PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2022,49(1):126~138
www.pibb.ac.cn

CKLF1 as a Potential Therapeutic Target for Ischemic Stroke*

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Abstract Ischemic stroke is a kind of stroke in which the blood supply to the brain is disrupted, resulting in serious neurological deficits. Among stroke patients, about 87% of the cases are ischemic stroke. Neuroinflammation is one of the main pathological conditions of stroke injury. CKLF1 is a non-classical CC-type chemokine discovered in 2001, which showed strong chemotactic activity on monocytes, neutrophils, and lymphocytes. CKLF1 is most abundant in the fetal brain, but absent in the healthy adult. Growing evidence shows that CKLF1 is reactivated and expressed in adult stroke animal models and participates in multiple processing of neuroinflammatory responses. CKLF1 could activate the polarization of microglia to produce inflammatory mediators and trigger inflammation in the injured brain. And then they drove peripheral immune cells such as neutrophils to recruit into the injured brain. These stimulated the greater immune responses and destroyed the fragile blood-brain barrier (BBB). We believe that CKLF1 plays an important role in the course of ischemic stroke. However, the development of its biological activity and drug discovery lacks systematic literature reports. Thus, we collected the published data and made this review, to briefly describe the role of CKLF1 in ischemic stroke, and explained its mechanism of aggravating ischemic stroke. Moreover, some potential anti-stroke drugs have been discovered, indicating that CKLF1 is a potential target for the treatment of ischemic stroke.

Key words CKLF1, ischemic stroke, chemotaxis, microglia, neutrophil, inflammation, inhibitor
DOI: 10.16476/j.pibb.2021.0256

Stroke is one of the world's leading causes of disability and death[1-2]. Cerebral vascular embolization causes ischemic stroke, and rupture may cause hemorrhagic stroke. Ischemic stroke accounts for about 87% of all stroke cases[3]. There are currently two available major treatments for ischemic stroke: intravenous recombinant tissue plasminogen activator (rtPA) and endovascular therapy (EVT) [4]. However, both of them were restrictedly used in clinical practice due to their narrow therapeutic time windows, only patients who received treatment within 4.5−6 h after the onset of stroke were able to obtain beneficial therapeutic effects[5]. Furthermore, rtPA also has a risk of causing intracranial hemorrhage, which could lead to the switch from ischemia to hemorrhagic stroke[6]. Therefore, there is an urgent need to develop new strategies for the treatment of stroke.

Neuroinflammation has been involved in ischemic stroke by clinical investigations. Inhibiting

* This work was supported by grants from The National Natural Science Foundation of China (81873026, 82074044, 81730096), Beijing Natural Science Foundation (7192135), Key R&D Program of Shanxi Province (201803D421006), and the CAMS Innovation Fund for Medical Sciences (CIFMS) (2016-I2M-1-004).
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Received: August 28, 2021 Accepted: December 22, 2021

or alleviating the inflammatory and immune response caused by ischemic stroke might provide a new direction for the treatment of stroke. After ischemic stroke, the balance between pro-inflammatory and anti-inflammatory cytokines is broken, immune cells are activated, and they play a role in various stages of ischemic stroke. Neutrophils, as a kind of peripheral immune cells, migrate rapidly after cerebral ischemia. This phenomenon is one of the typical characteristics of neuroinflammation[7]. Microglia are inherent immune effector cells in the central nervous system, and play an extremely important role in the physiological process of the central nervous system[8]. After ischemic stroke, microglia are activated and polarized to the M1 phenotype and release pro-inflammatory factors to aggravate brain damage[9]. Chemokines are one of the critical cytokines that induce immune responses by binding to their corresponding receptors on the membrane and participating in various immune and inflammatory responses[10-13]. Some chemokines (such as CCL2, CCL3, CCL4, CCL5, CXCL1, CKCL2, CXCL3) are considered to be pro-inflammatory cytokines[10], leading to further activation and migration of immune cells.

In 2001, Han *et al.* [14] used the suppression subtractive hybridization (SSH) method to isolate a new cytokine and named it chemokine-like factor 1 (CKLF1), it has 3 different RNA splicing isoforms namely CKLF2, CKLF3, and CKLF4. It was found that CKLF1 had broad chemotactic activity on various cells[15]. Previous evidence has shown that the level of CKLF1 is enhanced in various inflammations such as autoimmune diseases and allergic diseases[16]. In subsequent studies, we found that CKLF1 played an important role in brain development[17-18]. We detected an increase in the expression of CKLF1 in the rat transient middle cerebral artery occlusion/reperfusion (tMCAO) model[19]. We searched the database and found that there were two review articles on CKLF1 research published in recent years (references 15 and 16), one of which was published by our lab. However, there is no systematic literature on the relationship between CKLF1 and stroke. Therefore, we collated the previous related articles and made this review, hoping to reveal the role of CKLF1 in ischemic stroke.

1 The biological function of CKLF1

CKLF1 is a secretory protein, and its structure is slightly different from the CC chemokine family. It has only one continuous CC structure and no additional C-terminal cysteine[14]. Due to the similar structure, some members of the classical chemokine family can be compensated by others after missing, but CKLF1 cannot.

