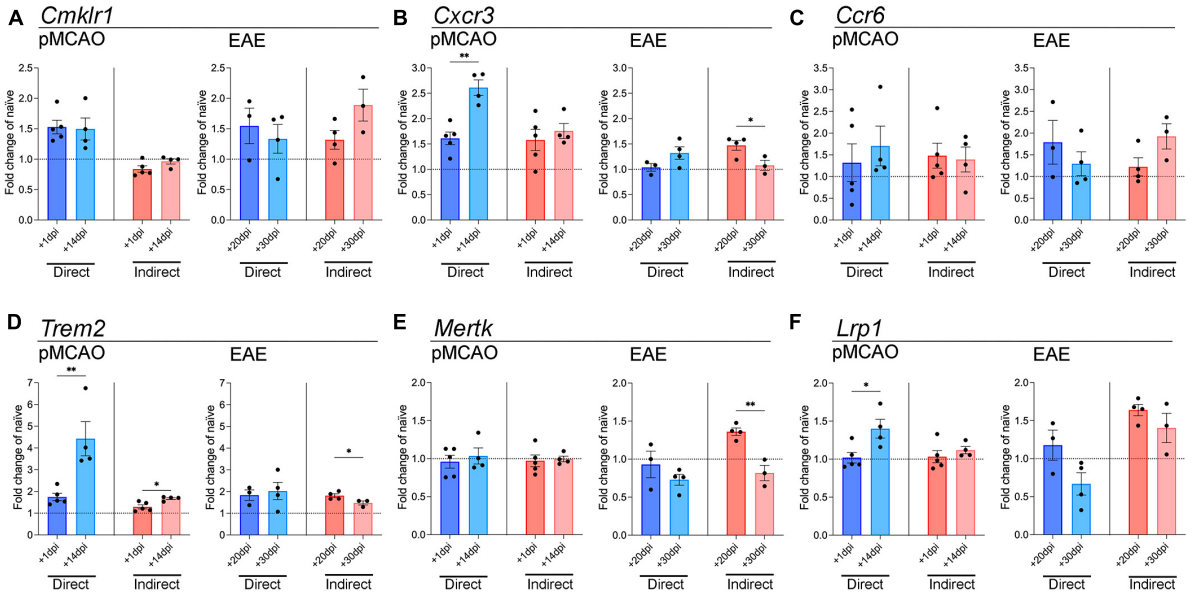

FIGURE 3
Gene expression analysis of chemokine receptor and phagocytosis genes in microglia. qPCR analysis of *Cmklr1* **(A)**, *Cxcr3* **(B)**, *Ccr6* **(C)**, *Trem2* **(D)**, *Mertk* **(E)**, and *Lrp1* **(F)** in adult microglia exposed to pMCAO or EAE myeloid cells. Direct: Direct contact and interaction between myeloid cells and microglia. Indirect: Indirect interaction between myeloid cells and microglia, only via soluble factors. Data are expressed as fold change of naïve microglia after normalization to *Gapdh* gene expression. The horizontal line at $y = 1$ indicates baseline expression in naïve microglia. Data are presented as mean ± SEM of 3–5 biological replicates. Each biological replicate is derived from 2–6 technical replicates. $*p \leq 0.05$, $**p \leq 0.01$, unpaired two-tailed *t*-test.

CD40 is upregulated with microglial activation and increases the release of cytokines and chemokines (Benveniste et al., 2004; Plastini et al., 2020). CD40 has a major role in EAE progression (Hart et al., 2005). Indeed, full activation of microglia in EAE depends on CD40, and lack of CD40 leads to lower T-cell proliferation and less severe disease (Ponomarev et al., 2006). The increase in microglial *Cd40* expression with exposure to peripheral myeloid cells from chronic EAE indicates that myeloid cells can induce *Cd40* expression, which might drive disease progression in the chronic stage through increased microglial activation and possibly T-cell proliferation.

Peripheral myeloid cells might have a more detrimental phenotype at the chronic timepoint following pMCAO, for example by providing "eat-me" signals to microglia, which in turn upregulate the expression of genes involved in phagocytosis. Thus, the increases in *Trem2* and *Lrp1* in microglia exposed to peripherally derived chronic pMCAO myeloid cells could be protective by eliminating detrimental infiltrating myeloid cells (Neumann et al., 2008). This is supported by several studies reporting an important role of Trem2 and Lrp1 in phagocytosis and neuroprotection in neurological disease (Takahashi et al., 2005; Fernandez-Castaneda et al., 2013; Kawabori et al., 2015; Chuang et al., 2016). Increased phagocytosis may also be detrimental if the targets are viable neurons (Neher et al., 2013; Shi et al., 2021), leading to a worsening of the ischemic damage at the chronic stage. However, phagocytosis of viable neurons depends primarily on Mertk (Neher et al., 2013; Shi et al., 2021), which was not upregulated in microglia following exposure to pMCAO peripheral myeloid cells in our study. Future studies using metabolomics and/or lipidomics, as well as scRNAseq, would help identify and quantify pathways and networks of cellular lipids and metabolites that change in microglia following exposure to myeloid cells at acute and chronic time points after EAE and pMCAO.

RNA sequencing studies reveal that peripheral myeloid cells from EAE mice upregulate multiple soluble cytokines and chemokines, especially in the acute phase of disease (Lewis et al., 2014; Yamasaki et al., 2014; Gao et al., 2023). This may explain the upregulation in microglial *Trem2*, *Mertk*, and *Cxcr3* that we observed with indirect contact with peripheral myeloid cells from acute EAE. Additionally, several studies demonstrated that infiltrating monocytes have very different transcriptional profiles at onset and peak phases of EAE compared to the recovery phase (Yamasaki et al., 2014; Gao et al., 2023). This fits well with our data, where we found microglia interacting with peripheral myeloid cells from acute EAE to have higher gene expression than with myeloid cells from chronic EAE, with the exception of *Cd40*. Also it was the exposure to acute EAE peripheral myeloid that altered microglial morphology to become more rounded, which is a hallmark of microglial activation (Stence et al., 2001; Woodburn et al., 2021). Overall, peripherally derived myeloid cells from acute EAE appear to be the most effective at inducing microglia activation, leading to changes in both gene expression and morphology.

This study prioritized investigating changes in gene expression, as a measure of whether peripheral myeloid cells were able to induce changes in naïve microglia using our *in vitro* assay. Future studies should aim to investigate whether the changes observed in microglial gene expression, persist as changes in protein levels. Moreover, as microglia will be activated in disease conditions (Woodburn et al., 2021), repeating the study with microglia isolated from EAE and pMCAO mice, to study the effect of activated peripheral myeloid cells on activated microglia, should be persued.

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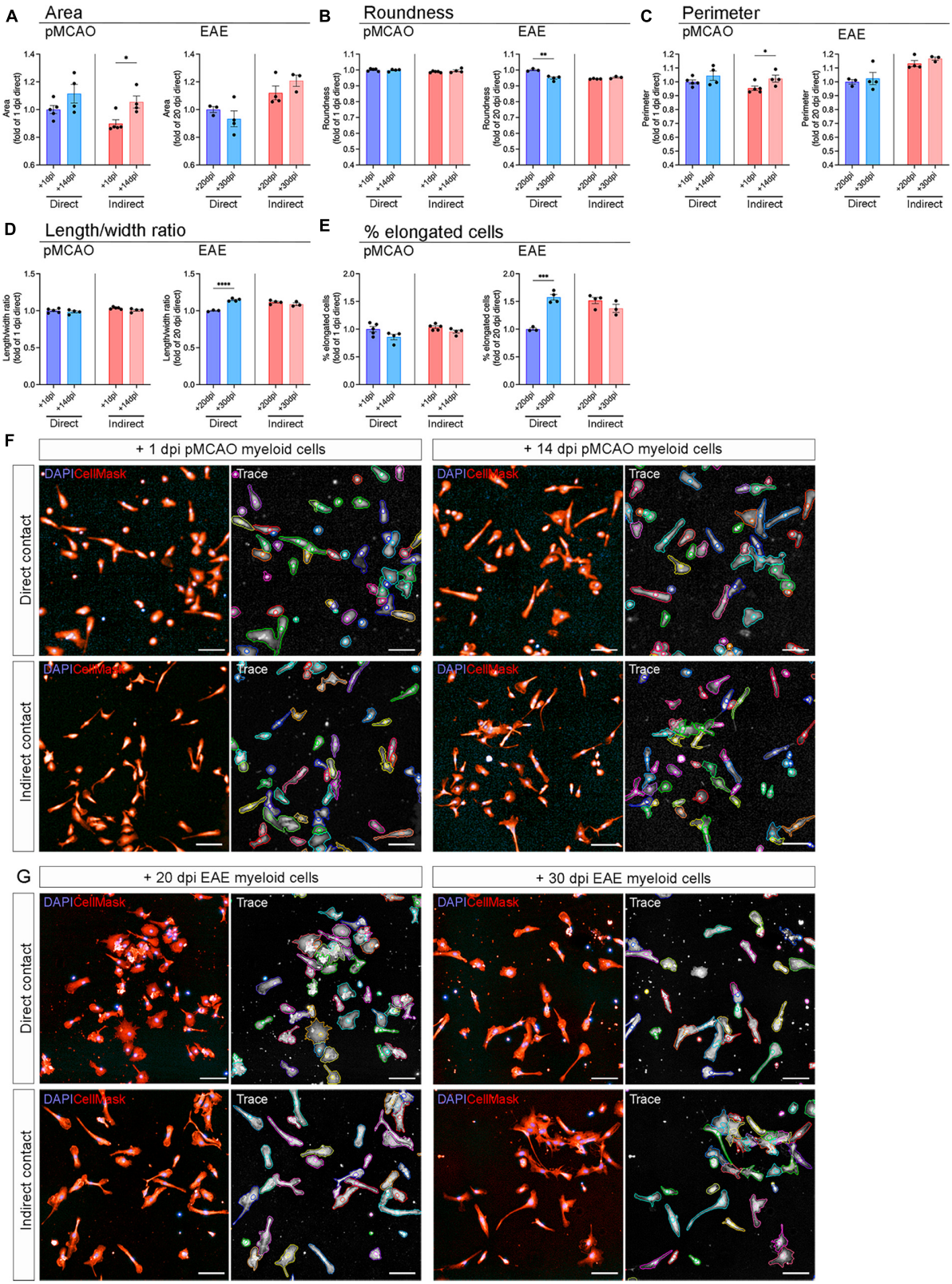

FIGURE 4
Microglial morphology after exposure to peripheral myeloid cells. Adult naïve microglia exposed to peripheral myeloid cells from pMCAO or EAE mice were evaluated for area **(A)**, roundness **(B)**, perimeter **(C)**, length/width ratio **(D)**, and percentage of elongated cells **(E)**. Data are normalized to naïve controls in each experiment and calculated as fold change of the acute timepoint with direct contact. Direct: Direct contact and interaction between myeloid cells and microglia. Indirect: Indirect interaction between myeloid cells and microglia, only via soluble factors. Data are presented as mean ± SEM of 3–5 biological replicates. Each biological replicate is the average of 1–8 technical replicates. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, unpaired two-tailed *t*-test. Representative images of microglia exposed to pMCAO **(F)** and EAE **(G)** myeloid cells with tracing generated by Harmony® high content analysis software. Scale bars = 100 μm.

Using naïve microglia in the present study was prioritized, as it allowed us to study the specific effect of activated peripheral myeloid cells, without involvement from intrinsic microglial activation. Additionally, it would be interesting to reverse the assay, to study the effect of microglia derived from different disease conditions on naïve peripheral myeloid cells.

