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Cell biology and clinical promise of G-CSF: immunomodulation and neuroprotection

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Abstract

In the light of the enthusiasm to use of recombinant human granulocyte colony-stimulating factor (G-CSF) for immunomodulation and neuroprotection, it should be remembered that the current knowledge is based on a century of laborious research. G-CSF is a pleiotropic cytokine playing a major role as regulator of haematopoiesis. Although the precise mechanisms of G-CSF are not known, there is growing evidence supporting the notion that G-CSF also exerts profound immunoregulatory effect in adaptive immunity and has a neuroprotective role in both cerebral ischemia and neurodegeneration. Here, we describe the immunomodulation and the neuroprotection that can be achieved with G-CSF, and summarize possible mechanisms of G-CSF as a potential therapeutic agent in autoimmune diseases and neurological disorders. Our understanding of these novel sites of action of G-CSF has opened therapeutic avenues for the treatment of autoimmune diseases and neurological disorders, and has translated the beneficial effects of G-CSF from basic experiments to clinical patients.

Keywords: G-CSF • immunomodulation • neuroprotection

Introduction

Granulocyte-colony stimulating factor (G-CSF) is a polypeptide growth factor that plays a role in the basal regulation of neutrophil production. G-CSF is also used for haematopoietic stem cells (HSC)

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mobilization into the peripheral circulation, thereby becoming crucial for the adoption of autologous peripheral blood stem cell transplantation in clinical practice. Recently, a series of studies have demonstrated novel sites of action of G-CSF in many other fields. Despite widespread availability of G-CSF for clinical use, many unanswered questions remain regarding the optimal clinical use of this potent agent. The knowledge gained from further investigations of the basic biology of G-CSF and from future clinical trials with G-CSF will be critical to determine its potential for rational clinical application. Here, we describe recent progress in immunomodulation and neuroprotection of G-CSF, and summarize possible mechanisms of action of G-CSF as a potential therapeutic agent in autoimmune diseases and neurological disorders.

The immunomodulation of G-CSF in adaptive immunity

G-CSF as a regulator of T cell responses

There is compelling evidence that G-CSF, well known as the haematopoietic growth factor of the myeloid lineage [1], also exerts profound immunoregulatory effects in adaptive immunity. G-CSF treatment enhances the total lymphocyte count in both bone marrow and peripheral blood and increases $CD3^+$, $CD4^+$ and $CD8^+$ T cells as well as $CD3^-CD16^+CD56^+$ NK cells, while the increase in $CD4^+$ and $CD8^+$ T cells results from $CD45RO^+$ memory T cells and from cells expressing the CD38 activation marker [2]. G-CSF not only alters T cell numbers, but also T cell functions through the shift of T cell subsets in both bone marrow and peripheral blood. G-CSF polarizes T cell differentiation from Th1 to Th2 cells and induces Th2 responses with the production of IL-4 and IL-10 [3–5], accompanied by a decrease in production of IFN-γ and IL-2 [6], thereby suppressing T cell proliferative responses to allogeneic stimulation [5]. In addition, G-CSF increased the production of TGF-β [7, 8], and decreased the production of TNF-α [8].

Besides, G-CSF treatment elevates a $CD4^+CD25^+$ T cell subset through membrane-bound and secreted TGF-β [9] or IL-10 [10]. These $CD4^+CD25^+$ T cells express Foxp3, and constitute functional regulatory T cells [11]. $CD4^+CD25^+$ regulatory T cells mobilized by G-CSF may represent a promising source of cell therapy for clinical application, because they can be expanded up to 40,000-fold with artificial antigen presenting cells (APC) and high-dose IL-2 *in vitro* [12]. Specifically, G-CSF also reduced the expression of stromal derived factor-1 (SDF-1), a CXCR4 ligand, in the bone marrow, thus favouring $CD4^+CD25^+$ regulatory cell trafficking from bone marrow to the periphery [11].

G-CSF as an inducer of tolerogenic dendritic cells (DC)

G-CSF was reported to induce tolerogenic dendritic cells (DC2, plasmacytoid DC) in the peripheral blood of normal human recipients [3, 13, 14], thereby reflecting an important role of G-CSF for the induction and maintenance of peripheral tolerance. Administration of G-CSF to normal stem cell donors selectively increased the number of DC2 in the peripheral blood, whereas counts of DC1 (immunogenic DC, myeloid DC) counts did not change [13]. Functional characterization of the DC subsets after G-CSF administration revealed that DC1 did not differ in their ability to stimulate allogeneic naive T cells, whereas DC2 behaved as poor stimulators but were not impaired in their capacity to induce a Th2 immune response [3, 13]. Downregulation of CD28/CD80/CD86 co-stimulatory signals was observed by use of G-CSF *in vivo* [15]. Besides the selective increase of DC2, *in vitro* studies demonstrated that serum collected after clinical administration of G-CSF contained high amounts of IL-10 and IFN-α, and promoted the generation of the regulatory DC derived from $CD14^+$ monocytes [16]. These regulatory DC-like cells showed an impaired ability to release IL-12p70 and poor stimulatory capacity [16]. Furthermore, co-culture of naive $CD4^+$ T cells with this DC population triggered generation of regulatory T cells which secreted the immunosuppressive cytokines TGF-β and IL-10 [16]. This novel mechanism of immune regulation effected by G-CSF may be therapeutically exploited for tolerance induction in autoimmune diseases.

