CC and CXC chemokines are pivotal mediators of cerebral injury in ischaemic stroke

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Summary
The definition of ischaemic stroke has been recently updated as an acute episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischaemia in the presence of a cerebral infarction. This "tissular" definition has highlighted the importance of pathophysiological processes underlying cerebral damage. In particular, post-ischaemic inflammation in the brain and in the blood stream could influence crucial steps of the tissue injury/repair cascade. CC and CXC chemokines orchestrate the inflammatory response in atherosclerotic plaque vulnerability and cerebral infarction. These molecules exert their activities through the binding to selective transmembrane receptors. CC and CXC chemokines modulate crucial processes (such as inflammatory cell recruitment and activation, neuronal survival, neangiogenesis). On the other hand, CXC chemokines could also modulate stem cell homing, thus favouring tissue repair. Given this evidence, both CC and CXC chemokines could represent promising therapeutic targets in primary and secondary prevention of ischaemic stroke. Only preliminary studies have been performed investigating treatments with selective chemokine agonists/antagonists. In this review, we will update evidence on the role and the potential therapeutic strategies targeting CC and CXC chemokines in the pathophysiology of ischaemic stroke.

Keywords
Stroke/prevention, chemokines, inflammatory mediators

Introduction
Stroke represents one of the leading causes of death and permanent disability in the adult population in industrialised countries (1). In particular, disability and dementia in stroke survivors have been estimated as a major social and financial burden that has been estimated to strongly influence political strategies of healthcare systems in the near future (1). Since cerebral ischaemia is the most common cause of stroke (about 85% of all causes of stroke), a strong effort has been done by basic and clinical researchers to better understand and prevent ischaemic cerebrovascular diseases. Recent pathophysiological findings have suggested a crucial role of inflammatory processes in carotid plaque vulnerability and reperfusion injury after acute cerebral ischaemia. Although ischaemic stroke is often associated with cardiac arrhythmias (mainly atrial fibrillation) or thrombotic disorders, this disease could be also caused by the rupture of a carotid plaque with the sudden occlusion of a cerebral blood vessel. Immediately after the loss of blood flow, the ischaemic cascade is rapidly initiated: the hypoperfusion leads to excitotoxicity and oxidative damage, which in turn exacerbate the initial injury with the induction of microvascular injury, blood-brain barrier (BBB) dysfunction, and post-ischaemic inflammation. In addition, inflammatory cell recruitment in the ischaemic brain during reperfusion might increase tissue injury (2). Cerebral damage and repair are regulated by soluble pro-inflammatory mediators (such cytokines, chemokines, adhesion molecules and matrix metalloproteinases [MMPs]) (3). In particular, chemokines have been shown to sustain inflammation from the risk of stroke (plaque vulnerability), till post-ischaemic reperfusion and neuroprotection. In the present review, we will update evidence on the role of chemokines and their corresponding receptors in ischaemic stroke pathophysiology. Their potential use as therapeutic targets will be also discussed.

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Chemokines are a subclass of cytokines, firstly described as “chemoattractants” inducing leukocyte chemotaxis. Considering the first two cysteines (N-terminal end) configuration, four subfamilies have been classified, respectively CC, CXC, XC, and CX3C chemokines (4, 5). On the basis of their functions, chemokines have been also defined as “homeostatic” chemokines (that are secreted constitutively and involved in immune surveillance and lymphocyte traffic) and “inflammatory” chemokines (that mediate pro-inflammatory signals and induce leukocyte recruitment to damaged or infected tissue). More recently, chemokines have been also shown to influence also angiogenesis and cellular differentiation (6). Chemokines exert their bioactivities through the binding with seven transmembrane domain receptors and the downstream activation of intracellular signalling pathways (7).

CC and CXC chemokines are crucial mediators of atherosclerotic plaque vulnerability (Table 1) (8, 9). In particular, they have been shown to induce inflammatory cell recruitment, activation and differentiation within atherosclerotic plaques (10), thus contributing to plaque vulnerability. CXC chemokines have been described as classical neutrophil chemoattractants in humans and mice (11, 12). On the other hand, CC chemokines mainly recruit monocyte/macrophages within inflamed tissues (13). More recently, CC chemokines (such as CCL3 and CCL4) have been described to also induce in vitro neutrophil migration (Fig. 1) (12, 14, 15). Neutrophils can also favour the extravasation of further inflammatory cell subtypes (such as monocytes) through the degranulation of chemotactic proteins (16). These recent studies showed that the inflammatory microenvironment importantly influences cell recruitment and activation, thus opening new investigation fields for pathophysiological research in the next future.