In conclusion, we developed an *in vitro* system that allowed us to investigate the communication between peripheral myeloid cells and microglia. We identified peripheral myeloid cell-induced changes in microglia not only dependent on the disease model, but also on the disease phase at which myeloid cells were isolated. This underscores that neuroprotective and neuroreparative therapies must be tailored to each condition, and no myeloid modulating approach fits all. Our assay can be used to identify potential targets in myeloid cells under specific disease conditions, opening up the possibility of modulating these *in vivo*, either genetically or pharmacologically, in future studies, to assess their specific role in disease development. Furthermore, we showed that our assay is a valuable tool for investigating myeloid cell interactions in a range of CNS neuroinflammatory conditions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of the University of Miami and the Danish Animal Inspectorate (J. no. 2019-15-0201-01620). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ET: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review and editing. BC: Methodology, Writing – review and editing. AW: Data curation, Methodology, Writing – review and editing. RB: Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Validation, Writing – review and editing. KLL: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mechanisms of immune response and cell death in ischemic stroke and their regulation by natural compounds

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Ischemic stroke (IS), which is the third foremost cause of disability and death worldwide, has inflammation and cell death as its main pathological features. IS can lead to neuronal cell death and release factors such as damage-related molecular patterns, stimulating the immune system to release inflammatory mediators, thereby resulting in inflammation and exacerbating brain damage. Currently, there are a limited number of treatment methods for IS, which is a fact necessitating the discovery of new treatment targets. For this review, current research on inflammation and cell death in ischemic stroke was summarized. The complex roles and pathways of the principal immune cells (microglia, astrocyte, neutrophils, T lymphocytes, and monocytes/macrophage) in the immune system after IS in inflammation are discussed. The mechanisms of immune cell interactions and the cytokines involved in these interactions are summarized. Moreover, the cell death mechanisms (pyroptosis, apoptosis, necroptosis, PANoptosis, and ferroptosis) and pathways after IS are explored. Finally, a summary is provided of the mechanism of action of natural pharmacological active ingredients in the treatment of IS. Despite significant recent progress in research on IS, there remain many challenges that need to be overcome.

KEYWORDS

ischemic stroke, inflammatory, immune cells, cell death, natural compounds

1 Introduction

Stroke is one of the major causes of death and disability worldwide (1). In the past three decades, the global incidence of stroke has increased by 70%, stroke mortality has increased by 43%, disability adjusted life spans lost due to stroke increased by 32%, and the economic burden of all countries has increased. Ischemic stroke (IS) is a severe insufficiency of the blood supply to the brain caused by thrombosis or embolism in the blood supply to the

cerebral vessels in the functional area of the brain, resulting in an insufficient oxygen supply to the brain that leads to neuronal death and brain function defects (2). Ischemia induces cell death and neuroinflammation by promoting the production of pro-inflammatory mediators. At present, the number of drugs that can be used to treat IS [such as tissue plasminogen activator (tPA)] is limited, the clinical effect is poor, and the adverse reactions are substantial (3). Therefore, there is an urgent need for further research on IS to find more effective and safe therapeutic agents to prevent or treat IS.

Systemic inflammation, immune responses, and cell death play a key role in the occurrence and development of stroke. Post-ischemic inflammation of the injured brain is characterized by the infiltration of blood immune cells, as well as the interactions between resident microglia and invading blood immune cells (4). After IS, microglia (as resident brain macrophages) and astrocytes are activated in the innate immune system, releasing numerous inflammatory factors (5). Inflammatory factors attract peripheral immune cells to infiltrate the lesion area (6). The mechanisms of action of immune cells are complex, and these cells also interfere with each other, thus forming a complex inflammatory network. This further aggravates systemic inflammation, increases neuronal death and infarct volume, and leads to poor neurological outcomes (7). During ischemia, the blood supply to brain tissue is disrupted, which subsequently promotes a series of pathophysiological reactions leading to different types of cell death, including pyroptosis, apoptosis, necroptosis, ferroptosis, and PANoptosis, the lattermost of which is the crosstalk between pyroptosis, apoptosis, and necroptosis (8). These types of cell death all play roles in the pathogenesis of IS and induce inflammation (9).

This review summarizes—in relation to the immune system, inflammation, and cell death—the mechanisms and pathways involved in IS. In the section on the immune system and inflammation, the pathways and functions of immune cells, including microglia, astrocytes, neutrophils, T lymphocytes, and monocytes/macrophages, in the post-IS inflammatory response are reviewed. In the section on cell death, we review the pathways that mediate pyroptosis, apoptosis, necroptosis, ferroptosis, and PANoptosis. The mechanisms of natural compounds, including salidroside, baicalin, astragaloside IV, and curcumin, in the treatment of IS are also reviewed. Finally, there is a discussion of the potential future directions in this field.

2 Role and pathway of immune cells in the inflammatory response to ischemic stroke

The expression of pro-inflammatory factors marks the beginning of the development of cerebral ischemic inflammation and involves various cell types. The inflammatory response after IS includes two processes, namely early neural injury and late neural repair. Post-stroke inflammation is especially complex, and the interaction of different types of immune cells is crucial as a mediator of neuroinflammation. The immune system can be divided into innate and adaptive immune systems. The innate immune system includes microglia, neutrophils, and astrocytes. Microglia are the first responders in ischemic tissue (10). Moreover, T cells in the adaptive immune system play a role in central nervous system injury and repair (11) (shown in Table 1; Figure 1).

TABLE 1 Modulators and pathways of immune cell functions.

Immune cells	Type of modulator	Modulator	Model	Regulatory pathway	Effect	Reference
Microglia	Transcription factors	NF-κB	LPS-treated BV2 microglia cells	TLR4/NF-κB	Promote the secretion of pro-inflammatory cytokines	(12)
			LPS-treated BV2 microglia cells	TXA2R/MAPK/NF-κB	Promote the secretion of pro-inflammatory cytokines	(13)
			LPS-treated BV2 microglia cells	MAPK/ERK/NF-κB	Promote the secretion of pro-inflammatory cytokines	(14, 15)
			MCAO mice and OGD/R-BV2 microglia cells	STING/IRF3/NF-κB	Upregulate protein levels of STING, cGAS, p-STING, p-p65, and p-IRF3 in microglia	(16)
			MCAO rats and OGD-treated BV2 microglia cells	Notch/NF-κB	Elevate Notch-1 and Delta-1 expression in microglia; increase mRNA expression of TNF-α, IL-1β and iNOS	(17, 18)
	Transcription factors	STAT family members	Hypoxia-BV2 microglia cells, MCAO/R rats, and OGD/R microglia	JAK/STAT pathway	Elevate the expression of NF-κB	(19, 20)
	Ion channel protein	HV1	Mice lacking Hv1 (Hv1-/-)	HV1/NOX/ROS	Elevate the expression of ROS	(21)
		Kv1.3	MCAO/R rats, OGD/R primary microglia, ICV-	/	Elevate the expression of pro-inflammatory cytokines, activate NLRP3 inflammasome	(22, 23)

(Continued)

TABLE 1 Continued

Immune cells	Type of modulator	Modulator	Model	Regulatory pathway	Effect	Reference
	Ion channel protein		LPS mice, microglia (adult brains)			
	Gene	H19	MCAO mice and OGD/R-BV2 microglia cells	H19 siRNA/HDAC1	Elevate the expression of pro-inflammatory cytokines	(24)
	Gene	miRNA-155	LPS-treated BV2 microglia cells	miR-155/SOCS1	Elevate the expression of pro-inflammatory cytokines,	(25)
	Cytokine	IL-4	Microglia/macrophage polarization BV2 cells	IL-4/ JAK1/STAT6	Alleviate neuroinflammation	(26)
	Transcription factors	PPARγ	pMCAO rats	IL-4R/ STAT6/PPARγ	Improve neurological function	(27)
	Transcription factors	Nrf2	tFCI rat	AMPK/Nrf2	Anti-inflammatory	(28)
			CUMS mice, LPS/ATP-treated BV2 cells	Nrf2/HO-1/NLRP3	Upregulate the expression of Nrf2, HO-1, downregulate the expression of NLRP3	(29)
	Transcription factors	STAT family members	tMCAO/R mice	STAT1/STAT6	Lead to neuronal survival, and neurological functional recovery	(30)
	Gene	miRNA-124	TBI rat	miRNA-124/TLR4	Inhibit TLR4	(31)
			MCAO mice	miRNA-124/STAT3	Inhibit astrocyte proliferation, decrease Notch 1 expression and increase Sox2 expression in astrocytes	(32)
	Gene	FAM19A3	MCAO mice	/	Attenuate cerebral ischemia	(33)
Astrogliosis	Receptor	$P2Y_1R$	tMCAO rats	$P2Y_1R$/NF-κB	Promote the secretion of pro-inflammatory mediators, activates the NF-κB pathway,	(34)
			TBI mice	TNF-α, IL-1β, IL-6 (Microglia)/ $P2Y_1R$	$P2Y_1R$ downregulation, GFAP and p-STAT3 upregulation	(35)
	Receptor	TLR-4	LPS-treated rodent brain astrocyte cultures	TLR-4/MyD88/ NF-κB TLR-4/MAPK/ JAK1/STAT1	Promote the secretion of pro-inflammatory cytokines, chemokines	(36)
	Protein	p38 MAPK	MCAO mice and primary astrocyte cultures	MAPK	Increase GFAP expression	(37)
	Protein	Notch	MCAO mice	Notch1/RBP-J	Increase GFAP expression and promote reactive astrogliosis	(38)
	Transcription factors	STAT family members	MCAO mice and OGD-treated primary astrocytes	DRD2/ CRYAB/STAT3	Promote the secretion of pro-inflammatory cytokines and astrocytic activation	(39)
	Protein	Complement system	Rat primary astrocytes	C3a/C3aR, C5a/C5aR	Decreases the production of cAMP and increase in intracellular calcium concentration	(40)
Neutrophils	Chemokine	CCL3	tMCAO mice	CCL3/CCR1 and CCR5	Recruit neutrophils	(41)
		CXCR1/2	MCAO/R rats	CXCL8/ CXCR1/2	Activate neutrophil, recruit neutrophil	(42)
		CCR5	MCAO rats	CKLF1/CCR5	Mediate neutrophils infiltration, migrate neutrophils may via Akt/GSK-3β pathway	(43)
		PPARγ	MCAO mice	RXR/PPARγ	Increase brain infiltration of N2 neutrophils	(44)

(Continued)

TABLE 1 Continued

Immune cells	Type of modulator	Modulator	Model	Regulatory pathway	Effect	Reference
	Transcription factors					
	Receptor	TLR4	t-PA induced HT rats	HMGB1/TLR4/NF-κB	Mediate neutrophil infiltration, disrupt BBB integrity	(45)
			Human neutrophils; FeCl3-induced CAT rats	TLR4/MyD88/MAPKs	Promote thrombogenesis, induce NETs formation	(46)
T cell $CD8^+$	Protein	FasL	MCAO mice	FasL/PDPK1	Promote cytotoxicity, apoptosis of neurons, and ischemic neurological dysfunction	(47)
Treg	Transcription factor	Foxp3	Rats, LPS-treated microglia/macrophages	IL-2/Foxp3/STAT5	Sustain Foxp3 expression and Treg-cell identity	(48)
	Chemokine	CXCL14	MCA and CCA rats, primary cortical	HIF-1α/CXCL14	Induce Treg differentiation, promote Treg accumulation, reduce infarct volume	(49)
	Cytokine	IL-2	Five different types of Treg cells from human umbilical cord blood	IL-2/JAK/STAT	Enhance IL-10 production and IL-10 mRNA expression	(50)
$CD4^+$ Th1	Transcription factor	T-bet	Gene knockout mice and wild-type mice	IL-2,IL-12/IL-12Rβ2/T-bet	Promote TH1 differentiation	(51)
Th2	Transcription factor	GATA3	$CD4^+$ splenic T cell from mice	IL-2/STAT5/GATA3	Drive Th2 differentiation, induce and maintain IL-4Rα,	(52)
Th17	Transcription factor	RORγt	Gene knockout mice and wild-type mice	IL-6/STAT3/RORγt	Direct Th17 differentiation, induce IL-17 and IL-17F expression	(53)
M1 macrophages	Receptor	P2X4R	MS-treated P2X4R knockout mice and wild-type mice	/	Increase IL-1β, IL-6, TNF-α mRNA levels,	(54)
M2a macrophages		IL-13	pMCAO mice and RAW 264.7 macrophages		Enhance the expression of M2a alternative activation markers (Arg1 and Ym1), increase IL-6 and IL-10 levels, decrease neuronal cell death	(55)