Whether this differentiation to Th2 cells is an indirect effect of APC which mediates a Th2 response

through G-CSF-mobilized DC2, or whether it is a consequence of a direct effect of G-CSF on T cells is still uncertain. Recent studies have demonstrated that monocytes from G-CSF-mobilized human donors suppressed T cell alloreactivity possibly through differential mechanisms, including IL-10-dependent pathway [17, 18], the inhibition of IL-12 [19] and TNF-α release [20] and downregulation of costimulatory molecules [15]. Monocytes from G-CSF-mobilized peripheral blood stem cell collections also inhibit T cell function by inducing $CD4^+$ T cell apoptosis *via* Fas–Fas ligand interaction [21]. However, other studies favour an indirect effect of G-CSF on the T cells *via* monocytes or DC. Most importantly, G-CSF receptor is expressed in mitogen-activated T cells and in unstimulated T cells [22, 23]. The expression of G-CSF receptor is further detectable on $CD4^+$ and $CD8^+$ T cells after G-CSF exposure at the single-cell level both *in vivo* and *in vitro* [24]. Purified $CD4^+$ T cells treated with G-CSF decreased IFN-γ and increased IL-4 production [23]. Thus, G-CSF appears to have a direct effect on T cells independent of monocytes or DC present in the co-culture. Taken together, these observations indicate that G-CSF, as a regulator of immune responses, has two pathways: (1) G-CSF selectively induces the generation of tolerogenic DC which promote the production of functional regulatory T cells and (2) G-CSF directly acts on T cells for Th2 cell differentiation through G-CSF receptor on the surface of T cells.

Mechanisms of action of G-CSF in immunomodulation

G-CSF, like each of the other CSFs, exerts its biologic activities through binding to G-CSF-specific, high affinity receptor, which subsequently triggers multiple signalling mechanisms (Fig. 1). In fact, G-CSF promotes neutrophil production and enhances neutrophil production and function by binding to its receptor [25]. The molecular mechanism by which G-CSF/G-CSF receptor signalling controls Th1 and Th2 differentiation as well as immune regulation is still poorly understood. It was reported that the T cell-specific transcription factor GATA-3 (GATA-3) was selectively expressed in Th2 cells, but not Th1 cells [26]. GATA-3 controls T helper cell differentiation, and directs to Th2 response [26, 27]. However, little is known about the regulation of GATA-3 expression. G-CSF treatment *in vivo* resulted in the upregulation of GATA-3 expression at both mRNA and protein levels accompanied by an increase of spontaneous IL-4 secretion [24]. GATA-3 activation in $CD4^+$ T cells seems to induce chromatin remodelling of the intergenic regulatory region for the IL-4/IL-13/IL-5 gene cluster [27], directly activating the IL-5 promoter [26] and exhibiting enhancer activity for IL-4 gene expression [28]. In addition to activating a Th2 program, GATA-3 directly inhibited the opposing Th1 immune response most likely by interfering with the IL-12 signal transduction pathway [29].

G-CSF can also induce the expression of suppressor of cytokine signalling 3 (SOCS3) [30, 31], a regulator of T cell activation and differentiation. SOCS3 has been shown to be preferentially expressed in Th2 cells, and to prevent IL-12-induced Th1 cell differentiation [32] and the secretion of IFN-γ and IL-2 [33]. If G-CSF triggers the induction of SOCS3 expression on DC, SOCS3-expressing DC might exhibit a tolerogenic DC phenotype, and drive myelin oligodendrocyte glycoprotein (MOG)-specific T cells to a strong Th2 differentiation *in vitro* and *in vivo* [34]. Mice lacking SOCS3 were more susceptible to experimental arthritis by mediating $CD4^+$ T cell activation [35]. Intracellular delivery of SOCS3 significantly reduced production of inflammatory cytokines and attenuated liver apoptosis and hemorrhagic necrosis, thus effectively suppressing the devastating effects of acute inflammation [36]. Therefore, SOCS3 seems to display benefits in a Th1-mediated autoimmune disease or inflammation. However, it should be noted that T cell-specific expression of SOCS3 deteriorated clinical and pathological features of allergic conjunctivitis [37]. Especially in patients with Th2 type diseases, the more severe the disease pathology, the higher the SOCS3 expression in T cells [32]. Thus, it is conceivable that Th2-specific expression of SOCS3 has a significant role in enhancing the pathophysiology of Th2-mediated immune diseases through producing IL-4, IL-5 and IL-10 [32]. Reduction of the expression level or inhibition of function in SOCS3 clearly reduced the severity of allergic conjunctivitis. These results at least in part provide a possible explanation for disease exacerbation in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), predicting that agonists of