CKLF1 is widely expressed in human lung, spleen, ovary, and testis, as well as fetal placenta, heart, brain, and other tissues and organs, but CKLF1 is barely detectable in the mature brain or heart[16]. Due to the wide distribution and strong chemotaxis, CKLF1 plays an important role in the hematopoietic[20-21], cardiovascular[22-23], respiratory[24], immune[25] and nervous system. This paper focuses on the function of CKLF1 in the nervous system.

1.1 CKLF1 in the nervous system

CKLF1 may be involved in the physiological activities of the brain. Previous studies have found that the expression of CKLF1 in the brain has two characteristics. One is highly expressed in the fetal brain and rarely expressed in the mature brain[18]. Wang *et al.* [17] found that CKLF1 stimulated the migration of SH-SY5Y cells and rat primary cerebral cortical neurons in a dose-dependent manner. Neuron migration is a very important part of the brain development process. The other feature is that CKLF1 is upregulated after cerebral ischemia but does not seem to be expressed in healthy brains[19]. Zhou *et al.* [26] found that CKLF1 was highly overlapped with neurons in the early stage of stroke, and did not co-localize with glial cells, suggesting that CKLF1 comes from neurons. Inhibition or specific knockdown of *CKLF1* can reduce the expression of CKLF1 in the damaged brain[7, 27-28]. Kong *et al.* [29] discovered that C19 peptide (sequence: FNPSGPYQKKPVHEKKEVL, a C-terminal antagonistic peptide of CKLF1) could reduce the rate of infarction and edema after focal cerebral ischemia in rats. Further studies have found that the administration of anti-CKLF1 antibodies could promote glucose metabolism, inhibit neuronal apoptosis, protect the blood-brain barrier (BBB),

thereby protecting the brain from focal cerebral ischemia injury[30-31]. These indicated that antagonizing CKLF1 could play a potential neuroprotective effect.

1.2 CKLF1 in the cardiovascular system

CKLF1 acts as a double-edged sword for the cardiovascular system. On the one hand, it can mobilize stem cells to differentiate to form new cardiomyocytes, and promote the proliferation and differentiation of vascular endothelial cells[23]. On the other hand, CKLF1 can cause cardiovascular disease. Generally speaking, cardiovascular diseases are mainly caused by atherosclerosis, hypertension and blood viscosity changes[32]. Vascular smooth muscle cells (VSMCs) are one of the major components of vessel wall, which have the function of supporting blood vessels and maintaining vascular tension[33]. The abnormal proliferation and migration of VSMCs are the key factors leading to atherosclerosis[34-35]. The upregulation of CKLF1 expression changes the balance between VSMCs proliferation and apoptosis, and cause abnormal aggregation and migration of VSMCs[36]. Zhang *et al.* [37] found that CKLF1 was overexpressed in atherosclerotic plaques and promoted VSMCs migration. Vascular restenosis is another reason for cardiovascular disease. CKLF1 could promote the expression of vascular cell adhesion molecule-1 (VCAM-1), leading to neointimal hyperplasia, which in turn causes to restenosis[38]. Therefore, the above results suggest that inhibiting the activity of CKLF1 may be a new therapeutic strategy for atherosclerosis and restenosis.

1.3 CKLF1 in the respiratory system

CKLF1 is closely related to lung tissue diseases, which is inseparable from the high expression of CKLF1 in the lungs. Tan *et al.* [24] injected CKLF1-expressing plasmid into the mice lung tissue. They observed obvious morphological changes in lung tissue: smooth muscle cell proliferation, epithelial cell shedding, edema, fibrosis, alveolar wall thickening, and diaphragmatic alveolar enlargement. These lesions increase the incidence of chronic obstructive pulmonary disease, asthma, lung cancer, and other diseases. In mild cases, they can cause coughing, chest pain, and difficulty breathing. In severe cases, they can even cause death. CCR4, as one of CKLF1 receptors, is thought to be related to asthma[39]. Increased eosinophils are a typical clinical feature of asthma. Tian *et al.*[40] showed that C19 peptide could treat asthmatic mice, inhibit the expression of CKLF1, reduce eosinophils in the airway and ameliorate lung inflammation.

1.4 CKLF1 in the hematopoietic system

The hematopoietic system includes the spleen, bone marrow, lymphocytes, monocytes, *etc*. These tissues and organs are the factories for human blood production. As reported previously, CKLF1 is highly expressed in the spleen, fetal liver and skeletal muscle[16]. Han *et al*. [21] found that CKLF1 could stimulate hematopoietic cells and promote the proliferation of bone marrow cells. Ke *et al*. [20] showed that CKLF1 could significantly promote the proliferation and differentiation of hematopoietic stem/progenitor cells. These results indicated that CKLF1 may have some therapeutic effects on hematopoietic dysfunction diseases.