nuclear factor NF-kappa-B (NF-κB), thromboxane A2 (TXA2R), Janus kinase (JAK), phosphorylated-JAK (p-JAK), lipopolysaccharide (LPS), voltage-gated proton channel (Hv1), nicotinamide adenine dinucleotide phosphate oxidase (NOX), reactive oxygen species (ROS), voltage-gated potassium channel (Kv1.3), NACHT, LRR and PYD domain-containing protein 3 (NLRP3), histone deacetylase 1 (HDAC1), suppressor of cytokine signaling 1 (SOCS1), nuclear factor erythroid 2-related factor 2 (Nrf2), AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor γ (PPARγ), interleukin-4 (IL-4), signal transducer and activator of transcription (STAT), sphingosine 1-phosphate receptor (S1PR), sphingosine 1-phosphate (S1P), transient focal cerebral ischemia (tFCI), transient middle cerebral artery occlusion-reperfusion (tMCAO/R), purinergic receptor type 1 ($P2Y_1R$), phosphorylated-nuclear factor NF-kappa-B p65 subunit (p-RelA), recombining binding protein suppressor of hairless (RBP-J), dopamine D2 receptor (DRD2), αB-crystallin (CRYAB), adenosine 3',5'-cyclic monophosphate (cAMP), glutamic acid-lysine-arginine (ELR), chemokine-like factor 1 (CKLF1), CC chemokine receptor 5 (CCR5), hemorrhagic transformation (HT), carotid artery thrombosis (CAT), 3-phosphoinositide-dependent protein kinase-1 (PDPK1), tumor necrosis factor ligand superfamily member 6 (FasL), right middle cerebral artery (MCA), bilateral common carotid artery (CCA), interleukin 12 receptor β2-chain (IL-12Rβ2), chromodomain helicase DNA-binding protein 4 (Chd4), IL-4 receptor alpha-chain (IL-4Rα), GATA-binding factor 3 (GATA3), interleukin-17F (IL-17F).
/, not applicable.

2.1 Brain immune cells

2.1.1 First line of defense: microglia

Microglia, as resident CNS macrophages, play a dual role of neurotoxicity and protection in IS. When brain injury occurs, numerous DAMPs and cytokines are released, and microglia are activated with the death of nerve cells in the central area of the infarct (5); this, together with the activation of macrophages from blood-derived monocytes, constitute the innate immune response, which is the first line of defense (56). During systemic inflammation, microglia are involved in tissue damage and repair, respectively (57).

Previously, the pro-inflammatory microglia phenotype was termed M1, while the anti-inflammatory phenotype was termed M2. Since then, microglia have been demonstrated to exist in a wide range of activated states. For example, several *in vivo* studies failed to even find pure M1 or M2 states. In the same cell and at the same point in time, these "microglial markers" have multiple overlapping phenotypes. Therefore, a binary M1/M2 characterization is not sufficient for defining the inflammatory characteristics of microglia (58), and a systematic and careful nomenclature will greatly benefit the biological study of microglia. The current view is that moderate and precise terms should be used to properly understand the state of microglia. Using markers (genes or proteins) to identify populations of cells may be a solution, but they cannot be used as a readout of cell function (59). Wahane et al. combined transcriptomics and single-cell RNA sequencing to reveal a wide range of microglial states after spinal cord injury. The transcription profiles were diverse, with each transcription profile comprising four transcription subtypes. Furthermore, RNA-seq showed a well-defined temporal trajectory of IAM (injury-activated microglia

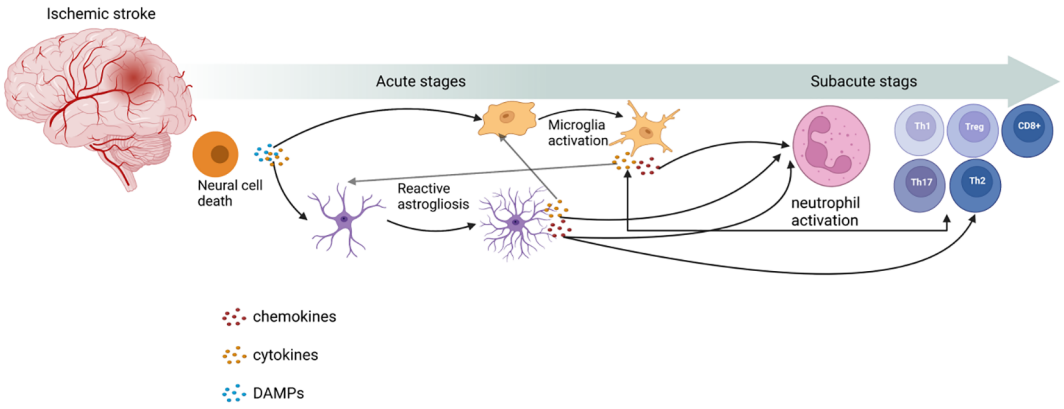

FIGURE 1
Role of immune cells in ischemic stroke. Post-IS, with the release of damage-associated molecular patterns (DAMPs) and cytokines from neuronal cell death, microglia activation and reactive astrogliosis occur. These processes can release cytokines and chemokines to activate neutrophils and T cells to proliferate and differentiate into different types. This image was created using BioRender.com.

and macrophages) gene programs over several days, including 3 days proliferation and motility, 7 days axon chemical attraction and ion channel activity, and 14 days extracellular matrix (ECM) recombination. The data also found that (i) phagocyte genes were still induced at 14 days to support the durable repair function of IAM; (ii) there was a high expression of ECM genes and nutrient factors; and (iii) the anti-inflammatory gene set signature was enriched at all stages. These three phenomena indicate that IAM has a long-lasting repair function. RNA-seq did not show significant changes in M1/M2 genes in bone marrow cells, and scRNA-seq further revealed their heterogeneous expression patterns in bone marrow subclusters. This further demonstrates the limitations of the conceptual dichotomy of pro-inflammatory and anti-inflammatory phenotypes (60).

The involvement of microglia in tissue damage reaches its peak 3–5 days after ischemic injury. At this stage, microglia play a harmful role, mainly by destroying the blood brain barrier (BBB), aggravating brain edema, and promoting neuronal apoptosis by producing and secreting many inflammatory mediators (61). The release of pro-inflammatory cytokines leads to secondary brain injury, while microglia have been shown to exhibit repair functions for nearly 14 days after injury (62). Microglia play a primarily protective role that promotes the regression of inflammation by secreting IL-4, IL-10, and transforming growth factor (TGF)-β, thereby indirectly preventing inflammation-induced damage to the blood brain barrier (BBB). The regulatory pathways of microglial cell polarization can be divided into four categories: 1) transcription factors; 2) receptors; 3) ion channels; and 4) gene modulators (shown in Table 1). Moreover, microglial phagocytosis is a double-edged sword in immune inflammation and stroke recovery. Microglia have been shown to exhibit phagocytosis, and their ability to clean neuronal debris reduces brain damage after stroke (5). Microglia invade the ischemic site of stroke earlier than macrophages, and they are the main phagocytes for the first three days after stroke (63). After ischemia, microglia infiltrate the injured brain tissue, engulfing living and dead neurons, myelin debris, apoptotic cell debris, endothelial cells, and leukocytes. During pathological cases, microglial phagocytosis can be initiated by specific "eat me" signals on specific cell types and their corresponding receptors (64).

As shown in Table 1, many pathways and mediators have been confirmed to regulate the activation of microglia. For example, NF-κB and IL-4 as regulators promote activation of microglia, respectively, and are the most extensively studied and clearly researched mediators. Some conflicting results have been found in studies on the regulatory effects of TLR and STAT, and further research is necessary to clarify their roles (65). Most of these signaling pathways overlap to varying degrees and do not appear to work independently, but rather synergistically, resulting in an inflammatory maelstrom (66). Many studies have shown that targeting microglia can effectively treat IS (67). Further research on microglial activation signal pathways after IS will help identify effective drugs that inhibit microglial activation and prevent neuroinflammation mediated by microglial activity. Therefore, in future studies, it is crucial to identify appropriate targeted intervention drugs according to the role of microglia at different times.

2.1.2 Dual regulatory effects of astrocytes

Astrocytes, the most abundant neuroglial cells in the brain, are essential housekeeping cells that maintain the central nervous system. Astrocytes play, as do microglia, a dual role in the pathophysiology of IS (68). After IS, damaged cells produce and release cytokines and DAMPs to stimulate receptors of astrocytes and change their phenotype. A few minutes after IS, due to reactive astrocyte proliferation, astrocytes respond to various inflammatory factors (including TGF-α, ciliary neurotrophic factor, IL-1, IL-6, and kallikrein-related peptidase 6) released by ischemic/hypoxic cells, and are subsequently activated and reproduced (69, 70). Reactive astrogliosis occurs in the peri-infarct region, and a glial scar is formed to maintain CNS homeostasis and wall off the lesion (71). During this process, astrocytes display cellular hypertrophy, proliferation, and increased expression of intermediate proteins, including glial fibrillary acidic protein (GFAP), vimentin, and

nestin (72). After experiencing reactive astrogliosis, astrocytes produce and release pro-inflammatory cytokines (such as IL-6, TNF-α, IL-1α, IL-2β, and IFN-γ), chemokines (such as CXCL1/10 and CCL2/3/5), important sources of ATP, and free radicals such as NO, superoxide, and peroxynitrites (73). Thus, the activation of microglia and infiltration of white blood cells are enhanced (74). Studies have discussed the two subtypes of reactive astrocytes as A1 and A2. The A1 subtype includes astrocytes induced by IL-1α, TNF-α, and complement component subunit 1q (C1q) secreted by activated microglia. A1 subtype astrocytes induce neuronal and oligodendrocyte death. The A2 subtype can secrete IL-2, IL-10, and TGF-β, thus accelerating the regression of inflammation (75). Additionally, the A2 subtype can play an inflammatory and neuroprotective role by secreting neurotrophic factors, neuropoietic cytokines, and growth factors (76).

The response of astrocytes to Injury is a major determinant of the outcome after stroke. The gene expression of A2 astrocytes dominates the expression of A1 astrocytes (77). The JAK/STAT3 signaling pathway was found to be an important switch controlling many molecular and functional changes in reactive astrocytes *in vivo* and *in vitro* (78). However, the roles of all these molecules and pathways need to be further validated in future research (79, 80). In addition, the complexity of the multiple roles of complement protein and receptor expression in astrocytes has only recently been studied. Further *in vivo* and *in vitro* research is needed to determine astrocyte pathways and effects to determine targeted treatment strategies.

2.2 Peripheral immune cells

2.2.1 Recruitment and infiltration of neutrophils

Increased numbers of leukocytes have been found to be a marker of the inflammatory response in IS. Among various types of leukocytes, neutrophils are the first to respond to ischemic brain injury (81). An *in vivo* study found that neutrophils were found in leptomeninges and cerebral parenchyma 6 hours and 12 hours, respectively, after permanent middle cerebral artery occlusion (pMCAO) (82). Their recruitment reached a peak on days 1-3 and gradually decreased over time (83). Neutrophils produce extensive weblike structures of DNA (neutrophil extracellular traps, NETs) that reached their peak 3-5 days after the transient middle cerebral artery occlusion (tMCAO) (84). These NETs have been associated with inflammation (85). After cerebral ischemia, neutrophils undergo conformational changes due to the presence of many adhesion molecules, which helps them to migrate through blood vessel walls to the brain tissue. Activated microglia and astrocytes release chemokines (such as CXC and CC) to promote neutrophil activation. These chemoattractants bind to the C-C chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 1 (CXCR1) on the surface of neutrophils, making neutrophils the first blood-derived immune cells to migrate to damaged brain tissue (86). Neutrophils are attracted to the ischemic region by chemokines, and then infiltrate damaged brain tissue soon after injury, which aggravates inflammation (87).

Traditionally, neutrophils have been considered the main mediators of harmful inflammatory responses in IS (88). However, a significant amount of evidence suggests that neutrophils can obtain different phenotypes. As in the case of microglia, it is believed that some neutrophil subsets show different characteristics. The response phenotype of neutrophils to the ischemic environment, and the interaction between neutrophils and endothelial adhesion molecules has shifted from protective N2 to the injurious N1 phenotype (89). *In vivo* and *in vitro* studies have shown that PPARγ and TLR4 mediate the N2 phenotype of neutrophils (90, 91). However, research on the functional changes and biomarkers of the N1/N2 phenotypes of neutrophils after IS are not sufficient. This also leads to a shortage of known pathways. Therefore, further research is needed on the role of neutrophils in the inflammatory response after IS.