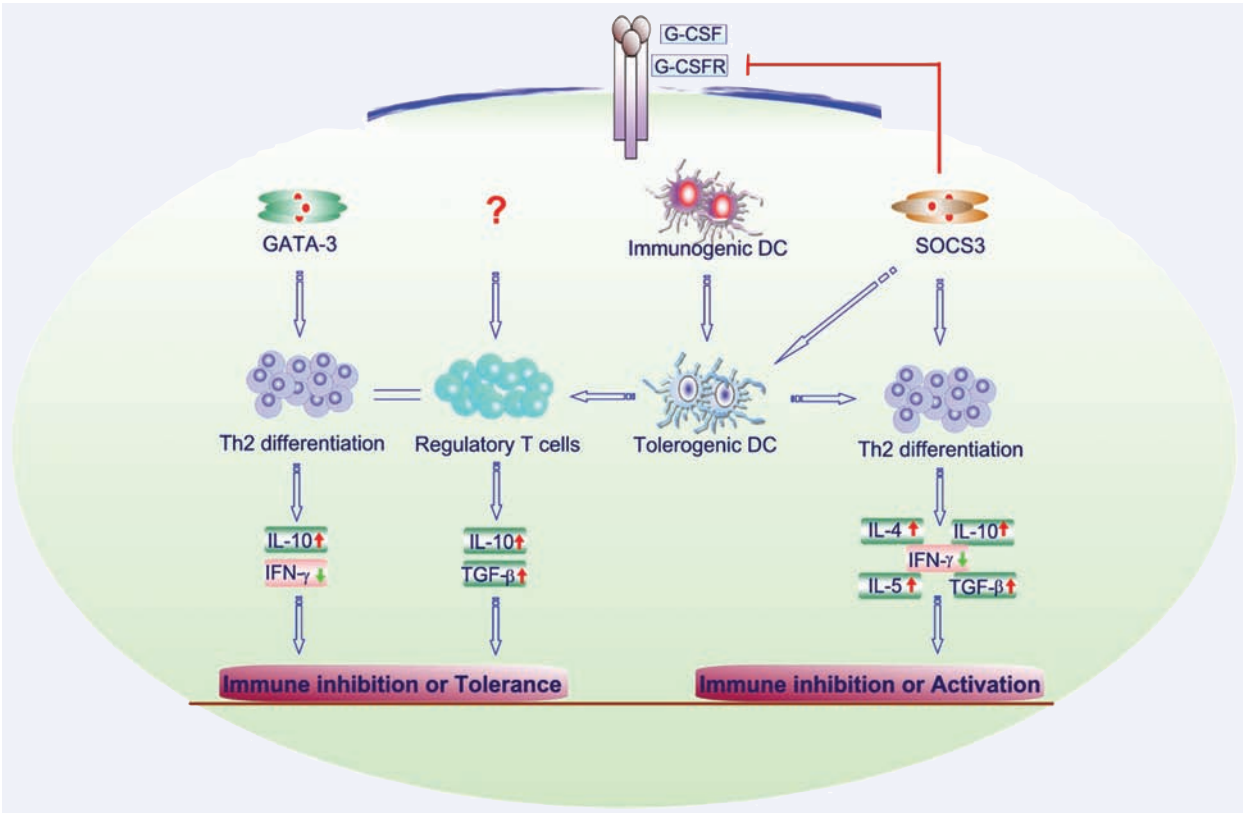

Fig. 1 Possible mechanisms of immunomodulation of G-CSF in adaptive immunity. G-CSF induces the expression of both GATA-3 and SOCS3, which control T helper cell differentiation, and directs to Th2 response. G-CSF directly induces the generation of tolerogenic DC, or indirectly drives the production of tolerogenic DC through inducing SOCS3 expression. Tolerogenic DC have the capacity to induce a regulatory T cells or/and Th2 immune responses. Despite our limited knowledge about the molecular mechanisms involved, it is clear that G-CSF treatment results in increase in the number of regulatory T cells and the differentiation of Th2 cells. G-CSF-induced SOCS3 in turn limits G-CSF receptor signalling.

SOCS3 may reduce unwanted clinical sequelae of inflammation processes.

G-CSF up-regulated rapidly the expression of SOCS3, which in turn potently inhibited G-CSF receptor-mediated signal transduction [31]. Further study indicates that the inhibitory action of SOCS3 on G-CSF signalling involves the direct binding of SOCS3 to the activated G-CSF receptor and identifies Tyr 729 of G-CSF receptor as being the recruitment site for SOCS3 [31]. Overexpression studies have suggested that SOCS3 interacts with the CSF receptor and inhibits G-CSF-induced Stat3 activation, indicating a negative role of G-CSF in the survival and growth of neutrophils [38]. To evaluate the implications of SOCS3 recruitment to Tyr 729 of G-CSF receptor on immunomodulation in autoimmune diseases, further studies are necessary.

Therapeutic potential of G-CSF in autoimmune diseases

In experimental models, G-CSF was shown to protect mice from graft-versus-host disease (GVHD) by orienting T cells to Th2 response [6]. G-CSF exhibited therapeutic potential in experimental autoimmune encephalomyelitis (EAE) [39, 40], since G-CSF-treated mice showed limited demyelination and reduced recruitment of T cells to the central nervous system (CNS), accompanied by reduced IFN-γ and increased IL-4 and TGF-β1 production [40]. Treatment of non-obese diabetic (NOD) mice with G-CSF seemed to prevent the spontaneous development of autoimmune diabetes by recruiting immature $CD11c^{lo}$ $B220^{+}$ plasmacytoid DC with high levels of IFN-α, but low levels of IL-12p70 [9]. Another study

performed in NOD mice showed that G-CSF protected from autoimmune diabetes by preventing the loss of the regulatory T cells and promoting their expansion in the spleen [41]. Adoptive transfer of DC isolated from G-CSF-treated mice into naive NOD recipients also exhibited protective capacities by accumulating $CD4^+CD25^+$ regulatory T cells [9]. Potentially beneficial effects of G-CSF have also been demonstrated in a hapten-induced colitis model with Th1-associated mucosal damage. Pre-treatment with G-CSF (125 mg/kg twice per day) over 5 days before hapten challenge drastically attenuated the degree of colitis through selective downregulation of Th1-associated cytokines [42]. In an adjuvant arthritis model in Lewis rats, G-CSF application reduced the severity of disease which was associated with a decrease of IFN-γ secretion [43]. Importantly, in patients suffering from RA, the administration of G-CSF seemed to suppress clinical signs of ongoing disease in a dose-dependent manner [44]. Altogether, these data demonstrate a promising rationale for the clinical application of G-CSF in human autoimmune diseases.

However, we must not ignore the fact that G-CSF administration has been associated with disease exacerbation in some patients with multiple sclerosis (MS), RA and SLE [44–46]. In MS patients, a possible mechanism for G-CSF-related exacerbation is that G-CSF enhanced the adhesion of encephalitogenic T cells to extracellular matrix components [46]. In RA patients, G-CSF exacerbated inflammatory joint disease by increasing the production and mobilization of myeloid lineage cells from the bone marrow and inducing the trafficking and local activation of these cells in peripheral tissues [47]. These aggravating effects of G-CSF were abrogated when G-CSF was administered together with cyclophosphamide or high-dosage corticosteroids. In SJL/J EAE mice with EAE induced by immunization with proteolipid protein 139-151 (PLP139-151), G-CSF administration caused worse clinical scores compared to controls not receiving G-CSF [48]. It seems as if disease severity in EAE cannot be completely explained by the alterations of Th1- and Th2-derived cytokines from peripheral lymphocytes. G-CSF administration has also been shown to exacerbate collagen-induced arthritis (CIA) in mice [49] and passive-transfer model of CIA in rats [50].