2 The mechanism of CKLF1 aggravating ischemic stroke

Previous studies have found that CKLF1 seems to be little in the normal adult brain, but is rapidly expressed after ischemic stroke, and peaked on the second day[19]. Knockout of *CKLF1* can produce a neuroprotective effect[41]. Mounting evidence points out that CKLF1 exhibited significant chemotactic effects on neutrophils, lymphocytes, and monocytes both *in vivo* and *in vitro*[14]. We have proved that specific knockdown of *CKLF1* can reduce neutrophil infiltration[7]. In addition, microglia as the resident immune cells in the brain, play a crucial role after ischemic stroke[42].

2.1 CKLF1 is involved in ischemic stroke through CCR4/CCR5

CCR4 is mainly distributed in Tregs, NK cells, microglia and astrocytes, and plays a role through the migration and recruitment of immune cells under pathological conditions. The main ligands are CCL-17, CCL-22 and CKLF1[43-44]. Chen *et al*. [41] reported that CKLF1 might activate M1 microglia through CCR4 to produce inflammatory factors and aggravate early cerebral ischemia injury. In another study, the combination of CKLF1 and CCR4 activated the NLRP3 inflammasome and caused excessive release of inflammatory factors[28].

CCR5 is one of the important receptors in the CC chemokine family. In addition to being a therapeutic

target for AIDS, it is also a potential target for neurodegenerative diseases. Score *et al.*[45] had found that the lack of CCR5 led to the aggravation of neurological deficits in mice stroke model. In contrast, another research found that *CCR5*$^{-/-}$ mice received bilateral common carotid artery occlusion have fewer neurological deficits[46]. Maraviroc, as an anti-HIV infection drug, is the only CCR5 receptor inhibitor approved by the FDA and has achieved good clinical curative effects[47]. Joy *et al.*[48] determined the active therapeutic effect of Maraviroc on ischemic stroke. In this article, they mentioned that stroke patients with *CCR5Δ32* (the deletion of 32 base-pairs) had better prognosis for cognitive and motor functions than those without. In normal brains, CCR5 is highly expressed in microglia and extremely low in neurons. After ischemia, the expression of CCR5 in cortical neurons around the infarct core area was significantly increased, even higher than that of microglia. In the previous article, we reported that neurons produced CKLF1 in ischemic stroke. Therefore, our team explored the relationship between the increase of CKLF1 and CCR5 after cerebral ischemia. The co-immunoprecipitation test showed that CKLF1 and CCR5 interact, and specific knockdown of *CKLF1* could reduce the combination and reduce ischemic injury[7].

2.2 CKLF1 promotes neutrophil migration

Neutrophils, one of the leukocytes, are a major component of the innate immune system and the body's first line of defense against the invasion of pathogens, as well as one of the main participants in acute inflammation. Normally, neutrophils are reserved in the bone marrow. When stimulated by inflammatory factors, they mature rapidly and are recruited to the inflammatory area within a short time to produce immune effects[49].

The BBB is composed of capillary endothelial cells, pericytes, tight junction proteins, and astrocyte end-feet[50], and it is the interface of the central nervous system and blood exchanging material information. The BBB can block the entry of macromolecules and neurotoxic substances from plasma into the brain and maintain the microenvironment homeostasis of the central nervous system[51]. Ischemic stroke can cause edema, leading to serious damage to endothelial cells and rupture of the BBB[50]. Neutrophils migrate to the ischemic area of the brain after BBB disruption and interact with endothelial cells, which in turn lead to more severe BBB dysfunction and aggravate brain damage[52].

CKLF1 is an inducible chemokine stimulated by ischemic stroke, and the inhibition of CKLF1 improves neurological function. Hence, whether CKLF1 contribute to the aggravation of stroke injury by recruiting neutrophils infiltration? Our group investigated the relationship between CKLF1 and neutrophils in ischemic stroke. Kong *et al.*[53] treated the rats with anti-CKLF1 antibody, the results showed that inhibition of CKLF1 could improve neurological deficit and inhibit neutrophil infiltration. Furthermore, anti-CKLF1 antibody inhibited the expression of p38, ERK, and JNK in MAPK pathway, suggesting that the neutralization of CKLF1 activity reduced the recruitment of neutrophils in the ischemic brain by affecting the MAPK pathway. Chen *et al.*[7] further found that CKLF1 bound to the CCR5 receptor on neutrophils to activate Akt/GSK-3β pathway, further leading to neutrophil infiltration. Moreover, specific knockdown of *CKLF1* significantly reduced the degree of neutrophil infiltration and ameliorated brain injury in rats. These results indicated that CKLF1 promoted the neutrophil infiltration into the ischemic brain area, thereby exacerbated stroke outcome, which provide a new therapeutic clue for the treatment of ischemic stroke.

2.3 CKLF1 activated microglia to M1 phenotype

Normally, microglia are in a highly branched "resting state", and establish an efficient and rapid neural dynamic surveillance network through numerous cellular processes[54]. Microglia are rapidly activated by stimuli and exhibit different biological characteristics depending on the temporospatial difference of ischemia. Microglia can be polarized into two phenotypes: (1) Pro-inflammatory M1 phenotype releasing inflammatory cytokines such as nitric oxide (NO), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α). CD16/32 are markers of M1 type. (2) Anti-inflammatory M2 phenotype releasing anti-inflammatory cytokines such as interleukin-10 (IL-10), transforming growth factor (TGF-β), arginase-1 (Arg-1), CCL-22. CD206 is a marker of M2 type[42]. Regulating the balance of M1/M2 microglia may be of great significance to the protection of ischemic stroke.