2.2.2 Conflicting roles of T lymphocytes

T lymphocytes play an important role in the process of nerve damage and repair in the late stage of IS. In the acute phase of IS, T cells chiefly react in an antigen-independent manner and are closely related to the development of the infarct volume. After 3-7 days, the T cell response gradually transforms into antigen-dependent antigen recognition (92). Brain-derived antigens are recognized by T cell receptors (TCRs) on the surface of naïve T cells. Then, T cells migrate to the brain parenchyma through cell adhesion molecules (such as P-selectin, E-selectin, VCAM-1, and ICAM-1) and chemokines. Ultimately, adaptive immune responses exacerbate ischemia-reperfusion (I/R) injury. *In vivo* studies involving ischemic rats demonstrated that by day 3 after ischemia, many T cells infiltrated the peripheral areas around the lesion and surrounded the infarct center, and the number of T cells increased between days 3 and 7 (93). According to different functions, T cells have multiple types marked by CD3 expression, including $CD8^+$ cytotoxic T lymphocytes (CTL), $CD4^+$ T helper (Th) cells, regulatory T cells (Treg), and gamma delta (γδ) T cells (94). The different roles of different types of T cells are already known, but the specific mechanism of T cell function after IS still needs further research.

CD^{4+} T cells, as the main effector T cells, regulate brain inflammation by producing cytokines (95). The signals derived from T cell and co-stimulatory T cell receptors and extracellular cytokines determine the phenotype of Th cells. Cytokine signals are received through multimeric receptors and propagated largely through Janus kinase/signal transducer activator of transcription (JAK/STAT) signaling pathways (96). Th cells can be divided into Th1 and Th17 (pro-inflammatory), and Th2 and Treg (anti-inflammatory) based on their cytokine secretion profile. Th1 and Th17 cells produce IL-1, IFN-γ, IL-17, IL-22, and other cytokines. Th2 and Treg cells produce IL-4, IL-10, and TGF-β (97). Different types of $CD4^+$ T cells have their own specific transcription factors that play a crucial role in their differentiation, maintenance, and function (98).

$CD8^+$ T cells can play a cytotoxic role through antigen recognition of the TCR and subsequent release of granzyme and perforin, forming pores on target cells and inducing apoptosis (99). Selvaraj et al. investigated the role of $CD8^+$ T cells in stroke by

establishing a tMCAO mouse model. The results showed that $CD8^+$ T cells had an adverse effect in the chronic phase after stroke. At 30 days, there was an increase in the number of ipsilesional $CD8^+$ T cells, revealing its association with deterioration in mouse functional outcomes (100). In recent years, there have been studies on CD^{8+} T cells inducing neuronal apoptosis through the FasL/PDPK1 pathway, but their mechanism of action after IS remains unclear.

γδ T cells do not require antigen recognition to activate and are detected in infarcts 6 hours after ischemia. During the onset of IS, γδ T cells mainly secrete cytokines such as IL-17, IL-21, IL-22, and IFN-γ through receptors to protect the barrier from infection and exacerbate inflammation (101). Arunachalam et al. found that $Vγ6^+/CCR6^+$ γδ T cell subtypes are the main source of IL-17 (102). However, few studies have paid attention to the signaling pathways present in γδ T cells after activation by ligands that bind to their receptors. The low number of γδ T cells, difficulty in extraction, and lack of cell lines may be the reasons for this lack of research.

2.2.3 Double-edged sword: monocytes/macrophage

The role of monocytes and macrophages in ischemic stroke is the same as that of microglia. Post-IS, pro-inflammatory monocytes infiltrate the inflamed brain, where they differentiate into macrophages that are morphologically indistinguishable from the local microglia (103). In contrast to the rapid microglial response, macrophages are rarely detected within the first 48 hours. Their level gradually increases, with a peak during the first week after stroke (104). On day 3 after stroke, the phenotypes of monocytes were found to change from the predominantly pro-inflammatory M1 to the anti-inflammatory M2 phenotype, indicating a functional shift from an enhanced immune response to inflammation resolution (105). Transcriptomic analysis of macrophages has shown that infiltrated macrophages on day 5 after stroke promote an effervescent increase and inflammation resolution after ischemic stroke (106). M2 macrophages can be further subdivided. For example, studies have classified macrophages as M2a, M2b, and M2c (107), while other authors have also classified them into an M2d subtype (108). All four M2 macrophage subtypes acquired enhanced phagocytosis and expressed IL-10, contributing to the resolution of inflammation (61). In a mouse model of ischemic stroke, researchers found that inflammatory activity peaked at 72 hours. Microglia produce relatively high levels of reactive oxygen species and TNF, while monocytes are major IL-1β producers. Although microglia show enhanced phagocytosis activity after stroke, monocytes have a significantly higher phagocytosis capacity at 72 hours (104).

Most M2 macrophages derived from monocytes can protect the blood-brain barrier from ischemic damage through vascular remodeling, physical attachment, and regression of inflammation (109). M2a macrophages express various anti-inflammatory and neurotrophic factors, such as arginase 1 (Arg1) and insulin-like growth factor-1. M2c macrophages increase the expression of TGF-β, CD163, and sphingosine kinase. However, M2b macrophages increase the production of pro-inflammatory factors, including IL-1β, IL-6, and TNF-a, which may enhance inflammation and increase blood-brain barrier permeability early in IS (110). M2d macrophages secrete VEGF-A and TNF-α, all of which are detrimental to the blood-brain barrier integrity in IS (111).

2.3 Mutual coordination between immune cells in ischemic stroke

2.3.1 Crosstalk between microglia and astrocytes

The interaction between activated microglia and astrocyte has a critical role in the process of neuroinflammation (shown in Table 2). In the first 6 hours after cerebral ischemia, microglia are first activated by pathogens or injury through TLR4, and release inflammatory mediators (112, 113). At the same time, astrocytes dependently activate TLR2, TLR3, and TLR4 to respond (114). The "molecular signal" (IL-1, TNF-α, and C1q) released by microglia can convert astrocytes into a neurotoxic A1 phenotype. For example, Dr. Ben Barres' lab, using single-, dual-, and triple-gene knockout mice, pioneered the discovery that activated microglia secreting IL-1α, TNF-α, and C1q together are necessary and sufficient to induce A1 astrocytes (75). These neuroinflammatory reactive astrocytes lose many of their stereotypical physiological functions and secrete one or more unknown factors with strong toxicity to neurons and oligodendrocytes (115). Tarassishin et al. showed that human astrocytes and reactive astrogliosis are highly sensitive to IL-1β but unresponsive to lipopolysaccharide (LPS) stimulation. In human astrocytes, IL-1 induced both A1 and A2 responses (116). Glucagon-like peptide-1 receptor (GLP1R) is highly expressed in microglia, and is also expressed in astrocytes and neurons at reduced levels. Some studies have found that GLP1R agonists can directly prevent microglia-mediated astrocyte transformation into the A1 neurotoxic phenotype and have neuroprotective effects (117). The interleukin-1 family member interleukin-33 (IL-33) is produced by developing astrocytes, and it mainly signals to microglia and promotes synaptic phagocytosis of microglia under physiological conditions. IL-33 also drives microglia-dependent synaptic depletion *in vivo*. The transcriptomes of acutely isolated microglia from $IL\text{-}33^{-/-}$ animals showed 483 significantly altered transcripts, including reduced expression of NF-κB targets (e.g., Tnf, Nfkbia, Nfkbiz, and Tnfaip3) (118). IL-15 is also the mediator of crosstalk between astrocytes and microglia, thus aggravating brain damage after intracerebral hemorrhage. Shi et al. established a transgenic mouse model targeting IL-15 expression in astrocytes and found that the accumulation of microglia near astrocytes in the tissue around the hematoma increased after brain injury. The expression of biomarkers in M1 microglial cells increased significantly (119).

2.3.2 Crosstalk between glial cells and peripheral immune cells

Astrocytes are the bridge between infiltrating T lymphocytes and neurons during cerebral ischemia. *In vivo* knockdown of interleukin-15 (IL-15) in astrocytes alleviates ischemic brain

TABLE 2 Cytokines in mutual coordination between immune cells.

Cytokines	Main producer	Effect immune cell	Role in ischemic stroke
TNF-α	M1 microglia, Th1 cell	T cell, microglia, and astrocyte	Activate astrocytes, accelerate the polarization of Th1 cells, mediate endothelial necrosis, promote the destruction of BBB, promote M1 polarization
IL-1β	Monocytes/ macrophages M1 microglia	Astrocyte	Activate astrocytes, aggravate the dysfunction of BBB, stimulate the activation of microglia, and promote the apoptosis of damaged cells
IL-6	M1 microglia, astrocytes,	T cell, microglia, astrocytes,	Recruit and induce differentiation of Th17 cells, promote proliferation and activation of microglia and astrocytes, aggravate the damage effect
IL-12	M1 microglia	T cell	Accelerate the polarization of Th1 cells
IL-15	Astrocytes	T cell, microglia,	Increase the number of $CD8^+$T cells and activated brain infiltrating $CD4^+$T cells, promote the differentiation and accumulation of M1 microglia, and aggravate ischemic brain damage,
IL-17	γδ T cells, astrocyte, and Th17 cells	Neutrophil	Promote neutrophil recruitment to the ischemic hemisphere, upregulate neutrophil-mobilizing cytokines and chemokines,
Anti-inflammatory			
IL-4	$CD4^+$, Treg, Th2 cell	Astrocyte, microglia	Promotes M2 polarization of microglia, inhibits pro-inflammatory cytokines (IL-1β, TNF-α)
IL-10	M2 Microglia Th1, Th2, Treg and astrocyte	Microglia, T cell,	Mediate the function of Th2 cells to reduce infarction lesions, inhibit cell apoptosis, and drive M2 Microglia polarization,
IL-33	Astrocytes, Th2 cell	Treg cell, microglia	Expand Treg cell and induce IL-4 secretion, activate M2 microglial polarization, and reduce astrocytic activation

References are shown in the text.

damage. Decreased levels of $CD8^+$ T cells were also found in mice with knockdown of the IL-15 receptor α or blockade of cell-to-cell contact. Subsequent studies further confirmed the role of IL-15 from astrocytes on T cells. At the same time, a lower number of activated brain infiltrating $CD4^+$ T cells were also found in $Il15^{-/-}$ mice (120). Astrocytes, γδ T cells, and Th17 cells are the main sources of interleukin-17 (IL-17) after IS. The main function of IL-17 involves coordinating local tissue inflammation by upregulating pro-inflammatory and neutrophil-mobilizing cytokines and chemokines. Kang et al. established a mouse model with specific deletions of key components of IL-17 signaling in various immune cells. It was found that astrocytes were crucial in IL-17-mediated white blood cell recruitment (121). Astrocyte-derived CXCL-1 acts as a key mediator of IL-17-initiated neutrophil chemotaxis in stroke. IL-17 secreted by γδ T cells has also been reported to attract neutrophils to the site of injury (122). Subsequently, reactive microglia engulf neutrophils in the periphery of ischemic lesions, while the local microglia loss and dystrophy occurring in the ischemic core are associated with the accumulation of neutrophils, first in perivascular spaces and later in the parenchyma (123). Following IS, central nervous system injury can trigger the release of IL-33 from astrocytes. Ito et al. found that many Treg cells accumulated in the brain of mice dependent on IL-33 after IS. The chemokines CCL1 and CCL20 drive penetration into the brain. This helps with neurological recovery in the chronic phase of ischemic brain injury (124). In the MCAO mouse model, IL-33 treatment increased the number of Treg cells in the ischemic brain. IL-33 was shown to increase the levels of anti-inflammatory cytokines in serum and brain tissue (125). IL-33 also enhanced M2 polarization marker expression in microglia. Activation of the IL-33/ST2 axis led to polarization of M2 microglia, which provided protection for ischemic neurons in an IL-10 dependent manner (126).