Additionally, an unexpected modulation of disease severity was observed in a murine SLE model. Chronic treatment of MRL-lpr/lpr mice with low dose of G-CSF (10 mg/kg) increased glomerular deposition of immunoglobulins and accelerated lupus disease, whereas high-dose treatment with G-CSF (200 mg/kg) prevented lupus nephritis by local down-modulation of FcgRIII expression within the glomeruli [51]. At lower dose, G-CSF seems to function as a key regulator of B cell homeostasis *via* the production of B cell-activating factor (BAFF), a novel member of the TNF ligand superfamily that is important for B cell maturation and antibody production. This novel finding might in part explain the occurrence of exacerbations of B cell-mediated autoimmune diseases in animal models and humans. Despite some setbacks in clinical application of G-CSF, it is clear that G-CSF plays an important role in the regulation of adaptive immunity. For example, G-CSF blockade in established CIA mice markedly reduced disease manifestations and was found be as effective as TNF blockade [47]. Taken together, G-CSF may have dual effects as an important anti-inflammatory or proinflammatory cytokine under different conditions [52], thereby suggesting that it should be used with great caution in patients until these issues are resolved.

The neuroprotection of G-CSF in cerebral ischemia and neurodegeneration

The expression of G-CSF and G-CSF receptor in central nervous system

Besides T cells and DC, other cell types also express G-CSF following appropriate stimulation or even in resting state, including vascular endothelial cells, fibroblasts and mesothelial cells. Within the CNS, G-CSF is expressed by neurons in all brain regions including the hippocampus CA3 field, the hilus and subgranular zone of the dentate gyrus, the entorhinal cortex, the olfactory bulb, several cerebellar and brainstem nuclei where its receptor is expressed [53]. G-CSF receptor is also indeed expressed in dopaminergic neurons in the adult substantia nigra or mesencephalic cultures [54–56]. Besides in neurons, G-CSF is detected in astrocyte cultures after stimulation [57–60]. However, Schneider *et al.* [53] did not detect any astrocytic G-CSF expression

in vivo, not even in the acute cerebral ischemia. Our results showed that G-CSF is expressed in astrocytes, even in the resting state (data not published). Recently, G-CSF expression was also observed in reactive astrocytes in amyotrophic lateral sclerosis cases but not in controls [61]. It is certainly possible that G-CSF expression in astrocytes may be influenced by different culture conditions or other stimuli at different time points. No information is available that microglia expresses G-CSF, but expression of G-CSF receptor could be detected on microglia in subacute cerebral infarcts in humans [62].

Importantly, G-CSF transcripts were induced 485-fold at 4 hrs and 65-fold at 16 hrs in ischemic lesions after middle cerebral artery occlusion (MCAO) compared with control brains [63]. Furthermore, an increase in G-CSF mRNA expression was not only seen in the ischemic lesion but also in the non-ischemic frontal cortex after focal cerebral ischemia [63]. Similarly, there was a dramatic upregulation of G-CSF (more than 100-fold) elsewhere in the ipsilateral hemisphere 2 hrs after MCAO, and an accompanying induction of G-CSF in the contralateral hemisphere. At 6 hrs following ischemia, G-CSF expression became more clearly localized to the ischemic hemisphere, and, at 20 hrs of reperfusion, G-CSF expression was no longer detectable [53]. In the human, G-CSF was significantly increased in cerebrospinal fluid from patients with amyotrophic lateral sclerosis compared with controls, and expressed in reactive astrocytes [61].

The upregulation of G-CSF was accompanied by a more modest induction of the G-CSF receptor after cerebral ischemia, more prominent in the ipsilateral than the contralateral hemisphere [53]. Immunohistochemistry demonstrated the co-expression and up-regulation of G-CSF and its receptor in neurons after MCAO and reperfusion. In human acute ischemic stroke, strong neuronal G-CSF receptor expression was encountered in the infarct area and the peri-infarct rim as compared to the contralateral cortex. In subacute infarctions, microglial G-CSF receptor predominated, whereas chronic infarction was characterized by the presence of G-CSF receptor-expressing reactive astrocytes [62]. We observed that G-CSF receptor was expressed in glial fibrillary acidic protein $(GFAP)^+$ astrocytes in ischemic regions, but not in non-ischemic regions (Fig. 2c, unpublished data), revealing that G-CSF and its receptor likely function as an autocrine adaptive system within the CNS.

Penetration of the blood-brain barrier

A prerequisite for obtaining any effect of G-CSF within the CNS is that G-CSF penetrates the blood-brain barrier (BBB) upon being administrated in the periphery. The amounts of iodinated G-CSF (^{131}I–G-CSF) in brain and serum were measured at 1, 4 and 24 hrs after intravenous injection in non-injured rats and the brain/serum ratios of ^{131}I–G-CSF and ^{131}I-albumin were calculated as an index of BBB permeability. Injection of G-CSF showed a higher brain/serum ratio at different time points, indicating that systemically given G-CSF is able to pass the intact BBB [53]. The most striking effect of peripherally administered G-CSF on the brain was seen in the dentate gyrus, where G-CSF increased the number of newly generated neurons under ischemic conditions but also in non-ischemic, sham-operated animals. In addition, both immunofluorescent staining and western blot showed that receptor for G-CSF was expressed in the capillaries of adult rat brain, suggesting that G-CSF entry to the brain may be mediated *via* receptor-mediated transport on cerebral microvessels [64].

Mechanisms of G-CSF in neuroprotection

G-CSF mobilizes haematopoietic stem cells to the injured brain

Administration of G-CSF is known to mobilize HSC from the bone marrow into the peripheral blood (Fig. 3). G-CSF application resulted in a significant decrease in infarct volume and enhanced survival rate, which may be mediated by the mobilization of autologous HSC in experimental cerebral ischemia [65, 66]. Our results demonstrated that subcutaneous injection of G-CSF increased the mobilization of circulating $CD34^+$ cells which were seen around the perivascular in ischemic hemisphere, indicating that $CD34^+$ cells mobilized with G-CSF can home from the circulating blood into the ischemic brain tissues [67]. Other studies have also showed that ischemic brain specifically attracted peripheral transplanted bone marrow stromal cells (BMSC) [68–70].