Table 1: Role of CC and CXC chemokines in ischaemic stroke.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Receptor</th>
<th>Risk of stroke</th>
<th>Post-ischaemic inflammation</th>
<th>Neuroprotection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2/MCP-1</td>
<td>CCR2</td>
<td>Induction of monocyte recruitment within atherosclerotic plaques [30].</td>
<td>Up regulation in ischaemic brain, CSF and serum [32, 37, 38].</td>
<td>Induction of migration of bone marrow stromal cells [44] and newly formed neuroblasts [45].</td>
</tr>
<tr>
<td>CCL3/MIP-1α</td>
<td>CCR1; CCR5</td>
<td>Induction of monocyte and neutrophil recruitment at inflammatory sites [14, 15, 52].</td>
<td>Up regulation in ischaemic brain [48].</td>
<td>Promotion of HUCB cell migration in ischaemic regions [39]. Inverse correlation with cerebral deficits [38].</td>
</tr>
<tr>
<td>CCL5/RANTES</td>
<td>CCR1; CCR3; CCR5</td>
<td>Promotion of atherogenesis in humans and mice [10, 56, 59].</td>
<td>Smaller infarct volume in CCL5 knockout mice [60]. Migration of peripheral blood mononuclear cells into the brain [61]. Induction of others pro-inflammatory cytokines [62].</td>
<td>Increase of infarct volume in CCR5 knockout mice [68].</td>
</tr>
<tr>
<td>CCL7/MCP3</td>
<td>CCR1; CCR2; CCR3</td>
<td>Not known.</td>
<td>Up regulation in the ischaemic cortex [70].</td>
<td>Not known.</td>
</tr>
<tr>
<td>CCL20/MIP-3α</td>
<td>CCR6</td>
<td>Not known.</td>
<td>Up regulation in the ischaemic brain after stroke [71, 72].</td>
<td>Not known.</td>
</tr>
<tr>
<td>CCL21/SLC</td>
<td>CCR7; CCR3</td>
<td>Not known.</td>
<td>Up regulation in ischaemic brain; possible modulation of microglia activation [74].</td>
<td>Not known.</td>
</tr>
<tr>
<td>CXCL1</td>
<td>CXCR2</td>
<td>Mouse neutrophil chemoattractant [77].</td>
<td>Up regulation in ischaemic brain and CSF in humans and mice [64, 82]. Protection of astrocytes from ceramide-induced apoptosis [32].</td>
<td></td>
</tr>
<tr>
<td>CXCL8/IL-8</td>
<td>CXCR1; CXCR2</td>
<td>Neutrophil chemoattractant [75]. Up regulation in carotid plaques [11].</td>
<td>Elevated levels in serum and CSF [76, 77]. Improvement of neurological deficits with CXCL8 receptor inhibition [79].</td>
<td></td>
</tr>
<tr>
<td>CXCL12/SDF1</td>
<td>CXCR4; CXCR7</td>
<td>Up regulated in atherosclerotic plaques [86, 87].</td>
<td>CXCR7 up regulation in ischaemic brain after stroke [89]. Increase of recruitment of monocytes in the ischaemic area [90]. Increase of recruitment of protective endothelial cell progenitors and neuroblasts [91–94]. Promotion of neuronal progenitor survival [97].</td>
<td></td>
</tr>
</tbody>
</table>
In the CNS, chemokines and their cognate receptors regulate both physiological and pathological processes. Physiologically, they are involved in neurotransmitter release and in CNS development. Fractalkine (CX3CL1) interferes with excitatory postsynaptic current, causing current reversible depression (17). The expression of CXCL12 and CXCR4 is involved in neurogenesis processes, such as neural progenitor/stem cell migration, proliferation, and differentiation (18). Chemokine receptor expression has been shown in astrocytes and microglia (10, 19). On the other hand, CC and CXC chemokines might influence immunemediated neurological diseases, including multiple sclerosis (MS) (20). Alterations in the development and maintenance of the oligodendrocyte lineage and myelination have been shown in CXCR2-knockout mice, suggesting a crucial role of this receptor in neuronal pathophysiology (21). Moreover, CC chemokines have been indicated to directly contribute to neurodegeneration and disease progression in the EAE model. Evidence from studies using CCR2-knockout mice showed that severity and progression of experimental autoimmune encephalomyelitis (EAE) was increased in the absence of this receptor (22). On the other hand, the relevance of chemokines in the cerebral nervous system diseases has been confirmed in humans. In cerebral tissue of MS patients, high expression levels of chemokines (such as CCL2–5, CCL7, CCL8, CXCL1, CXCL10, and CXCL12) and their receptors (CCR1, CCR2, CCR3, CCR5, CCR8, CXCR2, and CXCR3) has been shown (23). Furthermore, CXCL13 may play a prognostic role in MS patients. High cerebrospinal fluid (CSF) concentration of CXCL13 in patients affected by clinically isolated syndrome (CIS) correlate with a higher risk to develop MS (24). Potential involvements of CC and CXC chemokines have been identified also in Alzheimer’s disease (AD). The accumulation of monocytes and microglial cells at perivascular sites of beta-amyloid deposition and in senile plaques was shown to be mediated by CC and CXC chemokines (CCL2 and CXCL8, respectively) (25). CC and CXC chemokines were also shown as possible inflammatory mediators in neuro-inflammation following traumatic brain injury (Table 1) (26).

Therefore, considering their potential activities in recovery and repair in later stages of brain injury (27), experimental evidence suggests that CC and CC chemokines could be promising therapeutic targets to prevent inflammation in the different phases (from plaque rupture to cerebral repair) of ischaemic stroke disease. For this reason, in the following paragraphs, we will focus on the role of CC and CXC chemokines in the physiopathologic processes of brain ischaemia.