The crosstalk between M1 microglia and Th1/Th17 cells plays a pro-inflammatory role and contributes to brain injury. The crosstalk between M2 microglia and Th2/Treg cells plays an anti-inflammatory role and helps with brain recovery. M1 microglia secrete IL-12 and TNF-α, which induce Th1 cells, and these two types of cells work together to promote inflammation. The M1 polarization promoted by Th1 cytokines (TNF-α and IFN-γ) is associated with classic activation (127). M1 microglia secrete IL-6 and IL-23, which recruit Th17 cells and induce their differentiation (128). Th2 cells secrete IL-4 and IL-10, while Tregs secrete IL-10, further driving M2 polarization, inhibiting inflammation, and promoting tissue repair. IL-33 is suppressed in human stroke, resulting in an insufficient Th2-type response driven. In human T cells, IL-33 treatment induced IL-4 secretion while reducing astrocyte activation and increasing the number of M2 microglia (129).

3 Cell death in ischemic stroke

After ischemia, hypoperfusion of brain tissue leads to a decrease in oxygen, ATP, and glucose, which leads to cell death over time. Ischemic tissue can be functionally divided into irreversibly injured infarcted core tissue and peripheral ischemic penumbra tissue. The infarct core is composed of dead or dying tissues and is located in the central area of the infarct area. In the penumbra, this depletion hampers cellular physiological functioning but does not induce an irreversible change. Neuronal death in IS involves a variety of cell death pathways. Apoptosis, pyroptosis, necroptosis, and PANoptosis are four key cell death pathways (Table 3; Figure 2).

TABLE 3 Cell death and pathways.

Cell death	Model	Type of modulator	Modulator	Pathway	Reference
Pyroptosis	MCAO/R mice, primary microglial	PRR	NLRP3	NF-κB/NLRP3	(130)
	MCAO/R rats, OGD/R-treated SH-SY5Y cells	PRR	NLRP3	NLRP3/Caspase-1/GSDMD	(131)
	MCAO rats, OGD/R-treated neurocytes	PRR	AIM2	lncRNA MEG3/miR-485/AIM2	(132)
	MCAO/R rats,	Protein	GSDMD	GSDMD/caspase-1	(133)
	MCAO rats	PRR	NLRP1	miR-9a-5p/NLRP1	(134)
	ICH mice	Adaptor protein	ASC	Asc/GSDMD/Caspase-1	(135)
Apoptosis	MCAO mice, OGD/R-treated primary cultured mouse embryonic cortical neurons	Pro-apoptotic protein	P53	p53/Bcl-2/Bax	(136)
	CIR rats	Pro-apoptotic protein	P53	p53/Bax/Cytochrome C/Caspase-3	(137)
	MCAO/R rats	Pro-apoptotic protein	ERK	ERK/JNK/p38/Bim	(138)
	MCAO mice, OGD/R-treated PC12 cells	Pro-apoptotic protein	ERK	ERK1/2/CREB/BCL-2	(139)
	FI/R mice	Pro-apoptotic protein	JNK	JNK/Bim, Bax	(140)
	MCAO/R mice, SH-SY5Y cell	Pro-apoptotic protein	NF-κB	NF-κB/Bim/caspase-3	(141)
	MCAO rats	Pro-apoptotic protein	Notch/HIF-1α	Notch/HIF-1α/Bcl-2/Bax	(142)
	OGD-treated cortical cultures, TNFR1 knock-out mice	Receptor	TNFR1	TNFR1/TNF-α	(143)
Necroptosis	MCAO rats	Kinase	RIPK1/RIPK3	RIPK1/RIPK3/MLKL	(144)
	I/R rats and H/R-treated H9c2 rat cardiomyoblast cells	Kinase	RIPK1/RIPK3	TNF-α/RIP1/RIP3/MLKL	(145)

middle cerebral artery occlusion (MCAO), oxygen-glucose deprivation/reperfusion (OGD/R), pattern recognition receptors (PRR), NOD-like receptors containing pyrin domains (NLRPs), absent in melanoma 2 (AIM2), long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3), gasdermin D (GSDMD), microRNA-9a-5p (miR-9a-5p), intracerebral hemorrhage (ICH), Ag phosphatidylinositol 3-kinase (PI3K), cerebral ischemia-reperfusion (CIR), extracellular signal-regulated kinase (ERK), cyclic AMP-responsive element-binding protein (CREB), focal ischemia and reperfusion (FI/R), genetically deficient mouse embryo fibroblasts (MEFs), mesenchymal stem cells (MSCs), ischemia/reperfusion (I/R), hypoxia/reoxygenation (H/R).

3.1 Pyroptosis, apoptosis, necroptosis, and PANoptosis

3.1.1 Pyroptosis

Pyroptosis is a form of regulatory necrosis mediated by caspase-1 and is mainly seen in the ischemic penumbra. Pyroptosis can be divided into inflammatory and non-inflammatory pathways (146). Inflammatory pathway is the main pathway, one is the classical inflammatory pathway mediated by caspase-1, the other is the non-classical inflammatory pathway mediated by caspase-11. Inflammatory pathway of pyroptosis is an effective inducer of pro-inflammatory pathways in IS, occurring after the assembly and activation of inflammasomes (147). Inflammasomes contain pattern recognition receptors (PRRs), adapter proteins, and caspase family members (148). The adapter protein ASC has a cysteine protease recruitment domain (CARD) and a pyrin domain (PYD) (149). The structural characteristics of ASC provide support for binding of procaspase-1 to receptors. After IS, DAMPs secreted by necrotic cells in the ischemic core region are recognized by PRRs. Then, procaspase-1 autocrine signaling produces cleaved caspase-1. Cleaved caspase-1 mediates microglial pyroptosis with the release of a large number of pro-inflammatory factors (IL-1 and IL-18) that induces neuronal death (150). Caspase-1 cleaves gasdermin D (GSDMD) into N-GSDMD, which binds directly to the plasma membrane and forms pores, releasing large amounts of cytosolic content to promote inflammation. The non-classical inflammatory pathway is one in which caspase-11 mediates the "non-canonical inflammasome" to participate in IL-1 and IL-18 processing and cell death (108). Studies had shown that ccaspase-11 (mouse-derived) also cleaves GSDMD, leading to focal ptosis under LPS stimulation (151). In addition, caspase-11 has been found to promote inflammation by regulating caspase-1 expression by promoting K^+ efflux (152). Non-inflammatory pyroptosis pathways are pathways in which caspase-8 is involved. It has been found that catalytic caspase-8 promotes the assembly of ASC-procaspase-1, in which caspase-8 acts as a scaffolding protein (153). In addition, caspase 8, procaspase 1, and cleaved caspase-1 were upregulated in an MCAO/R model (154). Similar to caspase-1, caspase-8 can also

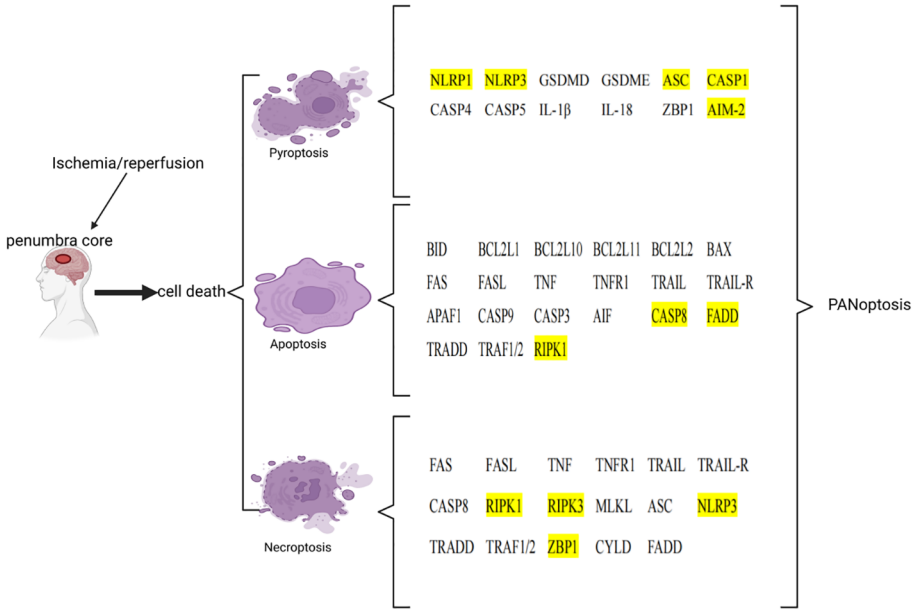

FIGURE 2
List of key genes involved in pyroptosis, apoptosis, and necroptosis in ischemic stroke. The genes involved in PANoptosis are marked in yellow. This image was created using BioRender.com.

cleave gasdermin family proteins to induce pyroptosis. Under hypoxia conditions, nuclear transcription of GSDMC increases and caspase-8 cleaves GSDMC into N-GSDMC to induce pyroptosis after TNF-α stimulation (155). Further studies have identified a principal axis of pyroptosis extending from ROS-initiated DR6 endocytosis to caspase 8-mediated GSDMC cleavage (156).

3.1.2 Apoptosis

Apoptosis is the most common form of programmed cell death in multicellular organisms that elicits no inflammatory response. It is the main mechanism of neuronal loss after IS and can be triggered either through the intrinsic or the extrinsic pathway. The intrinsic pathway is caused by DNA damage or endoplasmic reticulum stress, and the extrinsic pathway is mediated by the activation of the death receptor family members (157).

The intrinsic pathway involves a non-receptor-mediated signaling cascade (158). After IS, excitotoxicity produced through the mitochondrial pathway can mediate Ca^{2+} overload, leading to cell apoptosis. Glutamate binds to N-methyl-D-aspartate receptors (NMDARs), resulting in an overload of Ca^{2+} in neurons (159). Ca^{2+} activates the interaction of calpain with the Bcl-2 family proteins. Eventually, proapoptotic proteins are upregulated and mitochondrial permeability transition pores are formed (160), allowing for the release of apoptogens. The Bcl-2 protein family members (pro-apoptotic) regulate changes in the mitochondrial permeability, the release of cytochrome c, and contribute to apoptogen formation by binding with apoptotic protease activating factor-1 (Apaf-1) (161). Finally, activation of caspase leads to degradation of nuclear DNA, thus promoting cell apoptosis (162).

Following IS, the activation of immune cells during inflammation results in the release of a variety of factors (including pro-inflammatory cytokines) that trigger neuronal cell death via the extrinsic apoptotic pathways (163, 164). The extrinsic cell apoptosis pathway is triggered by the ligation of tumor necrosis factor (TNF)-family death receptors on the cell surface during external stimuli (165). After the receptor is bound, it recruits the adapter protein [Fas-associated death domain protein (FADD)] to create a death-inducing signaling complex with procaspase-8, which activates caspase-8 (166). Caspase-8 activates the downstream effector caspase, mediating apoptosis by direct proteolytic cleavage or indirectly by catalyzing the Bcl-2 protein family members (167). Velier et al. established a pMCAO rat model and found that proteolytic processing yielding the active form of caspase-8 was active 6 hours after stroke (168).

3.1.3 Necroptosis

Necroptosis, which is a lytic-programmed cell death with the ability to cause inflammation, is independent of caspase transmission. Similar to apoptosis, necroptosis is triggered by the ligation of specific death ligands to TNF-family death receptors or by pro-caspase inhibitors (169). This process leads to de-ubiquitination of receptor interacting protein kinase 1 (RIPK1) by the de-ubiquitination enzyme CYLD (170). RIPK1 activates the kinase RIPK3 within a cytoplasmic high molecular weight complex called a necrosome. RIPK3 phosphorylates and activates the mixed lineage kinase domain-like protein (MLKL), forming the homotrimer necrosomes (171). The accumulation of necrosomes leads to increased permeability of plasma membranes and organelles. This leads to membrane damage and subsequent cell death (172, 173). The phosphorylation of MLKL and the formation

of necrotic bodies are therefore considered as cellular markers of necrosis (174). After cerebral I/R injury, perivascular M1-microglia secrete TNF-α and its receptor TNFR1 on the endothelium, which serve as the main mediators triggering endothelial necroptosis (175). Necroptosis promotes the release of DAMPs, driving an inflammatory response. *In vivo* and *in vitro* studies found that RIPK3 promoted NLRP3 inflammasomes and the IL-1β inflammatory response independently of MLKL and necroptosis (176). In another study, it was found that MLKL signaling also activated NLRP3 inflammasomes and induced IL-1β secretion to promote inflammation. MLKL-induced NLRP3 inflammasome formation and IL-1β cleavage occur before cell lysis (177).