Which signalling molecules attract peripheral $CD34^+$ cells and direct their migration to damaged areas? Cerebral ischemia increased CXCR4 receptor

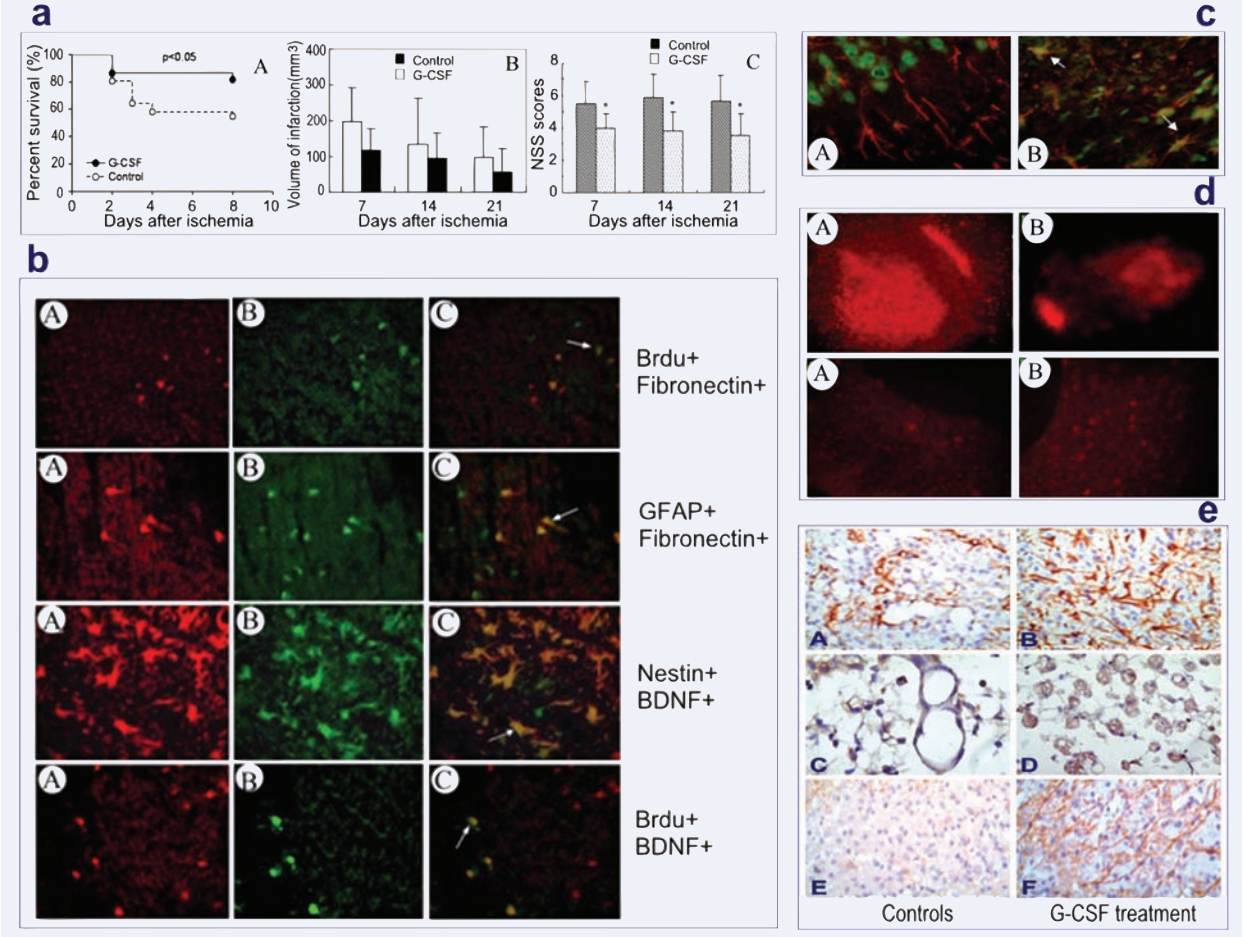

Fig. 2 G-CSF-mediated neuroprotection in cerebral ischemia. (**a**-**A**) Survival rate of rats with cerebral ischemia treated with G-CSF and with saline as control, (**a**-**B**) Infarction volume and (**a**-**C**) Neurological Severity Score. (**b**) Double-labelled immunofluorescent staining of brain slices obtained from G-CSF-treated rats at day 7 after MCAO. Red images correspond to Brdu, GFAP or nestin and green images to fibronectin or BDNF. Yellow images reveal double-labelled positive cells. (**c**) G-CSF receptor expression in $GFAP^+$ astrocytes in ischemic region (**B**), but not in non-ischemic region (**A**). Red images correspond to GFAP and green images to G-CSF receptor. Yellow images show double-labelled positive cells. (**d**) Area of cell death stained with PI (up) and number of $bcl\text{-}2^+$ cells (down) at 7 days after hippocampal slice cultures in the absence (**A**) or presence (**B**) of G-CSF. (**e**) Expression of nestin, vWF and MAP-2 expression brain sections. The immunohistochemistry of nestin (**A** and **B**), vWF (**C** and **D**) and MAP-2 (**E** and **F**) are performed on brain slices obtained from G-CSF-treated rats and control rats at day 7 after MCAO.

ligand SDF-1 expression in regions adjacent to the infarct area, indicating that SDF-1 within the brain could be a chemoattractant for peripheral $CD34^+CXCR4^+$ cells [71]. Thus, we speculate that the expression of the CXCR4 receptor on haematopoietic $CD34^+$ cells may act as signalling mechanism for the adhesion and migration of HSC to ischemic tissue. It is clear that $CD34^+$ cells isolated from adult peripheral blood express the CXCR-4 receptor, suggesting that haematopoietic $CD34^+$ cells can undergo directional migration towards SDF-1 in regions adjacent to the infracted area.