Chen *et al.*[55] demonstrated that under the exposure of CKLF1, the immunofluorescence intensity of CD206 decreased and the expression of

TGF-β and Arg-1 reduced in BV2 microglia. It was proved that CKLF1 could enhance the M1 phenotype polarization and inhibit the M2 phenotype of microglia *in vitro*. Based on these results, the mice MCAO model was validated and the same conclusion was obtained[41]. It has been demonstrated that CKLF1 could regulate microglia polarization toward M1 type both *in vitro* and *in vivo* and aggravate the extent of brain injury in the acute phase of ischemic stroke. CKLF1 binds to CCR4, and then activates M1 microglia polarization and promotes the release of IL-1β and TNF-α. These process might activate the NF-κB pathway (one of the classical pathways of the inflammatory response[56]), and the activated pathway further promotes the release of inflammatory cytokines and forms a vicious cycle which eventually leads to the aggravation of brain injury.

3 Establishment of a drug screening platform targeting CKLF1

CKLF1 has broad-spectrum chemotaxis on leukocytes and can cause inflammation in various tissues. In recent years, CKLF1 has been found to play a significant role in cerebral ischemia/reperfusion injury and has attracted much attention in ischemic stroke research. CCR4, CCR5 are the functional receptors of CKLF1, which are involved in the inflammatory response[44]. Inhibition of CKLF1 could effectively improve neurological deficits, suggesting that screening compounds with neuroprotective effects by targeting CKLF1 may facilitate the development of new drugs against ischemic stroke. Our lab has long been devoted to CKLF1 research and has set up a drug screening platform targeting CKLF1. A variety of compounds antagonizing CKLF1 were screened out through the platform.

The C27 peptide on CKLF1 (ALIYRKLLFNPSGPYQKKPVHE-KKEVL) could interact with CCR4[57]. Li *et al*. [58] transfected HEK293 cells with human pCDI-CCR4 and constructed a HEK293-CCR4 overexpression cell strain, and then establish a high-throughput screening method on this cell strain to discover antiasthma activity drugs. They screened more than 2 000 compounds and obtained the 3-piperazine substituted coumarin compound 41 with oral activity. In the calcium transient and chemotaxis tests, compound 41 effectively reduced the calcium transient and chemotactic index in HEK293 cells, suggesting that compound 41 might interfere with the binding of C27 to CCR4. Mice treated with compound 41 had less NF-κB expression. These results illustrated that compound 41 might inhibit the activation of the NF-κB pathway by blocking the interaction of CKLF1 to CCR4, thereby reducing inflammatory injury. Since compound 41 showed a certain anti-inflammatory effect and inflammation plays an essential role in ischemic stroke, our lab optimized its structure and obtained a series of derivatives to discover new and effective anti-inflammatory drugs. We found that in the carrageenan-induced rats' pleurisy model, Sephadex-induced rats lung inflammation model, xylene-induced mice ear swelling model, and lipopolysaccharide (LPS)-induced RAW264.7 cell model[59-61], IMMLG5521 (a derivative of coumarin) had better anti-inflammatory activity than compound 41.

Moreover, to search potential neuroprotective agents, we established an *in vitro* ischemic cell culture model with PC12 cells[62]. Transient oxygen and glucose deprivation (OGD) in cells simulate the decrease of tissue blood flow after thrombosis, while restoration (R) represents the recovery of tissue blood flow. Among many derivatives of compound 41, IMM-H004 (7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one) was found to have potential neuroprotective effects. In cells, IMM-H004 protected PC12 cells from OGD/R-induced damage and decreased the oxidative stress of PC12 cells[62]. In another research, IMM-H004 reduced the apoptosis of cultured cortical neurons and PC12 cells caused by Aβ[63]. IMM-H004 could also inhibit the activation of LPS-induced BV2 microglia[64], which can simulate the pathological damage caused by microglia after neuroinflammation. In animals, we used classic cerebral ischemia models such as transient middle cerebral artery occlusion/reperfusion (tMCAO), permanent middle cerebral artery occlusion (pMCAO), and transient global cerebral ischemia, and found that IMM-H004 could alleviate brain neurological deficits[28, 65-69].

In general, IMMLG5521 and IMM-H004 showed anti-inflammatory activity in various inflammatory models both *in vivo* and *in vitro*. These results proved that the drug screening platform targeting CKLF1 was feasible. To better clarify the role of CKLF1 *in vivo*, we have established two kinds

of mice with gene defects: (1) *CKLF1* knockout of C57/BL6 mice and SD rats, technical support provided by Professor Zhang (Institute of Laboratory animal sciences, Chinese Academy of Medical Sciences & Peking Union Medicine College). (2) specific knockdown by adeno-associated virus (AAV) transfer *CKLF1* shRNA. Chen *et al*.[7] injected AAV into SD rats by stereotaxic microinjection and found that the expression of CKLF1 in the ischemic brain was decreased, indicating that AAV-CKLF1 specifically knocked down CKLF1 in the brain. The results showed that the expression of inflammatory cytokines in the KD group was significantly suppressed. The brain infarction of *CKLF1*$^{-/-}$ mice was lower than that of the wild-type group after MCAO[41]. It indicates that inhibition of CKLF1 can protect against cerebral ischemic damage. The use of these gene-deficient animal models can explain the role of CKLF1 in various diseases more intuitively, it also perfects the animal experiment part of our drug screening platform.