3.1.4 Ferroptosis

Ferroptosis refers to a new form of cell death caused by an increase of iron ion-dependent lipid peroxide (178). It is characterized by the accumulation of iron-regulated lipid peroxidation and caused by an imbalance of lipid metabolism, the depletion of glutathione (GSH), and the abnormal metabolism of iron. Excessive accumulation of iron is the key feature of ferroptosis, and most iron comes from damaged or aged red blood cells. Fe^{2+} produced by erythrocyte degradation can be oxidized to Fe^{3+}, and Fe^{3+} binding transferrin (TF) mediates endocytosis through transferrin receptor (TFR)1 (179, 180). After the endocytosis of TF-TFR1, Fe^{3+} is released from TF and reduced to Fe^{2+} by six-transmembrane epithelial antigen of the prostate 3 (STEAP3). Finally, unbound iron is easily absorbed by neurons, resulting in intracellular iron accumulation (181). When iron is overloaded, Fe^{2+} generates a large number of lipid-active oxygen radicals through the Fenton reaction. Fe^{2+} can also participate in the synthesis of lipoxygenase and then catalyzes lipid peroxidation (182).

Lipid peroxidation is a critical process of ferroptosis (183). Ferroptosis shows obvious lipid peroxidation stress and cell membrane damage. Polyunsaturated fatty acid (PUFA)-phospholipid (PL) species are the most sensitive to peroxide because they contain highly active hydrogen atoms in their methylene bridge. In ferroptosis, acyl-CoA synthetase long-chain family member 4 (ACSL4) catalyzes fatty acids to form acyl coenzyme A and promotes fatty acid oxidation or lipid biosynthesis (184). Next, lysophosphatidylcholine acyltransferase 3 (LPCAT3) inserts the composite into the membrane phosphatidylethanolamine (PE). The ferroptotic signal is then activated (185). These lipids can be peroxidized under the catalysis of lipoxygenase (LOX) or under the induction of ROS (OH^-) produced in the Fenton reaction. The resulting lipid peroxide can attack the proximal PUFA, causing a chain reaction and ferroptosis (186).

The manifestations of ferroptosis is the depletion of GSH and the inactivation of glutathione peroxidase 4 (GPX4). GSH is a tripeptide containing a sulfhydryl group, and it is composed of glutamic acid, glycine, and cysteine. It can combine with free radicals to repair cell membrane damage caused by lipid peroxide and it can clear ROS (187). GPX4 is a selenium enzyme. It can reduce oxidized lipids (L-OOH) (such as cholesterol and PL containing PUFA) to harmless lipid alcohols (L-OH) by converting GSH into oxidized glutathione (GSSG) (188). Therefore, GSH can also regulate GPX4 activity. In the process of ferroptosis, the accumulation of oxidation-reduction active iron consumes GSH reserves through the Fenton reaction, and then inhibits the activity of GPX4, leading to an overwhelming antioxidant reaction (189). The lack of GPX4 in turn leads to the accumulation of iron.

3.1.5 PANoptosis

Pyroptosis and apoptosis both involve the activation of members of the caspase protease family. Studies have found that the activation of caspase-1 triggers pyroptosis and apoptosis (190). As has already been mentioned, RIPK3 and MLKL are crucial for the occurrence of necroptosis. They can also mediate the formation of NLRP3 inflammasomes and trigger pyroptosis. This widespread crosstalk between pyroptosis, apoptosis, and necroptosis led to a new form of programmed cell death called "PANoptosis". PANoptosis is an inflammation-regulated cell death pathway. These cell death pathways are interconnected through the shared regulatory proteins called the PANoptosome. The PANoptosome is a cell death–inducing complex that is characterized by pyroptosis, apoptosis, and necroptosis molecules. It was identified as an inducer and regulator of PANoptosis. Christgen et al. found that RIPK1, RIPK3, caspase-8, NLRP3, ASC, and FADD interacted to form PANoptosomes (191). These proteins can be divided into sensors (ZBP1 and NLRP3), adapters (ASC and FADD), and catalytic effectors (RIPK1, RIPK3, caspase-1, and caspase-8) based on their functions (192). Lee et al. found that AIM2 regulated the innate immune sensors pyrin and ZBP1 to drive inflammation signal transduction and PANoptosis. The results confirmed that AIM2 mediated the assembly of multi-protein complexes, known as the AIM2 PANoptosome (193). Another study found a RIPK1 PANoptosome complex in an *in vivo* model of bacterial pathogen infection, which regulates all three branches of PANoptosis (194). In addition, during influenza virus infection, ZBP1 recruited RIPK3 and caspase-8 to activate ZBP1-NLRP3 inflammasomes. The formation of ZBP1-NLRP3 inflammasomes mediates PANoptosis by assembling the ZBP1 PANoptosome (195). Yan et al. confirmed the existence of PANoptosis in *in vitro* and *in vivo* models of ischemic brain injury through researching literature (196). In a following study, they demonstrated the occurrence of PANoptosis-like cell death in *in vivo* and *in vitro* models of ischemia/reperfusion injury (197). In summary, these studies indicate the presence of PANoptosis in ischemic brain injury. However, more research is needed to broaden our understanding of the basic processes of neuronal cell death and molecular targets, and to identify key molecules that regulate PANoptosis, which will lead to the development of new therapies.

4 Regulation mechanisms of natural compounds

The pathology of ischemic brain injury is an exceptionally complex pathological process involving a variety of cytotoxic

factors and inflammatory cells in the CNS as well as in the peripheral circulatory system. Inflammation and cell death are the two main factors in IS. Inflammation and cell death, which are caused by ischemia, overlap and are interrelated. Due to the complexity of these factors and their interactions, it is very difficult to develop effective treatment methods based on the "one drug, one target" strategy, which leads to adverse outcomes in stroke treatment (198). A substantial number of studies have shown that some natural compounds (such as salidroside, baicalin, astragaloside IV, and curcumin) have protective effects on IS with few side effects (shown in Table 4).

4.1 Salidroside

Salidroside (Sal) is the main bioactive component in Rhodiola rosea. In many studies of IS *in vitro* and *in vivo* in cells and animals, salidroside has demonstrated strong biological activity. Sal can significantly reduce the brain infarct size and cerebral edema by inhibiting inflammatory signaling. Sal reduces the levels of pro-inflammatory cytokines and chemokines in tissues or serum, such as TNF-α, IL-2, IL-6, IL-8, IL-1β, MCP-1, and MIP-1α (199). After cerebral ischemia, inflammatory transduction mainly depends on NF-κB, mitogen-activated protein kinases (MAPK), phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt), and phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) signaling pathways. Chen et al. found Sal effectively reduced the levels of IL-6, IL-1β, and TNF-α by blocking the RIP140/NF-κB pathway (200). Sal also significantly inhibited activation of NF-κB, blocked degradation of tropomyosin-related kinase B (IκBα), and reduced p-MAPK levels (JNK, p38 and ERK1/2) (201). In a further study, it was demonstrated that Sal inhibited CD11b and inflammatory mediators through PI3K/Akt/HIF signaling. Sal significantly upregulated HIF subunits (HIF1α, HIF2α, and HIF3α) and the HIF downstream target (erythropoietin). Sal reduced CD14, CD44, and iNOS mRNA (202). Zhang et al. demonstrated that Sal reduced inflammation and brain damage through PI3K/PKB/Nrf-2/NFκB signaling transduction. Sal induced NeuN and inhibited NF-κB p50 subunit and other pro-inflammatory mediators. It prevented a significant decrease in the proportion of p-PKB/PKB in the brain (203). These studies imply that Sal may inhibit inflammatory signaling through the Nrf2, HIF, MAPK, PI3K/Akt, PI3K/PKB, and NF-κB signaling pathways. In addition, Sal acts on immune cells to recover the damage caused by IS. A recent study reported that Sal significantly inhibited the release of inflammatory factors derived from microglia. To study microglia polarization, M1 phenotypic markers (CD16, CD32, iNOS, and CD11b) and M2 phenotypic markers (CD206, Arg1, TGF-β, and YM1/2) were analyzed. The results showed that Sal promoted the transformation of microglia from the M1 phenotype to the M2 phenotype to enhance the phagocytosis of microglia. At the same time, Sal-treated M1 microglia promoted oligodendrocyte differentiation (204). Sal has been shown to effectively reduce VCAM-1, ICAM-1, P-selectin, and E-selectin, as well as neutrophil recruitment in the ischemic brain (205).

Apoptosis is one of the main mechanisms of brain injury, and Sal has been found to have significant anti apoptotic effects. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic factors. BDNF has a protective effect on ischemic brain injury. Zhang et al. indicated that Sal produced its anti-apoptotic effects by regulating the BDNF-mediated PI3K/Akt apoptosis pathway in a DNA-binding-dependent and -independent manners (206). Sal has been shown to inhibit the downregulation of Bcl-2, the upregulation of Bax, and the release of mitochondrial cytochrome c into the cytosol. Sal attenuated the activation of caspase-3, -8, and -9, and ultimately protected cells from apoptosis (207). Another study demonstrated that Sal induced activation of the mitogen-activated protein kinase kinase (MAPKK)/extracellular signal-related protein kinase (ERK) pathway, thereby reducing cell apoptosis (208). Shi et al. showed that Sal decreased the expression of Bax and restored the balance between pro-apoptotic and anti-apoptotic proteins (209).

These studies indicate that Sal has anti-inflammatory and anti-apoptotic effects (Figure 3). In addition, Sal also demonstrated excitotoxicity inhibition and anti-oxidant effects, and reduced damage to the BBB. Therefore, as an effective neuroprotective agent, it can be developed as a potential drug for treating stroke.

4.2 Astragaloside IV

Astragaloside IV (AS-IV) is one of the main active ingredients from Astragalus (Astragalus membranaceus (Fisch.) Bunge., Leguminosae, Huangqi in Chinese). AS-IV has been shown to significantly reduce neuronal apoptosis. AS-IV can suppress the activation of key factors in the death receptor pathway. AS-IV was found to inhibit mRNA upregulation of Fas, FasL, Caspase-8, and Bax/Bcl-2. AS-IV also inhibited the protein levels of caspase-8, Bid, cleaved caspase-3 and cytochrome C (210). AS-IV can regulate the Nrf2 signaling pathway. Yang et al. found that AS-IV induced Nrf2 through the downstream signaling pathways (MAPK pathway) to prevent cell apoptosis. AS-IV inhibited the CXCR4 receptor and downregulated the activation of the p-JNK/JNK pathway, thereby inhibiting the expression of Bax/Bcl-2 and ultimately increasing Nrf2/Keap1 signaling (211). Another study confirmed that AS-IV regulated cell apoptosis through the PI3K/Akt/GSK-3β pathway (212). The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor. Its activation can increase the intracellular calcium concentration and contribute to cell apoptosis (213). AS-IV alleviated brain injury by inhibiting cell apoptosis induced by CaSR activation (214). Excitotoxicity by glutamate and mitochondrial dysfunction are common causes of cell apoptosis. AS-IV protects the integrity of mitochondria by promoting the combination of Akt and hexokinase II (HK-II). This helps to protect neurons from cell apoptosis and DNA damage (215). The PKA/CREB pathway regulates mitochondrial activity. AS-IV protects primary neurons from IS-induced apoptosis by regulating the PKA/CREB pathway and protecting mitochondrial function (216).

AS-IV also promotes the conversion of immune cells to an anti-inflammatory phenotype after IS, thereby reducing brain

TABLE 4 Regulation mechanisms of natural compounds.