How do haematopoietic $CD34^+$ cells contribute to the improvement of neurological function after cerebral ischemia? One possibility is that G-CSF-mobilized $CD34^+$ cells integrate into the tissue, replace damaged cells and reconstruct the neural circuitry. Another reasonable hypothesis is that the interaction of $CD34^+$ cells with the host parenchymal cells in ischemic tissue leads parenchymal cells to produce

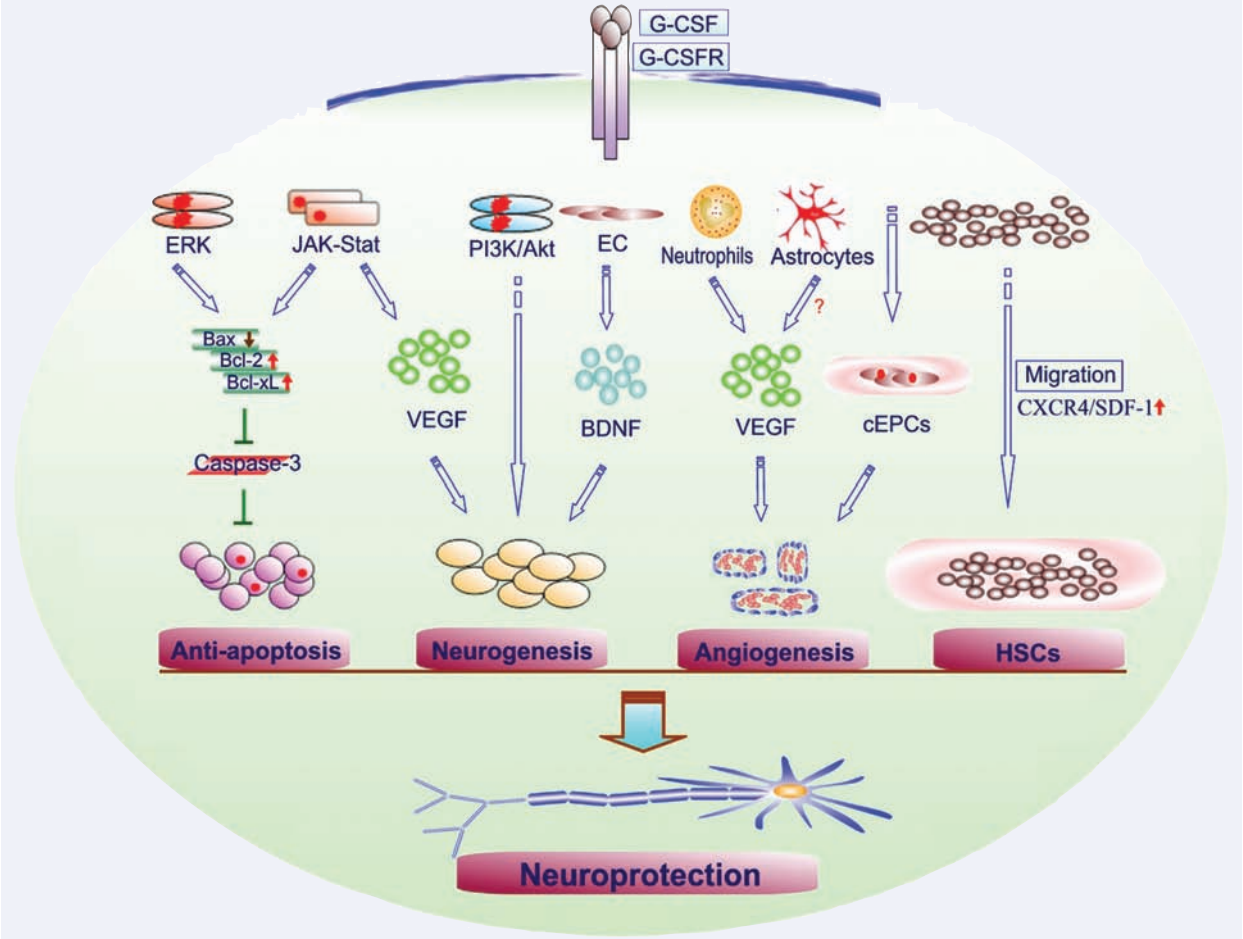

Fig. 3 Possible mechanisms for neuroprotection of G-CSF in cerebral ischemia and neurodegeneration. G-CSF provokes multiple intracellular signal transductions including Jak/Stat, ERK and PI3K/Akt in neuroprotection. (**1**) Anti-apoptosis: G-CSF mediates anti-apoptotic pathway through ERK or/and JAK/Stat signalling activation and subsequent upregulation of bcl-2 and inhibition of caspase-3; (**2**) Neuronal differentiation: Stat regulates VEGF expression, or G-CSF activates endothelial cells to release BDNF. VEGF, BDNF and activated PI3K/Akt promote neurogenesis (**3**) Angiogenesis: G-CSF stimulates neutrophils or astrocytes to secrete VEGF, or directly mobilizes circulating endothelial progenitor cells (cEPCs) to promote angiogenesis within the CNS; and (**4**) The mobilization of autologous hemopoietic stem cells: G-CSF triggers the mobilization of autologous hemopoietic stem cells that migrate into ischemic brain, and thus significantly improve lesion repair.

trophic factors that contribute to the recovery of neural functions [72]. We found that the level of fibronectin in brain of rats treated with G-CSF was augmented compared with control rats [67]. It has been noted that fibronectin promoted survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain [73]. Fibronectin-deficient mice showed increased neuronal apoptosis and infarction area following transient focal cerebral ischemia [74]. To mimic the ischemia-reperfusion injury in experimental animals, we employed hippocampal slice cultures that were first treated with oxygen and glucose deprivation (OGD) and then with oxygen-glucose re-supply, finding that fibronectin significantly increased the neurite outgrowth of OGD hippocampal slices and ameliorated the ultrastructure damage of OGD hippocampal slices [67]. Blockade of fibronectin *in situ* with an anti-fibronectin antibody dramatically decreased neurite outgrowth [75], suggesting that fibronectin may play an important role in axon regeneration.