**CC chemokines in ischaemic stroke**

**CCL2/macrophage chemoattractant protein (MCP)-1**

Several studies in human beings and animal models suggested an active role of CCL2 in atherosclerotic plaque vulnerability (28, 29). This chemokine has been shown as a crucial mediator of monocyte/macrophage recruitment and activation within atherosclerotic plaques through the binding with its cognate receptor CCR2 (30). Although large clinical studies are still needed to verify this strategy, CCL2 inhibition could represent a very promising therapeutic approach to reduce carotid plaque vulnerability and related ischaemic stroke (31). CCL2 might play a pivotal role also in post-ischaemic microglial activation. CCL2 has been shown to be released in the ischaemic brain after transient focal ischaemia (32). In animal models of ischaemic stroke (performed by transient occlusion of the middle cerebral artery [MCAO]) CCL2 mRNA expression was increased in the ischaemic cortex from 6 hours (h) with peak at day 2 after ischaemia (32, 33). A gradual de...
crease in CCL2 levels was observed five days after cerebral ischaemia (32, 33). This temporal profile massively increased macrophage infiltration at days 2–7, confirming the crucial role of CCL2 as the most potent mediator of monocyte/macrophase infiltration in the ischaemic brain. Importantly, CCL2/CCR2 interaction could result in the recruitment of certain monocyte subsets during stroke. In mice, different monocyte subsets have been recently identified, showing that mainly the monocyte “inflammatory population” (that might differentiate into tissue macrophages and dendritic cells), which is positive for CCR2, is regulated by CCL2 (34, 35). Conversely, a second murine monocyte subset (not well investigated, but negative for CCR2) appears to be resistant to CCL2-mediated inflammation. Since the role of Gr1+/Ly6C<sup>low</sup>CCR2<sup>-</sup>CX3CR1<sup>high</sup> monocytes is still unclear, further studies are needed to better understand their pathophysiological relevance in inflammatory diseases. Similarly to mice, CCL2 could differently regulate different monocyte subset functions during stroke also in humans. In fact, rare populations (such as CD14<sup>high</sup>CD16<sup>-</sup> and CD14<sup>low</sup>CD16<sup>+</sup>), while the blood count of the most abundant CD14<sup>high</sup>CD16<sup>-</sup> monocyte did not change after a stroke (36). Since CCL2 could induce different functions (also potentially beneficial) in these cells, the role of this chemokine on monocytes during stroke represents a very interesting field for researchers.

Increased levels of CCL2 have been detected in the CSF and serum in the early hours after an ischaemic stroke (37, 38). However, these studies enrolled a very low number of patients. Thus, a confirmation by larger trials is needed. Similarly to atherosclerotic plaques, the selective inhibition of CCL2 could also be considered as a promising secondary prevention strategy to reduce macrophage recruitment and associated post-ischaemic damage.

The potential CCL2-mediated cerebral injury after stroke was supported by independent studies, showing that chemokine-knockout animals were protected from the disease. In particular, smaller infarct size and decreased inflammatory cell infiltration were demonstrated in CCL2-deficient mice (39, 40). Analogous effects were observed using chemokine receptor-antagonist treatment (41) or CCR2-deficient mice (42). These studies showed that CCL2/CCR2 axis might modulate BBB permeability and edema through the reduction in pro-inflammatory mediators and leukocyte infiltration (42). Confirming these results, CCL2 overexpression was associated with an enlarged ischaemic brain damage (43).

In vivo and in vitro studies indicated that CCL2 might contribute to the migration of bone marrow stromal cells (MSCs) within the ischaemic brain. These cells expressing neural phenotypes could reduce neurological deficits after stroke in rodents (44). Moreover, CCL2 might also promote the migration of newly formed neuroblasts from neurogenic regions (the subventricular zone and the posterior peri-ventricle region) to the damaged cerebral regions after focal ischaemia (45).

Interestingly, experimental evidence suggests that CCL2 might play a double role in ischaemic stroke, increasing the infarct damage in earlier phases and promoting later neurogenesis.

CCL3/macrophage inflammatory protein (MIP)-1α

CCL3 is an active mediator in cerebral post-ischaemic inflammation (12, 46). This chemokine has been shown to exert its pro-inflammatory activities through the binding to selective CC chemokine transmembrane receptors (CCR1 and CCR5) on inflammatory cell surface (12, 47). In neonatal rat brain, acute hypoxia determined an increase in tissue concentrations of CCL3, showing a peak from 8 to 72 h after injury. CCL3 upregulation has been shown to be associated with monocyte and microglial accumulation in injured brain (48). In adult rat models of cerebral ischaemia, the upregulation of CCL3 after cerebral ischaemia has been confirmed (49, 50). Interestingly, resident microglial cells and astrocytes could synthesise and release this factor (50). The role of CCL3 as potential therapeutic target in ischaemic stroke was tested in mice. Intracerebroventricular injection of viral macrophage inflammatory protein (vMIP)-II (a broad spectrum chemokine antagonist) reduced infarct volume in a dose-dependent manner (51). Accordingly, the intra-cerebro-ventricular injection of mouse CCL3 increases infarct volume (50).