4 Pharmacological characteristics of CKLF1 inhibitors in the treatment of ischemic stroke

Clinical data and animal models indicate that CKLF1 is a protein closely related to the inflammatory process. The up-regulation of CKLF1 expression can be detected in diseases such as asthma, atherosclerosis, and stroke[19, 36, 39-40]. Our lab found that CKLF1 aggravated cerebral ischemia/reperfusion injury in animals. On this basis, we constructed a drug screening system targeting CKLF1. And some compounds have been found to antagonize CKLF1 or its receptor, such as C19 peptide, compound 41, IMMLG5521, IMM-H004, *etc*[29, 58]. Among these, we found that the therapeutic effect of the drug IMM-H004, which targets CKLF1, is better than the existing anti-stroke drugs (tPA, urokinase, edaravone, GM1, *etc*.)[27-28, 62, 69-70]. These inhibitors or antagonists can produce some similar pharmacological effects in stroke. We summarize the pharmacological effects of CKLF1 inhibitors that have been discovered so far, hoping to provide a reference for the development of safe and effective anti-stroke drugs.

4.1 Inhibit microglia/macrophage polarization toward M1 phenotype

Microglia are the only resident immune cells in the brain. They come from the same source as peripheral macrophages and have similar biological functions and phenotypes[71]. Microglia are highly sensitive and can form an efficient surveillance system to monitor the homeostasis of the brain's internal environment. Once the injury is monitored, microglia and macrophages constitute the body's fast line against ischemic brain injury, quickly gather at the injury site, produce an immune response, and release cytokines to recruit other immune cells into the injury area. Microglia change their shape and release different types of cytokines to participate in the immune response. It is generally believed that activated microglia have a pro-inflammatory M1 phenotype and an anti-inflammatory M2 phenotype[42]. At the early stage of the injury, microglia/macrophages located in the ischemic penumbra will first be polarized into M2 type, phagocytized dead neurons and cell debris, secreted anti-inflammatory cytokines such as TGF-β, IL-10, *etc*. and promote tissue repair and regeneration. However, with the extension of ischemia time, the infarct area spreads to the penumbra, causing the transformation of some microglia and newly aggregated macrophages to the M1 phenotype, releasing inflammatory cytokines like IL-1β, TNF-α. It leads to the generation of inflammatory cascade, damages normal neurons, and aggravates brain damage.

In the previous studies, we found that the expression of CKLF1 in rats and mice increased rapidly in a short period of time after tMCAO[19, 41]. There is a correlation with the activation of microglia. Therefore, the expression of M1 and M2 microglia biomarkers in the ischemic area were detected. It was found that the immunofluorescence intensities of the M1 markers (CD16/32) increased, and the mRNA expression levels of iNOS, CD16, CD32 upregulated, while the immunofluorescence intensity of M2 marker (CD206) decreased, and the mRNA expression levels of Arg1, CCL-22, and TGF-β downregulated. In mice with CKLF1 knockout, the activation of the M1 microglia/macrophages were inhibited, and the rate of cerebral infarction and edema reduced[41]. These results indicated that CKLF1 aggravated ischemia/reperfusion injury and promoted the polarization of microglia/macrophages to the M1 phenotype.

IMM-H004 is an antagonist of CKLF1, which can inhibit the binding of CKLF1 and CCR4 receptors

both *in vitro* and *in vivo*, thus alleviating the injury of ischemic stroke. IMM-H004 can reduce the activation of BV2 microglia induced by LPS or OGD/R[55, 64]. The expression of CKLF1 was up-regulated in OGD/R-induced BV2 microglia injury, and the expression of TNF-α and IL-1β were also increased. After the treatment with IMM-H004, TNF-α and IL-1β significantly reduced, while the protein expressions of anti-inflammatory cytokines Arg-1 and TGF-β increased[55]. In the model of BV2 microglia induced by LPS, LPS up-regulated the expression of iNOS, TNF-α, and IL-1β. And the up-regulation of these inflammatory cytokines could be inhibited by the administration of IMM-H004. NF-κB is an important regulatory factor in animals, which is involved in various inflammatory and immune response processes. NF-κB can induce a variety of cytokines (IL-1, IL-2, IL-6, IL-8, TNF-α, TNF-β, IFN-β), and also regulated by IL-1 and TNF-α[56]. Western blot analysis showed that IMM-H004 inhibited the expression of NF-κB in the two cell injury models[55, 64]. These results suggested that IMM-H004 might reduce the release of inflammatory factors by inhibiting the activation of the NF-κB signaling pathway, thereby inhibiting the polarization of BV2 microglia to the M1 phenotype.