Components	Experimental model	Effective dosage	Treatment time point and path	Targets	Results
Salidroside	Rats (MCAO), SH-SY5Y (I/R)	20 or 40 mg/kg	Before 30 minutes surgery (administrate orally)	↓ TNF-α, IL-1β, IL-6 and Bcl-2 ↑ RIP140, Bax, p-IKKα, p-IKKβ, p-IκBα, and p-p65	Anti-inflammation, inhibit RIP140/NF-κB pathway, anti-apoptosis,
	BV2 microglial cells (LPS)	75, 150, and 300 μM	After LPS	↓ MCP-1, MIP-1α, and IL-8 ↓ d-p-IκBα, p-NF-κB ↓ p65, p-JNK, p-p38 and p-ERK1/2	Anti-inflammation, inhibit migratory ability of BV2 cells,
	Rats (2/1h MCAO/R)	50 mg/kg	After MCAO/R (i.p.)	↓ TNF-α, IL-1β, IL-6, CD14, CD44, iNOs, CD11b, ↑ NeuN, p-Akt, HIF1α, HIF2α, HIF3α, EPO	Anti-inflammation, inhibit PI3K/Akt signaling
	Rats (pMCAO)	100 mg/kg	7 days i.p.	↑ NeuN, Nrf2, HO-1, p-PKB ↓ NF-κB p50, IL-6, TNF-α	Anti-inflammation, activate PI3K/PKB signaling pathway
	Mice (tMCAO/R 1h)	2.5, 5, 10, and 20 mg/kg/day	Give immediately after R (CVI) once/day for 5 days	↓ TNF-α, IL-1β, IL-2, IL-6, and IL-8 ↑ CD206, Arg1, TGF-β, and YM1/2	Anti-inflammation, promote M2 microglial polarization inhibit M1 microglial polarization
	Rats (MCAO/R) HUVEC (OGD/OGD-OGR)	50 mg/kg (rat), 10μM (cell)	After MCAO and OGD/OGD-OGR	↑ CD46, CD59 ↓ ICAM-1, VCAM-1, P-selectin, and E-selectin ↓ C1q, C2-mRNA, C3 protein level ↓ Bcl-2/Bax	Anti-inflammation, anti-apoptosis, suppress endothelial activation, inhibit neutrophilic recruitment
	MCAO mice (2/24h MCAO/R) neurons cells (OGD/R)	25, 50, and 100mg/kg	3 days/once (i.p.)	↑BDNF, p-PI3K, p-AKT ↑p-Bad, Bcl-2 and Bcl-xl	Anti-apoptosis, inhibit BDNF/TrkB/AKT/FoxO1 pathway, decrease accumulation of FoxO1,
	PC12 cells (H_2O_2 12h)	100μM	Pretreat	↑ Bcl-2 ↓ Bax ↓ cytochrome C release ↓ caspase-3, caspase-8 and caspase-9	Anti-apoptosis
	NGF-differentiated PC12 cells (H_2O_2 90 min)	128 μM	Pretreat 24h	↓ caspase-3, p-ERK1/2	Anti-apoptosis, activate ERK pathway
	Rats (2/24h MACO/R)	12mg/kg	Pretreat 7 days/once	↓ ROS, Bax ↑ Bcl-2	Anti-apoptosis, anti-oxidative effect,
Astragaloside IV	Rats (MCAO/R, after 1h MCAO to achieve R)	12.5 mg/kg, 25 mg/kg, and 50mg/kg	7 days/once after surgey (i.g.)	↓Fas, FasL, and Bax/Bcl-2 ↓caspase-8, Bid, cleaved caspase-3 and cytochrome C	Anti-apoptosis, inhibit death receptor pathway
	Rat primary cultured astrocyte (OGD/R)	16 μM, 32 μM, and 64 μM	After 4 h OGD	↓Bax/Bcl-2, Keap 1, ↑Nrf2, p-JNK/JNK	Anti-apoptosis, anti-oxidative effect, inhibit CXCR4/JNK pathway, and upregulate Keap1/Nrf2 pathway
	Mice (PBI), NSCs	200 mg/kg	3 days/once after stroke (i.v.)	↓IL-17, caspase 3, and number of NeuN/TUNEL ↑p-PI3K, p-Akt, and numbers of DCX/BrdU ↑numbers of $Wnt2^+$ cells	Anti-apoptosis, upregulate Akt/GSK-3β pathway, upregulate Wnt/β-catenin pathway
	Mice (photothrombosis), NSCs	2 mg/kg	3 days/once after stroke (i.v.)	↑DCX/BrdU and Sox2/Nestin ↓IL-17 ↑Wnt2, β-catenin, and GSK-3β,	Promote neurogenesis, activate NSC proliferation, upregulate Wnt pathway
		20 mg/(kg)			

(Continued)

TABLE 4 Continued

Components	Experimental model	Effective dosage	Treatment time point and path	Targets	Results
	Rats (MCAO/R) PC12 cells (OGD/R)		During reperfusion (i.p.)	↓cleaved caspase-3, AIF, and CaSR ↓Bax/Bcl-2	Anti-apoptosis, decrease the apoptotic rate, and inhibit calcium overload,
	Rats (MCAO) HUVECs (OGD/R)	40 mg/kg	Immediately after MCAO (i.g.)	↓EphrinA3 ↑miRNA-210	Activate HIF/VEGF/Notch pathway, stimulate angiogenesis
	Rats (tMCAO)	40mg/kg	14 days/once after MCAO (i.p.)	↑PPARγ, BDNF, IGF-1, and VEGF ↓CD86, iNOS, TNF-α, IL-1β, IL-6 ↑CD206, Arg-1, YM1/2, IL-10, TGF-β	Anti-inflammatory, promote M1 microglia to M2 through PPARγ pathway, promote neurogenesis and angiogenesis through PPARγ pathway
	Rats (MCAO)	40 mg/4 ml/kg	14 days/once (i.p.)	↑BDNF, TrkB	Promote neurogenesis, upregulate BDNF/TrkB pathway,
	Mice (MCAO) Primary cortical neurons (OGD/R)	15 and 30 mg/kg	/	↓cytochrome C, TUNEL-positive cells, glutamate, and caspase-3 ↑NAD^+ and ATP,	Anti-apoptosis, promote HK-II binding to mitochondria through Akt, protect mitochondrial integrity
	Primary cerebral cortical neurons (OGD/R)	6.25, 12.5 and 25 μmol/L	At the start of OGD/R	↓Cleaved caspase-3, ↑ATP, p-CREB, PKA	Anti-apoptosis, activate PKA/CREB pathway, protect mitochondrial,
Baicalin	Rats (pMCAO 24h)	30 or 100 mg/kg	2 and 12 h twice after the onset of ischemia (i.p.)	↓COX-2, iNOS, MPO, cleaved caspase-3, TUNEL-positive cells	Anti-apoptosis, reduced cerebral infarct area and infarct volume
	Rats (pMCAO 24h)	100 mg/kg	2 and 12 h twice after the onset of ischemia (i.p.)	↓TLR2/4, NF-κB, TNF-α, IL-1β ↓NF-κB p65, iNOS, COX-2	Anti-inflammation, reduced cerebral infarct area and infarct volume, inhibit activity of iNOS, COX-2
	Rats (pMCAO 24h)	100 mg/kg	2 and 12 h twice after the onset of ischemia (i.p.)	↓MMP-9 ↓expression of occludin	Anti-inflammation, reduced brain edema and BBB permeability
	Rats (MCAO/R), 2/24h, P12 cells (OGD/R)	100 mg/kg	24h	↓Drp-1, ↑ MFN2	Anti-apoptosis, enhanced mitophagy,
	Seven-day-old baby rats (left common carotid artery ligation)	120 mg/kg	After hypoxia for 2 h(i.p.)	↑p-Akt, GLT-1	Anti-apoptosis, upregulate GLT-1 via the PI3K/Akt pathway
	Rats (MCAO/R), primary astrocytes (OGD/R)	50 mg/kg	30 min before R (i.p.)	↓Mitochondrial succinate dehydrogenase	Anti-apoptosis
Curcumin	Rats (MCAO/R) 2/22h	200 mg/kg	30 min after I/R (i.p.)	↑p-Akt, p-mTOR, ↓LC3-II/LC3-I, IL-1, TLR4, p-38, and p-p38 ↓IL-6, TNF-α, and iNOS	Anti-inflammation, regulate TLR4/p38/MAPK pathway, mediate PI3K/Akt/mTOR pathway, improve neurological functions and reduce cerebral infarction,
	Rats (MCAO/R)	25 mg/kg	After MCAO (i.p.)	↑Bcl-2, Sirt1 ↓MMP, p53 and Bax ↓IL-6, TNF-α	Anti-inflammation, anti-apoptosis, reduce mitochondrial dysfunction, reduce infarct volumes and brain edema
	Mice (dMCAO/R) BV2 microglia (LPS, IFN-γ)	150mg/kg	0 h and 24 h after reperfusion (i.p.)	↓IL-6, TNF-α, IL-12p70 ↓CD16, CD32, iNOS ↑Arg-1 and YM1/2	Anti-inflammation, inhibit M1 microglia polarization, promote M2 microglia polarization
	Rats (MCAO)	300 mg/kg			

(Continued)

TABLE 4 Continued

Components	Experimental model	Effective dosage	Treatment time point and path	Targets	Results
			7 days/once after stroke (i.p.)	↑BrdU-positive cells, ↑BrdU/DCX-positive cells ↑NICD	Activate Notch pathway, improve neurofunctional recovery, promote neurogenesis
	Rats (MCAO)	300 mg/kg	30 min after MCAO (i.p.)	↓NF-κB, ICAM-1, MMP-9, ↓caspase-3	Anti-inflammation
	Mice (MCAO/R) N2a cells (OGD/R)	100, 200, 300 and 400 mg/kg	After occlusion 1 h (i.p.)	↓Bax, cleaved caspase-3 ↑Bcl-2,	Anti-apoptosis, alleviate mitochondrial dysfunction,
	Rats (MCAO/R)	50 mg/kg	5 days/once before MCAO (i.p.)	↑Sirt1, Bcl-2 ↓Ac-p53, Bax, cytochrome c ↓IL-6, TNF-α	Anti-apoptosis, anti-inflammation
	Primary cortical neurons (OGD/R)	0.25-10 μM	Add to culture medium	↓LDH, caspase-3, p-JNK ↑flotillin-1, p-ERK1/2	Attenuate cell death, regulate flotillin-1 and MAPK/ERK pathway,

References are shown in text.

nuclear receptor-interacting protein 1 (RIP40), phosphorylation (p-), lipopolysaccharide (LPS), degradation and phosphorylation (d-p-), phosphorylated protein kinase B (p-Akt), hypoxia-inducible factor (HIF) subunits (HIF1α, HIF2α, HIF3α), erythropoietin (EPO), intraperitoneal (i.p.), reperfusion (R), caudal vein injection (CVI), oxygen-glucose deprivation followed by restoration (OGD-OGR), superoxide dismutase (SOD), glutathione-S-transferase (GST), malondialdehyde (MDA), human umbilical vein endothelial cell (HUVEC), ultraviolet B (UVB), sunburn cells (SBCs), 8-hydroxy-2'-deoxyguanosine (8-OHdG), transient MCAO (tMACO), Kelch-like ECH-associated protein-1 (Keap 1), oxygen glucose deprivation/reoxygenation (OGD/R), C-X-C motif chemokine receptor 4 (CXCR4), photochemical brain ischemia (PBI), neural stem cells (NSCs), injected intravenously (iv.), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), glycogen synthase kinase-3β (GSK-3β), doublecortin (DCX), S-phase marker 5-bromo-2′-deoxyuridine (BrdU), pheochromocytoma (PC12), calcium–sensing receptor (CaSR), apoptosis-inducing factor (AIF), peroxisome proliferator-activated receptor γ (PPARγ), vascular endothelial growth factor (VEGF), brain-derived growth factor (BDNF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), wingless/integrated (Wnt), hexokinase II (HK-II), protein kinase A (PKA), cyclic AMP response element-binding protein (CREB), permanent middle cerebral artery occlusion (pMCAO), intraperitoneally injected (i.p.), myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), toll-like receptor 2 and 4 (TLR2/4), nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), streptozotocin (STZ), oxygen-glucose deprivation/reperfusion (OGD/R), mitofusin-2 (MFN2), glutamate transporter 1 (GLT-1), phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), NAD-dependent protein deacetylase sirtuin-1 (Sirt1), distal middle cerebral artery occlusion (dMCAO), arginine-glutamic acid dipeptide repeats protein (Arg-1), Notch intracellular domain (NICD), silent information regulator 1 (Sirt1), acetylated p53 (Ac-p53).