Since CCL3 is considered as a classical monocyte chemoattractant within inflammatory sites (52), its activity on mononuclear cells has been well accepted. On the other hand, CCL3 has been recently shown as a potent neutrophil chemoattractant in mice and humans. In particular, CCL3 induces neutrophil adhesion and transmigration through the secondary release of inflammatory mediators (53). Also in humans, CCL3-mediated neutrophil recruitment has been shown in the presence of other pro-inflammatory molecules, such as tumour necrosis factor (TNF)-α, or insulin (14, 15). These studies indicate that CCL3 could influence the migration of different inflammatory cell subsets within both injured brain and atherosclerotic plaques. Although it is still not clear in humans whether this chemokine is released by the infarcted area or by circulating inflammatory cells, CCL3 serum levels are increased in patients with acute ischaemic stroke as compared to controls (38). Moreover, CCL3 levels displayed an inverse correlation with functional deficits (estimated by Barthel Index at 28 days), suggesting its potentiality as a short-term prognostic marker of post-stroke functional disability (38). This aspect suggests a potential role of CCL3 in neuroprotection. CCL3 could directly increase neuroprotective processes, such as the promotion of human umbilical cord blood (HUCB) cell migration in ischaemic regions (46). This could determine a potential therapeutic anti-apoptotic activity against stroke (54).

CCL4/MIP-1 beta

CCL4 exerts its activities by selectively binding the transmembrane receptor CCR5 (14). Its prominent activity has been described as the induction of monocyte recruitment at inflammatory sites (13). Recent evidence showed that CCL4 mRNA expression was upregulated in downstream portions of human carotid plaques as compared to upstream in both patients symptomatic or asymptomatic for is-
chaemic stroke (11). In vitro, CCL4 significantly increased reactive oxygen species (ROS) production and adhesion of THP-1 cells to human umbilical vein endothelial cells (55). These studies indicate that CCL4 could be involved in monocyte recruitment within atherosclerotic plaques, thus contributing to their vulnerability. Moreover, a study by Tatar et al. also investigated the potential prognostic role of CCL4 in a cohort of hypertensive patients. In this study, which enrolled 551 subjects, elevated serum CCL4 levels have been shown to independently predict stroke and cardiovascular events in an average follow-up period of 37.2 ± 19.9 months (55). Further clinical studies are needed to confirm the potential pathophysiological role of CCL4 as a potential active factor in stroke. Possible CCL4-mediated neuroprotective activities remain unexplored.

**CCL5/regulated on activation, normal T-cell expressed and secreted (RANTES)**

The involvement of the pro-inflammatory chemokine CCL5 in atherosclerosis and ischaemia/reperfusion syndrome has been widely investigated during the last five years. CCL5 selectively binds three different transmembrane receptors (CCR1, CCR3 and CCR5) on cell surface (56). We recently showed that genetic deletion of CCR5 but not CCR1 inhibits development of atherosclerosis in ApoE-knockout mice under high-cholesterol diet (56). This study indicates that mainly CC chemokines selectively binding CCR5 could regulate atherogenesis. Accordingly, inhibition of CCL5 with the antagonist 44AANA-47-RANTES markedly reduces atheroprosessing and myocardial reperfusion injury in mice (57, 58). The beneficial effect of CCL5 inhibition was mediated by the reduction of monocyte/macrophage recruitment within inflammatory plaque and myocardium (57, 58). The beneficial effect of CCL5 inhibition was also confirmed by Koenen et al., using stable peptides disrupting CCL5-CXCL4 interactions (59). The inhibition of CCL5-mediated activities could be a promising approach to reduce leukocyte infiltration and related post-ischaemic cerebral injury. In a murine model of focal cerebral ischaemia, CCL5 knockout mice showed significantly smaller infarct volumes and significantly decreased BBB permeability compared with wild-type mice (60). Importantly, a similar beneficial effect was observed in wild-type mice transplanted with the bone marrow of CCL5 knockout mice (60). This study clearly shows that the release of CCL5 from blood cells is a crucial mediator of reperfusion injury also in cerebral ischaemia and reperfusion. In particular, the mechanisms suggested to be involved were the CCL5-induced promotion of leukocyte recruitment and platelet adhesion in the cerebral microvasculature (60).

In vitro, CCL5 has been shown to induce migration of peripheral blood mononuclear cells across the activated BBB by interactions with receptors (CCR1 and CCR5) and adhesion molecules (α4β1 integrin/FN CS-1 and αLβ2 integrin/ICAM-1) (61). On the other hand, CCL5 could also increase cerebral damage through the secondary induction of other potent pro-inflammatory cytokines (such as interleukin [IL]-6) (62).

In humans, the measurement of CCL5 serum levels as a potential parameter in ischaemic stroke raised several controversies. In the first 30 days after the acute event, CCL5 serum concentrations have been found increased in patients symptomatic as compared to asymptomatic for ischaemic stroke (11). However, considering the entire cohort of patients, no correlation between serum CCL5 and the likelihood to be symptomatic was found (11). Conversely, the increase in CCL5 serum levels was not confirmed by Zaremba et al., who serially measured CCL5 serum levels in the first week after ischaemic stroke (38). However, CCL5 serum levels (detected two days after stroke) positively correlated at 28 days with the Barthel Index, a scale used to evaluate the patient performance in basic activities in daily living (38).