4.2 Inhibit neutrophil infiltration

Neutrophils have a short half-life, so they usually stay in the bone marrow. Normally, neutrophils do not appear in the brain due to the presence of the BBB[49]. After cerebral ischemia, they will mature under the stimulation of cytokines released by damaged neurons and microglia in the brain, and complete the recruitment to the infarct area in a short time. Neutrophils will first attach to endothelial cells and stimulate the up-regulation of ICAM (intercelluar adhesion molecule)-1 and VCAM (vascular cell adhesion molecule)-1 on the endothelial cells. At the same time, neutrophils Mac-1 will also be up-regulated, which promotes neutrophils to penetrate through endothelial cells and tissues[72]. The MAPK pathway is one of the common intersection pathways of signal transduction pathways such as cell proliferation, stress, inflammation, differentiation, and apoptosis. ERK, p38, and JNK are several of these sub-families, each performing its duties. It has been reported that long-term activation of MAPK p38 after cerebral ischemia might induce neuronal injury[73]. Phosphorylated p38 would produce chemotaxis and promote the migration of neutrophils. While inhibiting its phosphorylation could affect but not completely inhibit chemotaxis[74].

After stroke, neutrophils penetrate the ischemic area through the BBB and produce an immune effect[75]. As mentioned in the previous research, CKLF1 aggravated early cerebral ischemia/reperfusion injury and promoted the release of inflammatory factors in the brain. Neutrophils were regulated by inflammatory factors. In other words, CKLF1 can act on neutrophils directly or indirectly affect neutrophil infiltration by regulating the expression of inflammatory factors. Kong *et al*. [53] measured the infarct size, MPO activity, the expression of TNF-α and IL-1β, and other indicators in tMCAO rats, and discovered that the expression of TNF-α and IL-1β were significantly increased after cerebral ischemia. MPO activity indicates the degree of neutrophil infiltration, the increased activity proved that neutrophils were recruited to the ischemic area. After treatment with anti-CKLF1 antibodies, the infarct volume was reduced and the nerve function was significantly improved. Moreover, the expression levels of TNF-α and IL-1β, and MPO activity were lower than the model group, indicating that the anti-CKLF1 antibody could reduce the expression of inflammatory factors and inhibit neutrophil infiltration. The p38, pJNK, and pERK levels were significantly increased in the model group, showing that MAPK signal pathway was activated after cerebral ischemia. The administration of anti-CKLF1 antibodies could reduce the level of phosphorylation and inhibit the activation of this pathway. To demonstrate that these effects were caused by the reduction of CKLF1, Chen *et al*.[7] injected AAV-HIF-1α-shRNA (CKLF1) into rat brain to cause specific knockdown of *CKLF1* in the brain region, and MCAO was performed. The levels of CKLF1, TNF-α, IL-1β, MPO activity, pJNK, and pERK in the infarct zone decreased to varying degrees, but p38 did not change significantly. Both anti-CKLF1 antibody and specific knockdown of *CKLF1* reduced the mRNA and protein expressions of ICAM-1, VCAM-1. In summary, CKLF1 inhibitors may inhibit neutrophil migration in several different ways. The first way is to down-regulate the expression of ICAM-1 and VCAM-1, the second way is to inhibit the activation of the MAPK pathway, and the third way is to decrease the inflammatory cytokines such as TNF-α and IL-1β to

reduce the stimulation of neutrophils.

4.3 Protect the BBB

The BBB is a natural barrier between the central nervous system and peripheral organs, it has the characteristics of selective permeability. This barrier can regulate with the material exchange between the central nervous system and blood, and maintain the homeostasis of the central nervous system. The physiological structure of BBB is roughly composed of: non-porous or few-pored endothelial cells, pericytes attached to endothelial cells, tight junction proteins, and end-feet of astrocytes[50]. Although its structure seems not complex, the physiological function is very subtle. The BBB can accurately control the entry and exit of substances, allowing nutrients (such as glucose, essential amino acids) to enter easily into the brain while keeping pathogens or harmful substances carried in the blood out. However, neurological diseases such as stroke, Alzheimer's disease, multiple sclerosis, epilepsy, traumatic brain injury, *etc*. will destroy the BBB and aggravate pathological damage[76]. Pericytes can interact with endothelial cells or astrocytes to stabilize the structure and function of BBB, and play a protective role in cerebral ischemia-reperfusion injury[77]. It has also been reported that pericytes can release sentrin/SUMO-specific protease 1 (SENP1), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor receptor-β (PDGFR-β), and these pro-regenerative molecules can promote revascularization[78-79]. Astrocytes are one of the common cell types in the central nervous system. Their enlarged end-feet are involved in the formation of the BBB, while the other side are in contact with neurons and can communicate between various types of cells[80]. AQP-4 is an important protein expressed at the astrocytes end-feet, which can maintain brain water balance and prevent edema[81].