Besides the increase of fibronectin, the numbers of brain-derived neurotrophic factor (BDNF) $^{+}$ cells in the marginal zone of the infarction were significantly increased in G-CSF-treated rats at 7, 14 and 21 days after ischemia compared to those of control rats [67]. Double labelling of 5-bromo-2–deoxyuridine (Brdu) revealed that fibronectin^{+} or BDNF^{+} cells in the marginal zone of the infarction were dividing (Fig. 2b). Double fibronectin- and GFAP-positive cells indicate that astrocytes in ischemic regions may be a major source of fibronectin production (Fig. 2b). BDNF-expressing nestin-positive cells (Fig. 2b), possibly partially through synthesizing and releasing neurotrophic factors such as BDNF, could play an important role in recovery of neurological dysfunction. Although G-CSF treatment enhances the translocation of HSC into the ischemic brain and improves lesion repair, a recent report rebuts that G-CSF decreased the migration of bone marrow-derived cells and increased intrinsic microglia at the ischemic penumbra, suggesting that extrinsic cells are not involved in G-CSF-induced reduction of ischemic infarction [76].

G-CSF activates anti-apoptotic pathway

G-CSF protected neurons against cell apoptosis, which appears to be mediated *via* the neuronal G-CSF receptor, since antibody against G-CSF receptor was capable to abolish the protection [53]. G-CSF exerts its neuroprotective effect over direct activation of the anti-apoptotic pathway by up-regulating Stat3, pStat3 and Bcl-2 in transient focal ischemia in mice [76]. Another study also found that the neuroprotective role of G-CSF was manifested through the JAK/Stat signalling pathway and subsequent activation of Bcl-2 [77], in which overexpression of Bcl-2 protected against post-ischemic cerebral neuronal death [78]. Schabitz *et al*. showed that G-CSF receptor existed not only on haematopoietic cells but also on neurons and glial cells, and that the neuroprotective effect of G-CSF is dependent on G-CSF receptor-mediated activation of the JAK/Stat pathway [53]. Under OGD of human cerebral-neuroblastoma hybrid cell line, G-CSF prevented caspase-3 activation and subsequent cell death. We found that the expression of Bcl-2 (Fig. 2d) was up-regulated in hippocampal slice cultures exposed to G-CSF and the expression of MAP-2 (Fig. 2e-E and F) was increased in brain section from ischemic rats receiving G-CSF treatment, indicating that G-CSF-mediated neuron survival may be related to Bcl-2-madiated neuronal protection.

In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuron death, neuroprotection of G-CSF was mediated by increased Bcl-2 expression and decreased Bax expression [55]. G-CSF also protected dopaminergic neurons against 6-hydroxydopamine (6-OHDA)-induced degeneration by activating the ERK pathway, but not the JAK/Stat pathway [56]. G-CSF-activated ERK signalling promoted cell survival *via* a dual mechanism by phosphorylating the pro-apoptotic protein Bad and by preventing 6-OHDA-induced Bcl-xL downregulation [56].

G-CSF also down-regulated cytochrome C release to the cytosol, Bax translocation to the mitochondria and cleaved caspase-3 levels in neurons. The activation of the Stat3 pathway was accompanied by increased cIAP2 expression in glial cells. After MCAO and dopaminergic neuron damage, G-CSF treatment increased both neuronal and glial survival by effecting different anti-apoptotic pathways, which reflects the multifactorial actions of G-CSF in anti-apoptotic pathways [79] (Fig. 3).

G-CSF drives neuronal differentiation

Neuronal death after ischemia may involve a combination of apoptotic and necrotic processes even at the level of the individual neuron. This raises a question of how G-CSF induces a neuroprotective effect for apoptotic or necrotic neurons. It has been clarified that the adult mammalian forebrain harbours neuronal stem cells and neuronal progenitor cells in the anterior subventricular zone (SVZ), rostral migratory stream, olfactory bulb core and dentate gyrus (DG). However, G-CSF can induce bone marrow stem cells proliferation and mobilization, and activate endothelial cell proliferation, which may help to establish a vascular niche for neuronal stem cells [80]. Importantly, G-CSF and its receptor were expressed in neurons of the SVZ and the DG [53]. G-CSF dose-dependently induced activity of the promoter of the mature neuronal marker β-III-tubulin with a maximal induction greater than that reached by the most standard neuronal induction protocol, including markers for neuronal differentiation (β-III-tubulin and neuron-specific enolase) and markers for mature glial cells (PLP and GFAP). G-CSF led to an increase in the population of cells expressing mature neuronal markers, indicating that G-CSF functions as regulator of the

differentiation of adult neuronal stem cells [53]. Administration of G-CSF in the subacute phase after cerebral infarction was effective for functional recovery through facilitating proliferation of intrinsic neuronal stem cells/progenitor cells [81]. In accordance with these observations, our previous studies reveal that nestin-positive cells were elevated in the marginal zone of the infarction of rats receiving G-CSF injection (Fig. 2e-A and B), suggesting that G-CSF may promote the differentiation of neuronal stem cells after ischemia/reperfusion lesion. Taken together with the recent evidence that G-CSF rescues dying neurons [53], G-CSF may potentially serve to promote brain recovery and repair by promoting the differentiation of neuronal stem cells. In addition, G-CSF enhanced the recruitment of progenitor cells from the lateral ventricular wall into the ischemic area of the neocortex and increased hippocampal neurogenesis not only in ischemic animals but also in the intact, non-ischemic region [53]. Based on this evidence, G-CSF may enhance structural repair and function even in healthy patients, and could offer a novel strategy for the treatment of patients with chronic stroke.

Little is known regarding the possible role of G-CSF in the adult brain neurogenesis (Fig. 3). Activated Stat translocated to the nucleus and regulates specific target gene expression, which allows cells to proliferate, differentiate and mobilize or to obtain enough trophic support for survival. On the other hand, G-CSF has been shown to directly activate endothelial cell proliferation to release BDNF [82], and establish a vascular niche that favours the proliferation of neuronal precursors [83]. Besides, Stat3 directly regulates vascular endothelial growth factor (VEGF) expression and hence angiogenesis in the adult brain [83, 84], operating an autocrine VEGF loop in neurogenesis through reciprocal interaction with VEGF and Stat activation [80]. G-CSF also induces the activation of PI3K/Akt pathway [85] which is involved in neurogenesis [86]. The inhibition of endogenous Akt activity by the PI3K inhibitor LY294002_reduced_neuronal differentiation and generation [87].