The association between the cardiovascular risk and some genetic polymorphisms of CCL5 and CCR5 has been recently investigated. In fact, not only CCL5, but also CCR5, is upregulated in the CNS after neurological insults (including cerebral ischaemia) and might influence CCL5 signalling in astrocytes, microglia and neurons (63). The A-403 allele variant in the promoter region of the Ccl5 gene resulted in increased gene expression as compared to G-403 and showed significant correlation with coronary artery disease (64) or cerebral infarction risk (65).

On the other hand, the del32 polymorphism of Ccr5 gene (a 32 base-pair deletion that results in a truncated nonfunctional receptor, which is present in homozygosis in 1–2% of the Caucasian population) was associated with lower levels of inflammatory markers (C-reactive protein [CRP], 1-antitrypsin, fibrinogen, and procollagen) lower carotid intima-media thickness and lower incidence of cerebrovascular disease (66). Although this aspect was more associated with cerebrovascular disease of cardio embolic origin, these genetic studies represent a very important approach to assess the complex interactions in the chemokine system (67). Following these observations, Sorce et al. recently showed surprising results in a mouse model of ischaemic stroke. CCR5-deficient mice showed larger cerebral infarct size, with increased neuronal death and neutrophil infiltration as compared to wild-type mice (68). This study might suggest a potential and unexpected neuroprotective role of CCR5 in ischaemic stroke. These results also indicate a crucial role for CCL5 or other CCR5 ligands, not only on inflammatory cells, but also on neurons. In fact, CCR5, which is also expressed in neurons, could modulate neuronal protection and survival (69).

**Other CC chemokines**

Few studies have been performed to clarify the involvement of other CC chemokines in ischaemic stroke. In murine models of permanent and transient occlusion of the MCAO, the induction CCL7/monocyte chemotactic protein (MCP)-3 was detected in the ischaemic cortex up to five days after the ischaemic injury (70). However, to present, no clinical studies evaluated the potential role of this molecule in humans.
Preliminary results have been published on another CC chemokine (CCL20/MIP-3α, which selectively binds CCR6). In a rat MCAO model, a marked induction of CCL20 and CCR6 mRNA expression was demonstrated after stroke (71, 72). CCL20/CCR6 downstream signalling pathways may accelerate the neuroinflammation of brain injury by favouring both peripheral immune cell infiltration and microglia activation (71).

Importantly, concomitant hypothermia suppressed this proinflammatory effect, suggesting a potential therapeutic strategy targeting these molecules (71).

CCL21/secondary lymphoid tissue chemokine (SLC) selectively binds CCR7 and has been shown to mediate the migration of circulating naive T lymphocytes to the lymph nodes (73). The expression of this chemokine is induced in the ischaemic brain, as well as in other conditions leading to cortical neuronal damage (74). CCL21 might directly modulate microglia-mediated neuronal injury, mainly through the binding with an alternative receptor (CXCR3), instead of CCR7 (74).

CXC chemokines in stroke

CXCL8/IL-8

CXCL8 (or its murine homolog CXCL2) is one of the most investigated chemotactants for neutrophils, a leukocyte subset mediating reperfusion tissue injury (75). Thus, this molecule has been supposed to play a crucial role in both atherosclerotic plaque vulnerability and ischaemic neuronal injury. CXCL8 mRNA expression has been found upregulated in carotid plaques of symptomatic as compared to asymptomatic patients for ischaemic stroke (11). In addition, post-stroke CXCL8 levels were found increased also in serum and CSF (76, 77), suggesting its intrathecal production and release in the circulation. In rats, the blood and brain levels of cytokine-induced neutrophil chemoattractant (CINC, belonging to CXCL8 family) proceeded and positively correlated with brain edema and cerebral neutrophil infiltration during early reperfusion (78). Accordingly, the pharmacologic inhibition of CXCL8 receptor with reparixin improved infarct size and neurological deficits in permanent and transient cerebral ischaemia in rats (79). This treatment also showed long-term benefits (up to 28 days after stroke) on clinical outcome and neurologic deficits (79), also suggesting a potential role for CXCL8 in neuroprotection. Little evidence is available on the correlation of IL8 haplotypes and/or single nucleotide polymorphisms (SNPs) with increased risk of myocardial injury or ischaemic stroke (80). Thus, this aspect remains to be verified.

Other CXC chemokines

Although preliminary results have been recently published, the potential role of CXC chemokines in stroke remains unclear. The increase of CXCL5 was demonstrated in CSF but not in serum of patients within the first 24 h of ischaemic stroke (81). Given the small sample size (23 patients and 15 controls), these data require experimental confirmation (81).