MMPs are one of the important proteases for cell movement, inflammation, and apoptosis, and can aggravate the destruction of the BBB and tissue edema[82]. The rupture of tight junction proteins caused by edema is one of the main reasons leading to the increase of BBB permeability. The change of BBB permeability allow the cells and chemicals in the blood to penetrate into the brain tissue, causing the activation of microglia to release inflammatory cytokines and recruit peripheral immune cells to migrate to the ischemic area[83]. The up-regulation of ICAM-1 and VCAM-1 in vascular endothelial cells would cause neutrophils to adhere and aggregate of and penetrate the brain parenchyma, thus triggering an inflammatory cascade reaction and further aggravating injury[84].

C19 peptide can interact with the CCR4 receptor, but its chemotactic activity is weaker than C27[57]. In previous studies, the increased expression of CKLF1 after cerebral ischemia/reperfusion would expand the infarct volume and increase the brain tissue water content. The C19 peptide could significantly reduce infarction and edema, and improve neurobehavior[29]. To further explore the effect of CKLF1 on the BBB, various indexes related to the BBB were measured in brain. Transmission electron microscopy examination of pathological tissue slices revealed that the end-feet of astrocytes swelled after cerebral ischemia/reperfusion, then the tight junction protein was squeezed and broken. Evans blue exudation was used to express the damage degree of the BBB. Anti-CKLF1 antibody and IMM-H004 could change the enlargement of astrocytes end-foot and reduce the exudation of Evans blue in a dose-dependent manner. The results of both experiments indicated that cerebral ischemia/reperfusion could cause structure damage and increase permeability of the BBB, and inhibition of CKLF1 reversed this phenomenon[31, 69]. Anti-CKLF1 antibody and IMM-H004 could down-regulate the protein expression of AQP-4 and MMP-9, and up-regulate the expression of tight junction proteins ZO-1 and occluding. These results proved that they have the effect of reducing edema.

To summary, CKLF1 inhibitors can not only inhibit the infiltration of inflammatory cells, but also reduce the edema caused by the astrocytes end-feet expansion, prevent the rupture of tight junction proteins, and might maintain the integrity of pericytes to protect the structural and functional integrity of the BBB.

4.4 Other functions

It was found that CKLF1 inhibitors or their receptor antagonists could inhibit neuronal and mitochondrial apoptosis to improve brain energy supply, and reduce the oxidative stress of cells caused by ROS, thereby achieving the goal of protecting the damaged brain and treating ischemic stroke[30, 62-63, 70].

When designing and developing anti-stroke drugs targeting CKLF1, the key is to inhibit the neuroinflammation caused by CKLF1. By inhibiting

the release of inflammatory factors caused by CKLF1, the excessive activation of immune cells such as microglia and neutrophils can be reduced, thereby inhibiting the adverse consequences of excessive inflammation and avoid further damage to the brain after the destruction of the BBB by peripheral stimulation.

5 Conclusion

The chemokine-like factor family is a subfamily that differs in structure from other classic chemokines. CKLF1 is the first revealed member of this family. Although CKLF1 has been exposed for two decades, the understanding of it is still in its infancy. According to published reports, CKLF1 was associated with various diseases, such as stroke, asthma, atherosclerosis, arthritis, and tumors. In several diseases, the expression of CKLF1 was abnormally elevated. Nowadays, the prevalence and mortality of ischemic stroke in China have shown an upward trend, but current therapies still have shortcomings. Maraviroc proved the feasibility of therapeutic drugs targeting chemokines and their receptors. The abnormally elevated expression of CKLF1 in the ischemic brain provides new clues for the treatment of ischemic stroke. However, reports on this aspect are still relatively fragmented and further exploration is needed.

In conclusion, our previous work illustrated that CKLF1 played an important role in ischemic stroke (Figure 1). As a new therapeutic target against ischemic stroke, CKLF1 has the following potential advantages: (1) High effectiveness. Whether knockout or pharmacological blockade, the deletion of CKLF1 significantly decreased the cerebral infarction rate after ischemic stroke, reduced motor dysfunction, and improved the long-term benefits of model animals[7, 29, 41]. (2) High safety. CKLF1 can be inducible to express in the injured brain area after ischemic stroke, but it is rarely expressed in non-ischemic areas[19], suggesting that CKLF1 has natural targeting to ischemic brain area as a target. (3) Long therapeutic time window. CKLF1 aggravates the neuroinflammation after ischemic stroke by activating microglia and promoting neutrophil infiltration, and the therapeutic time window is significantly longer than that of thrombolytic drugs. (4) Low risk of