The meaning of the symbol "↑" is "upregulated". The meaning of the symbol "↓" is "downregulated". /, not appicable.

damage. PPARγ is a nuclear transcriptional factor that is widely expressed in microglia (217). AS-IV can promote the polarization of M1 microglia to the M2 phenotype, which plays a long-term neuroprotective role in cerebral ischemia/reperfusion injury through the PPARγ pathway (218). In addition, AS-IV also promotes angiogenesis by activating the HIF/VEGF/Notch, Wnt, and BDNF-TrkB pathways after IS, increasing cell proliferation, migration, and neovascularization (219–221).

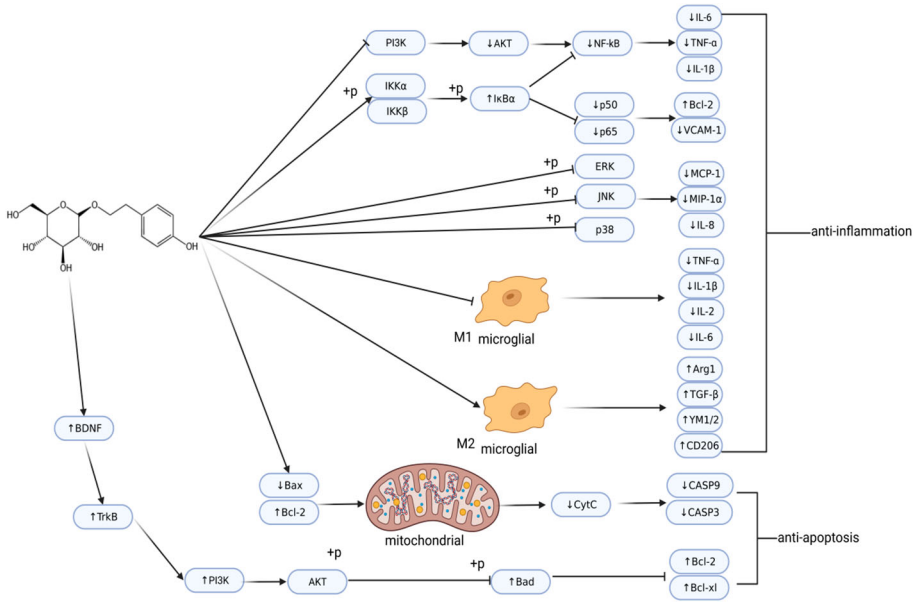

FIGURE 3
Regulatory network of salidroside in ischemic stroke. Post-IS, salidroside regulates inflammation and cell apoptosis. This image was created using BioRender.com.

4.3 Baicalin

Baicalin (BA) is a natural flavonoid compound isolated from the dried roots of Scutellaria baicalensis Georgi. BA alleviates the inflammatory reaction. BA was found to inhibit the TLR2/4 signaling pathway during cerebral ischemia, reducing expression of TLR2/4 and NF-κB in rat brain tissue. BA also attenuated the serum levels of TNF-α and IL-1β (222). In subsequent studies, Tu et al. also found that administration of BA after focal cerebral ischemia significantly reduced brain edema and BBB permeability. Overexpression of MMP-9 degraded the tight junction protein occludin, disrupting the integrity of the tight junction of the BBB (223). BA significantly downregulates the expression of MMP-9 protein and mRNA (224).

A growing body of evidence has shown the beneficial roles of BA in stroke management, such as anti-apoptosis. BA significantly inhibited neuronal apoptosis after cerebral ischemia injury in rats. Tu et al. found that BA significantly decreased MPO enzyme activity and iNOS and COX-2 mRNA expression in rat brain tissue, and significantly inhibited the expression of cleaved caspase-3 protein after IS (225). Li et al. showed that BA inhibited the expression of dynein related protein 1 (Drp-1). BA also reduced mitochondrial division and promoted the production of mitochondrial fusion protein 2 (MFN2) in an AMPK-dependent manner (226). Zhou et al. found that BA activated Akt phosphorylation and upregulated glutamate transporter 1 (GLT-1) expression through the PI3K/Akt signaling pathway. This inhibited cell apoptosis and reduced cerebral infarction volume and neuronal loss (227). BA also reduced mitochondrial succinate dehydrogenase (SDH)-mediated oxidative stress and reduced subsequent loss of glutamine synthetase (GS) (228).

4.4 Curcumin

Curcumin (CCM) is a compound mainly extracted from Curcuma longa. After IS, CCM can attenuate the inflammatory effect. The MAPK signaling pathway is regulated by TLR4 signaling and plays a key inflammatory role in IS. CCM alleviates inflammation of IS through the TLR4/p38/MAPK pathway. After CCM treatment, the protein levels of TLR4, p-p38, and IL-1 decreased, while the expression of IL-6, TNF-α, and iNOS increased (229). Another study demonstrated that CCM reduced inflammation by reducing levels of pro-inflammatory cytokines. Simultaneously, mitochondrial function was restored through an increase of MMP (230). In addition, CCM has a profound regulatory effect on the microglial response, promoting M2 microglia polarization and inhibiting the microglia-mediated proinflammatory response (231). However, further research is needed to confirm the involvement of curcumin and the specific mechanism of microglia phenotype regulation using stroke models. At the transcriptional level, the activation of NF-κB regulates ICAM-1, MMP-9 and caspase-3 expression (232). CCM decreased the expression of NF-κB, and subsequently attenuated the expression of the downstream mediators ICAM-1, MMP-9, and caspase-3 (233).

CCM exerts neuroprotective effects on IS and inhibits cell apoptosis. CCM reduces mitochondrial dysfunction and inhibits apoptosis by maintaining mitochondrial membrane potential and inhibiting the upregulation expression of Bax and downregulation of Bcl-2 (234). Silent information regulator 1 (Sirt1) is a class III group histone deacetylases that can protect the brain from ischemic damage (235). CCM was found to upregulate the expression of Sirt1 and Bcl-2 and downregulate the expression of acetylated p53 (Ac-p53) and Bax. Activating Sirt1 weakened cell apoptosis and promoted the neuroprotective effect of CCM (236). The MAPK signaling pathway regulates the expression of various pro-inflammatory cytokines and mediates apoptosis after ischemic injury. ERK1/2 and JNK are two of the main effectors of the MAPK signaling pathways (237). Lu et al. found that CCM reduced p-ERK1/2 and increased p-JNK protein levels. CCM also increased the level of flotilin-1 protein, thereby reducing cell death (238). CCM also improved neurofunctional recovery and promoted neurogenesis through Notch signaling after IS (239).

Therefore, based on the anti-inflammatory and anti-apoptosis effects of CCM, it may be a useful and promising neuroprotective agent against acute IS.

5 Conclusions and perspectives

Immunity, inflammation, and cell death play critical roles in the occurrence and development of stroke. This review summarizes the roles and mechanisms of immune cells and cell death pathways in IS. The immune cells discussed included microglia, astrocyte, neutrophils, T lymphocytes, and monocytes/macrophages. The cell death pathways discussed included apoptosis, pyroptosis, necroptosis, PANoptosis, and ferroptosis. This review also summarized the mechanisms of natural compounds in the treatment of IS. The natural compounds discussed include salidroside, baicalin, astragaloside IV, and curcumin.

Microglia and monocytes/macrophages form the first line of defense, but are involved in damage in the early stages of ischemic stroke. Microglia can induce increased damage to the A1 neurotoxic subtype of astrocytes. Neutrophils are recruited into damaged brain tissue, which can exacerbate inflammation. Subsequently, microglia and monocytes/macrophages show anti-inflammatory and repair functions. After T cells migrate to the brain parenchyma, they differentiate into different functional types. Hence, time-defined treatments targeting different phenotypes of immune cells may provide a clear protective strategy. At the same time, the interactions between immune cells cannot be ignored. The mutual coordination between immune cells is also caused by various inflammatory mediators. After IS, peripheral immune cells and brain immune cells form a complex inflammatory network. Treatments that target only one type of immune cell may be harmful or offset the benefits of another type of immune cell, resulting in an unsatisfactory stroke prognosis. Therefore, therapeutic strategies to modulate the immune system need to be further explored to determine effective treatment measures.

Compared to PANoptosis, the key molecular pathways involved in apoptosis, pyrotosis, ferroptosis, and necroptosis are clearer. Further research is needed on the molecular basis and key pathways of

PANopotosis after IS. Further research on the molecular and regulatory mechanisms of PANopotosis will have new impacts on the treatment of IS. For natural compounds in this review, mechanism research of Sal is the most extensive. All the natural compounds included in this review have therapeutic effects on inhibiting inflammation and cell apoptosis in IS. Salidroside, baicalin, astragaloside IV, and curcumin may be effective and promising candidates for the treatment of IS. However, they still have certain limitations, including whether they can show the same effect clinically as in research studies. Future research directions include 1) mechanisms for drugs to enter the central nervous system, 2) the ability to penetrate the blood-brain barrier and distribute widely in the brain, and 3) the side effects of drugs. With further research, the discovery of new drugs will lead to better treatment of IS for the benefit of public health.

Author contributions

NS: Conceptualization, Funding acquisition, Writing – review & editing. QC: Conceptualization, Writing – review & editing. ZG: Writing – original draft. JG: Writing – original draft. BL: Writing – review & editing. YG: Writing – review & editing. CC: Writing – review & editing. YJ: Writing – review & editing. NL: Writing – review & editing. MH: Investigation. TS: Writing – review & editing. LY: Writing – review & editing. HL: Writing – review & editing. HZ: Writing – review & editing. XZ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

IS	ischemic stroke
tPA	tissue plasminogen activator
PCD	programmed cell death
DAMPs	damage-associated molecular patterns
TGF	transforming growth factor
BBB	blood brain barrier
CD11b	integrin alpha-M
CD206	macrophage mannose receptor 1
Nrf2	nuclear factor erythroid 2-related factor 2
TLR	toll-like receptor
S1PR	sphingosine 1-phosphate receptor
GFAP	glial fibrillary acidic protein
C1q	complement component subunit 1q
pMCAO	permanent middle cerebral artery occlusion
NETs	neutrophil extracellular traps
tMCAO	transient middle cerebral artery occlusion
CCR5	C-C chemokine receptor 5
CXCR1	C-X-C chemokine receptor 1
TCRs	T cell receptors
I/R	ischemia-reperfusion
CTL	$CD8^+$ cytotoxic T lymphocytes
Th	$CD4^+$ T helper
Treg	regulatory T
γδ	gamma delta
JAK/STAT	Janus kinase/signal transducer activator of transcription
LPS	lipopolysaccharide
IL-15	interleukin-15
IL-17	interleukin-17
PRRs	pattern recognition receptors
CARD	cysteine protease recruitment domain
PYD	pyrin domain
NMDARs	N-methyl-D-aspartate receptors
Apaf-1	apoptotic protease activating factor-1
TNF	tumor necrosis factor
FADD	Fas-associated death domain protein
RIPK1	receptor interacting protein kinase 1
MLKL	mixed lineage kinase domain-like protein
Sal	salidroside

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MAPK	mitogen-activated protein kinases
PI3K/Akt	phosphatidylinositol 3 kinase/protein kinase B
PI3K/PKB	phosphoinositide 3-kinase/protein kinase B
IκBα	tropomyosin-related kinase B
BDNF	brain-derived neurotrophic factor
MAPKK	mitogen-activated protein kinase kinase
ERK	extracellular signal-related protein kinase
AS-IV	astragaloside IV
CaSR	calcium-sensing receptor
HK-II	hexokinase II
BA	baicalin
Drp-1	dynein-related protein 1
MFN2	mitochondrial fusion protein 2
GLT-1	glutamate transporter 1
SDH	succinate dehydrogenase
GS	glutamine synthetase
CCM	curcumin
Sirt1	silent information regulator 1

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