CXCL1/growth-regulated oncogene (GRO)-α levels were also found increased in CSF from patients with cerebral ischaemia, whereas no differences from controls have been observed in CXCL1 serum levels (82). The authors also showed a positive correlation between CSF levels of CXCL1 and the volume of brain computed tomography hypodense areas, suggesting an intrathecal origin of this chemokine (64). CXCL1 mRNA expression during ischaemic stroke was recently reduced in vivo by anti-atherosclerotic treatments (i.e. candesartan and pioglitazone) (83). This study indicates that CXCL1 could also represent a potential therapeutic target to reduce post-stroke inflammation and cerebral injury. However, some controversies on the role of CXCL1 in neuroprotection have been also raised (84). In fact, the stimulation of CXCL1/CXCR2 pathway in the presence of thrombin and a thrombin receptor agonist peptide has been shown to protect astrocytes from ceramide-induced apoptosis (84). This novel mechanism highlighted a potential dual role for CXC chemokines in tissue injury (increase of leukocyte recruitment and activation) and neuroprotection (improvement of neuronal survival).

Differently from other CXC chemokines, the binding with CXCL12/stromal cell-derived factor (SDF)-1 with its cognate receptors CXCR4 and CXCR7 has been mainly supposed to induce anti-atherosclerotic activities. This concept was supported by the beneficial role of CXCL12 in tissue reperfusion via the recruitment of mesenchymal stem cells (85). CXCL12 mRNA expression was detected in human atherosclerotic plaques as well as in developing and mature CNS (86–88). Importantly, CXCR7 expression was increased in the brain (mainly in ischaemic regions) after an ischaemic stroke, thus amplifying CXCL12-mediated functions (89). In the ischaemic brain, CXCL12 has been also shown to mediate both pro- and anti-inflammatory functions. In particular, CXCL12 induced the cerebral recruitment of monocytes (90), protective endothelial cell progenitors and neuroblasts (91–94). The recruitment of these cells was mainly mediated through the binding to CXCR4 (91–94). These data were recently confirmed by Wang et al., showing that the intracerebral recruitment of transplanted bone marrow-derived mesenchymal stem cells was mediated by CXCL12/CXCR4 interactions (95). In addition, CXCL12 and CXCR4 upregulation in murine olfactory ensheathing cells have been shown to promote axonal regeneration in vitro and in vivo (96), indicating that CXCL12 binding to CXCR4 on neuron precursor surface membrane induces promising neuroprotective results. On the other hand, CXCL12 might also promote neural progenitor cell survival via CXCR7 (97). This study suggests that probably CXCR4 and CXCR7 could activate parallel and protective pathways in ischaemic stroke. Importantly, CXCL12 has been shown to also induce deleterious effects. CXCL12 may be cleaved by the MMP-2 to form a highly neurotoxic molecule, SDF(5–67) that is not able to bind its receptor CXCR4 but recognises CXCR3. The CXCL12 cleavage in SDF(5–67) has been reported in the human immunodeficiency
virus (HIV)-infected brain and might contribute to neurodegeneration (98).

**Potential therapeutic approaches targeting CC and CXC chemokine pathways**

The pathophysiological role of chemokines and their receptors in several inflammatory diseases (including ischaemic stroke), makes them particularly attractive as therapeutic targets. To date, only few molecules targeting chemokines and their receptors have been approved by the U.S. Food and Drug Administration, like the inhibitor of CCR5 maraviroc (for the HIV prevention) or the CXCR4 antagonist mozobil (that induces mobilisation of hematopoietic stem cells from the bone marrow to the bloodstream). A large number of these molecules are now tested in phase I to III trials (99). None of these molecules has been recommended in ischaemic stroke.

Several approaches targeting the chemokine system have been considered:
1. modified chemokines;
2. synthetic small molecule receptor antagonists;
3. neutralizing antibodies;
4. chemokine antagonists produced by pathogens.

In addition, other medications have been shown to interfere with chemokine system. In the following paragraphs we will update these therapeutic strategies and their potential use in ischaemic stroke (Table 2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemokine target</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMIP-II</td>
<td>chemokine analogue peptide acting as antagonist against several chemokines and chemokine receptors.</td>
<td>Various chemokines, including CCL2, CCL3 and chemokine receptors (CCR1,2,3,4,5,8; CXCR3,4).</td>
</tr>
<tr>
<td>JWH-133</td>
<td>Synthetic cannabinoid 2 receptor agonist.</td>
<td>CXCL2</td>
</tr>
<tr>
<td>Estradiol</td>
<td>Synthetic cannabinoid 2 receptor agonist.</td>
<td>MIP-2; CCR7</td>
</tr>
<tr>
<td>Dimemorfan</td>
<td>sigma-1 receptor agonist.</td>
<td>CCL2</td>
</tr>
<tr>
<td>Candesartan</td>
<td>angiotensin AT1 receptor blockers.</td>
<td>CXCL1</td>
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<tr>
<td>Pioglitazone</td>
<td>thiazolidinediones.</td>
<td>CCL2; CXCL8</td>
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**Modified chemokines**