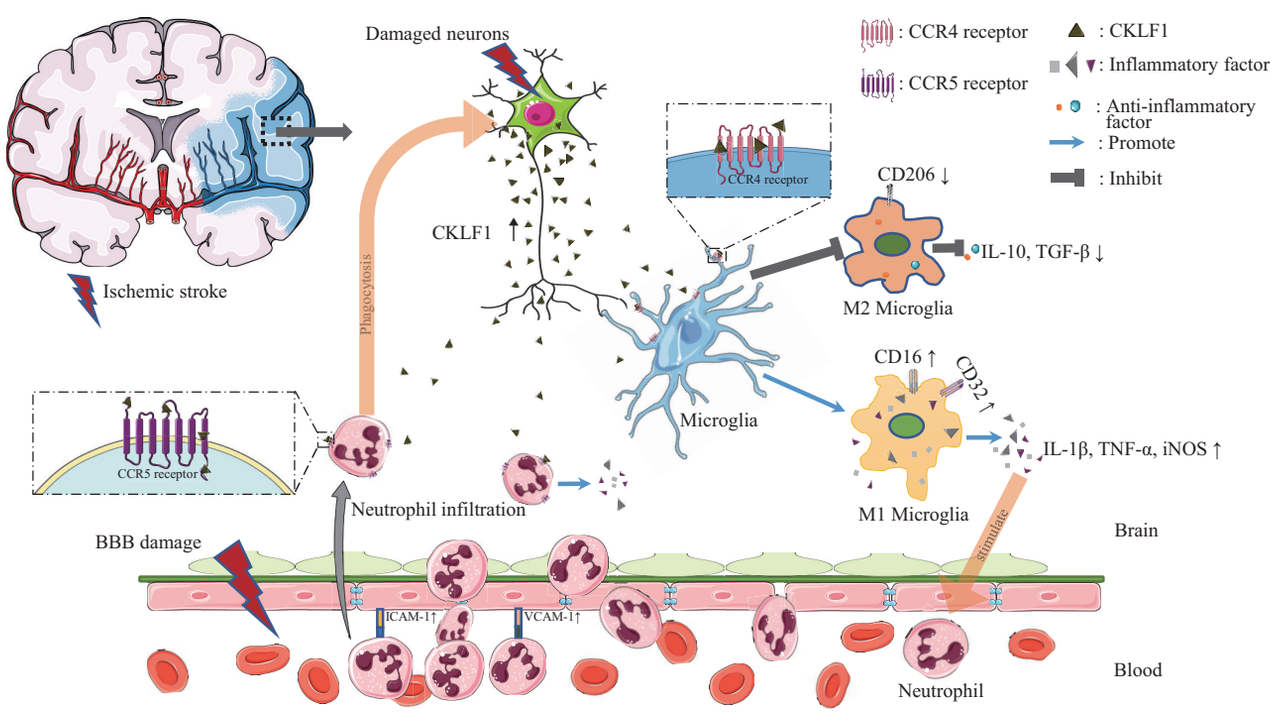

Fig. 1　The role of CKLF1 in ischemic stroke

When ischemic stroke occurs, damaged neurons release a large amount of CKLF1, causing neuroinflammation, and aggravating brain damage. CKLF1 activates microglia to polarize towards the M1 phenotype and releases inflammatory cytokines. Neutrophils feel the stimulation of CKLF1 and inflammatory cytokines and begin to infiltrate the ischemic brain area. These factors lead to the destruction of the blood-brain barrier and exacerbate brain damage.

hemorrhagic transformation. Previous studies have shown that the destruction of the BBB is the main pathogenies of hemorrhagic transformation. Moreover, the use of rtPA will also exacerbate the BBB destruction and increase the probability of intracerebral hemorrhage[85]. Inhibition of CKLF1 can significantly protect the BBB and ameliorate brain injury[31], indicating that it has a lower risk of hemorrhagic transformation. (5) Reduce cardiopulmonary complications. Ischemic stroke often increases the risk of infection[86]. It is reported that CKLF1 is closely related to lung inflammation[40]. Our research also found that CKLF1 inhibitors could significantly reduce cardiopulmonary injury after stroke and play a protective role in multiple organs[27]. (6) Mechanism is clear. CKLF1 could interact with CCR4 or CCR5 receptors on immune cells such as microglia and neutrophils to aggravate the neuroinflammatory response after stroke[7, 41]. Therefore, it has been proposed that CKLF1 is a new therapeutic target against ischemic stroke.

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CKLF1作为缺血性脑卒中治疗的潜在靶点*

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摘要　缺血性脑卒中是一种血液循环障碍疾病，可导致严重的神经功能缺损。卒中病人中约有87%的病例为缺血性卒中。神经炎症是中风损伤的主要病理状态之一。CKLF1是2001年发现的非经典CC型趋化因子，对单核细胞、中性粒细胞和淋巴细胞表现出很强的趋化活性。CKLF1在胎儿大脑中含量最高，但在健康成人阶段不存在。越来越多的证据表明，CKLF1表达在成年卒中动物模型中，并被重新激活，参与神经炎症反应的多个过程。然而，其生物活性和药物发现的发展仍缺乏系统的文献报道。因此，我们收集已发表的资料并做此综述，简要阐明CKLF1在缺血性脑卒中中的作用，并解释其加重缺血性脑卒中的机制。此外，还发现了一些潜在的抗卒中药物，表明CKLF1是治疗缺血性卒中的潜在靶点。

关键词　趋化素样因子1，缺血性脑卒中，趋化性，小胶质细胞，中性粒细胞，炎症，抑制剂
中图分类号　Q5　　**DOI**：10.16476/j.pibb.2021.0256

* 国家自然科学基金（81873026，82074044，81730096），北京市自然科学基金（7192135），山西省重点研发计划（201803D421006）和中国医学科学院医学与健康科技创新工程（2016-I2M-1-004）资助项目。
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收稿日期：2021-08-28，接受日期：2021-12-22