Some modified chemokines that block the receptor activation are especially effective to inhibit leukocyte recruitment. For example, the CCL2/MCP-1 analog 9–76 MCP-1, which is truncated in the NH2-terminal region, is a CCL2/MCP-1 receptor antagonist (100). In a mouse model of arthritis, the treatment with 9–76 MCP-1 antagonist has been shown to reduce the arthritis symptoms through inhibition of mononuclear cell infiltration (100). This beneficial effect of CCL2 inhibition has been confirmed using another CCL2 analog (P8A-MCP-1) to treat rats with adjuvant-induced arthritis (101). The potential use of CCL2 inhibitors in ischaemic stroke remains largely unexplored. Treatment with NR58–3.14.3 (a retroinverso analogue of a 12-mer peptide inhibiting broad spectrum [both CC and CXC] chemokine functions) attenuates the post-ischaemic inflammatory response, improving lesion volume and neurologic function in a rat model of cerebral ischaemia (102). Beside the CCL2/MCP-1 analogs, several mutants of CCL5/RANTES show powerful anti-inflammatory properties. Methionylated-RANTES (met-RANTES) could reduce inflammatory diseases, like atherosclerosis (103), myocarditis (104) arthritis (105) or liver fibrosis (106) by inhibiting CCL5 receptors CCR1, CCR3, and CCR5 (107, 108). Aminoxypentane-RANTES (AOP-RANTES), another chemokine analog was produced to reduce HIV infection by inducing the CCR5 internalisation (109). AOP-RANTES treatment has been shown to be protective in a model of airway inflammation (110). However, controversial outcomes were observed for the treatment of experimental glomerulonephritis (111, 112). The CCL5/RANTES analog 44AANA47-RANTES is mutated in the glycosaminoglycan-binding site and acts as a dominant negative inhibitor of CCL5/RANTES.
ligand (113). It has significant anti-inflammatory properties in several models of inflammatory pathologies, such as experimental autoimmune encephalomyelitis, atherosclerosis or myocardial ischaemia-reperfusion injury (57, 58, 113). However, treatment with CCL5/RANTES analogs in a model of adjuvant-induced arthritis after the induction of the disease did not improve the clinical signs of the disease (101). The use of modified CCL5 analogs remains to be explored in ischaemic stroke.

**Synthetic small-molecule receptor antagonists**

The most advanced therapeutic strategy to inhibit chemokine/chemokine receptor-induced inflammation consists in the treatment with synthetic small-molecule receptor antagonists. A large panel of small-molecules that target several chemokine receptors have been elaborated. The small chemical compounds could have several modes of action. They can competitively bind to the same site as the receptor, alter the tertiary or the quaternary structure of the protein ligand or interfere with the protein bioactivity. To date, small-molecule inhibitors for six receptors (CCR1, CCR2, CCR5, CCR9, CXCR2, CXCR4) have been investigated in patients (99). They are administered to potentially treat various diseases, such as HIV, MS, or rheumatoid arthritis.

Interfering with the chemokine heteromer formation may also represent an effective strategy to inhibit the chemokine signalling activation. For example, the peptide antagonist CKey2 (human) or mKey (mouse), by interfering with the CCL5-CXCL4 heteromer formation, could reduce the atherosclerosis development in hypercholesterolaemic mice (59). Till present, their use to treat ischaemic stroke has not been investigated yet.

**Neutralising antibodies**

The neutralisation of chemokine bioactivities with specific antibodies represents also potential therapeutic applications. To date, three neutralizing programs have been conducted. However, the clinical outcomes of these studies were not concluding. Anti-CXCL8/IL-8 antibody was tested in a chronic obstructive pulmonary disease trial with improvement of the transition dyspnea index but no benefit in lung function and health status of the patients (114). Another antibody against CCL2/MCP-1 was tested in a phase II trial for rheumatoid arthritis, but this treatment did not show any clinical improvement for the patients (115). Anti-CCR2 antibody was also tested to treat rheumatoid arthritis, but failed also to show efficacy (116). Although these results are disappointing, several anti-chemokine antibodies are currently under investigation to treat different inflammatory pathologies, such as atherosclerosis, cancer or multiple sclerosis (99).

In an ischaemic stroke animal model, treatment with CXCL8-neutralising antibody showed a neuroprotective effect, reducing brain oedema and cerebral infarct size (117). Encouraging results have been also observed with anti-rat MIP-3α antibody MAB540, (which reduced the ischaemic MIP-3α antibody MAB540, (which reduced the ischaemic area) (71), and with anti-CCL2 antibody (which decreased the BBB permeability after ischaemia/reperfusion) (118).

**Chemokine antagonists produced by pathogens**

Neutralising molecules produced by pathogens are very effective anti-chemokine agents. Indeed, to escape host detection and defense systems, pathogens produced antagonists that block cytokine signalling. Virus used different anti-chemokine strategies to evade the immune system like chemokine homologues acting as chemokine receptor antagonist, chemokine receptor homologues that function as chemokine scavengers, and secreted binding proteins that are extracellular chemokine scavengers (119, 120). Recently, anti-inflammatory and immuno-modulatory compounds secreted by ticks were identified (121). These molecules called 'evasins' allow the parasite to remain feeding during long period on the host without inflammatory response. Evasins are chemokine antagonists and reduces leukocyte recruitment at inflammatory sites (122). Evasins showed anti-inflammatory properties in several animal models, such as experimental colitis, myocardial reperfusion injury, or bleomycine-induced pulmonary fibrosis (77, 123, 124). Schistosoma mansoni, a trematode parasite, has been shown to secrete a chemokine-binding protein (called smCKBP) with anti-inflammatory properties (125). Treatment with recombinant smCKBP has been shown to reduce inflammation in mouse models of pulmonary inflammation, but not yet in ischaemic stroke (132). Importantly, beneficial effects on chemokine selective antagonism have been reported by Takami et al. (51). Authors demonstrated that the intracerebroventricular administration of viral macrophage inflammatory protein-II (vMIP-II), a chemokine analogue peptide encoded by Kaposi sarcoma-associated herpes virus, attenuated brain infarction in mice (51).

**Other drugs interfering with the chemokine system**

Different drugs might modulate chemokine and chemokine receptor expression in ischaemic tissues, thus interfering with post-infraction inflammatory cells cerebral infiltration. Treatments with cannabinoids were protective against ischaemic stroke. In a mouse model, the synthetic cannabinoid 2 receptor (CB2) agonist JWH-133 inhibited CXCL2-mediated neutrophil recruitment by activating p38 mitogen-activated protein kinase (MAPK) (126). Interestingly, the JWH-133 administration does not interfere with cell response migration toward N-formyl-methionyl-leucyl-phenylalanine (fMLP, a bacterial product). Thus, this treatment might selectively reduce neutrophil cerebral infiltration after ischaemic stroke, without reducing neutrophil response against bacterial infections. Considering that bacterial infections represent a major cause of death in acute stroke, these results could be of particular importance for clinical applications (127).
Estrogen is thought to reduce the risk and severity of stroke in adult females. In ovariectomised female mice, administration of estradiol (17-b-E2) or putative membrane estrogen receptor agonist (G1) decreased infarct volume (128).

In these mice, 17-b-E2 replacement markedly decreased MIP-2 mRNA in the brain, suppressing brain inflammation. In addition, 17-b-E2 administration increased CCR7 expression in the brain and normalised the count of cerebroprotective FoxP3(+) CD4(+) CD25(+) regulatory T cells (T_{reg} cells) in E2-deficient animals (128).

Treatment with dimemorfan (a sigma-1 receptor agonist with antitussive properties) in rats ameliorated infarct size through the reduction of glutamate accumulation and its downstream inflammatory events (129). Similarly, also antiplatelet drugs have been shown to inhibit post-stroke upregulation of inflammatory chemokines (such as CXCL18) in circulating leukocytes (130). In a murine model of focal cerebral ischaemia and reperfusion, treatment with rosiglitazone (1 h prior and 24 h after the MCA occlusion) reduced the infarct size and improved functional outcomes (131).

The main mechanisms underlying thiazolidinedione-mediated neuroprotection have been shown in a decrease in microglial activation, macrophage infiltration, and expression of pro-inflammatory molecules (132). The use of thiazolidinediones to prevent ischaemic stroke in humans has been investigated in human clinical trials, without confirming mouse results. In type 2 diabetic subjects, treatment with thiazolidinediones did not decrease the incidence of first stroke as compared to other oral hypoglycaemic drugs (133). Recently, these data were also confirmed by a retrospective cohort study enrolling 473,483 patients with type 2 diabetes mellitus (134). Clinical studies did not confirm the encouraging results on post-stroke outcomes (such as stroke recurrence), previously showed in mice. For instance, well-validated antiatherosclerotic drugs, such as angiotensin receptor blockers (telmisartan) failed to lower the rate of recurrent stroke and major cardiovascular events as compared to placebo in high-risk patients (135). Conversely, combination therapy with low-dose aspirin and dipyridamole improved cardiovascular outcomes in patients with disabling stroke (136). Potential benefits in both primary and secondary prevention of ischaemic stroke have been also shown for statin treatments (137). The potential direct activities of these treatments on the chemokine system remain to be evaluated in both human and mouse models of ischaemic stroke.

Conclusions

Recent findings demonstrated that both CC and CXC chemokines play a central role in inflammatory events underlying plaque vulnerability, cerebral damage and post-stroke neuroprotection. These molecules modulate a large spectrum of biological processes (such as inflammatory cell and stem cell infiltration, angiogenesis, BBB permeability, and neuronal survival). Depending on the concentrations and time of release, different CC and CXC chemokines have been shown to induce in the same time neuroprotective (i.e. CXCL12) and deleterious effects (i.e. CCL2, CCL3, and CXCL8). Although further studies are needed to explore the role of CC and CXC chemokines in neuronal post-ischaemic apoptosis, the chemokine system represents a promising target for selective therapies to prevent ischaemic stroke and reduce consequent disability. At present, only few studies in animal models of ischaemic stroke explored the potential benefit of anti-chemokine treatments. Although preliminary results are encouraging, further studies are needed to evaluate if anti-chemokine approach might safely reduce stroke. In fact, since chemokines are crucial mediators of the immune response, allergic and serious immunosuppressive reactions should be expected. In addition, the first studies in animal models did not sufficiently focus on the doses and duration of anti-chemokine treatments. This indicates that anti-chemokine treatments in humans will not represent an immediate therapeutic option for patients with ischaemic stroke. Although more studies are needed, we believe that anti-chemokine therapies might represent one of the most promising therapeutic approaches to reduce ischaemic stroke in the next decades.

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