G-CSF enhances angiogenesis

One week after unilateral hindlimb ischemia, administration of G-CSF significantly increased the laser Doppler blood perfusion index, number of angiographically detectable collateral vessels, and capillary density [88]. Local G-CSF administration into ischemic tissue contributed to neovascularization (such as the vascular surface area, the vascular branch points, the vascular length and the number of Brdu+ endothelial cells), and reduced the ischemic damage, thereby promoting the long-term functional recovery [89]. The question remains how G-CSF improves neovascularization. There is compelling evidence that circulating angiogenic cells are able to home to sites of vascular injury and further stimulate angiogenesis. However, the number of angiogenic cells in the blood is very low, limiting their accumulation to sites of ischemia. Capoccia *et al*. observed that G-CSF stimulated angiogenesis through the mobilization of monocytes into the blood with their subsequent recruitment to sites of ischemia and stimulation of angiogenesis [90]. On the other hand, G-CSF augmented the differentiation of bone marrow-derived stromal cells into endothelial cells of blood vessels, resulting in early recovery of blood flow in the ischemic limbs [91]. These data clearly show that G-CSF modulates the recruitment and incorporation of circulating endothelial progenitor cells into ischemic tissue, which requires a coordinated multistep process including mobilization, chemoattraction, adhesion, transmigration and *in situ* differentiation.

Recently, we also found that G-CSF can stimulate astrocytes to secrete VEGF that may promote angiogenesis within the CNS by paracrine pathway (data not published). Concomitant with an increase in neutrophil numbers in circulation, G-CSF increased plasma VEGF from neutrophils *in vivo* [92]. Blockade of the VEGF pathway abrogated G-CSF-induced angiogenesis, suggesting that G-CSF-induced angiogenesis is VEGF-dependent [92]. VEGF is an angiogenic protein with therapeutic potential in ischemic disorders, including stroke. The administration of VEGF to rats undergoing focal cerebral ischemia reduced infarct size and enhanced neurogenesis and cerebral angiogenesis *via* von Willebrand factor (vWF) staining [93]. *In vivo* experiments, our results show that administration of G-CSF enhanced the numbers of vWF-positive cerebral microvessels in the marginal zone of the infarction after ischemia/reperfusion lesion (Fig. 2e-C and D).

Evidence for neuroprotection of G-CSF

Recent studies have shown the presence of G-CSF/G-CSF-receptor system in the CNS, raising the

possibility that G-CSF should have important non-haematopoietic functions in the CNS. Growing evidence suggests that G-CSF plays a role in neuroprotection and neural tissue repair as well as in improving functional recovery [94]. Administration of G-CSF reduced infarction volume [76, 89] and mortality rate was significantly reduced in animals treated with G-CSF compared with control animals [67]. G-CSF-treated experimental models showed a better functional recovery from 2 weeks through 5 weeks after ischemia compared to the cerebral ischemia-only controls [67, 89]. G-CSF given in the subacute phase (days 11 to 20) effectively improved not only motor performance but also higher brain function, compared with acute-phase treatment (days 1 to 10) [81]. G-CSF injection showed a reduction in hemispheric atrophy at 35 days after cerebral ischemia and a significantly lower level of Evans blue dye extravasation compared to cerebral ischemia-only at 3 days, indicating a reduced BBB disruption [89]. G-CSF administration after transient ischemia also led to a decrease in the amount of edematous tissue present as measured by both structural magnetic resonance imaging (MRI) and brain water content [95]. Our results indicate that subcutaneous injection of G-CSF (10 μg/kg per day) for 5 days decreased mortality rate, reduced infarction volume and improved neurological functions after cerebral ischemia [67] (Fig. 2a). G-CSF not only decreased acute infarct volume in rats 4 hrs after onset of ischemia, but also improved recovery for a period of 10 days starting either 24 or 72 hrs after induction of ischemia, providing an experimental basis for application of G-CSF in the post-stroke recovery phase [96].

In a randomized controlled trial, seven patients with acute ischemic stroke received subcutaneous G-CSF injections (15 μg/kg per day) for 5 days within 7 days of onset. At 12-month follow-up, patients who had received G-CSF showed significant improvement in neurologic functions according to the clinical scales. MRI scans revealed no anatomic or structural changes, including cerebral haemorrhage. There was no significant difference with regard to infarction size at baseline and at 12-month follow-up [97]. Taken together, G-CSF offers some hope as therapy of stroke patients possibly through mobilization of endogenous stem cells [98]. Notably, a recent study reported that G-CSF treatment was only associated with transient early improvement in neurobehavioral outcomes after global ischemia complicated by mild hyperglycemia, but no long-term protection in global ischemia [99].

Based on the expression of G-CSF receptor in dopaminergic neurons, G-CSF demonstrates a neuroprotective role in neurodegeneration. G-CSF protected against MPTP toxicity in PC12 cells and primary neuronal midbrain cultures *in vitro* [54], against MPTP toxicity in older mice *in vivo* [54] and against MPTP-induced dopaminergic neuron death in a mouse model of Parkinson's disease [54, 55]. G-CSF also prevented dopaminergic neurons against 6-OHDA-induced degeneration [56]. In contrast, Henze *et al.* did not observe that G-CSF prevented MPTP-induced dopaminergic neuron death in primary neuronal midbrain cultures [100]. This apparent discrepancy could be due to the lower G-CSF concentrations (0.1–10 ng/ml) used in Henze's study compared with Meuer's work (30–50 ng/ml). In animals with spinal cord compression lesion, treatment with G-CSF (from day 7 to 11 post-injury) had higher the Basso–Beattie–Bresnehan scores and better recovery of hind limb sensitivity than controls injected with saline [101]. A small beneficial effect of G-CSF on functional outcome after traumatic brain injury in adult mice was observed between G-CSF-treated and control groups [102].

G-CSF-Bridge between immunomodulation and neuroprotection

The inflammation within the CNS is a common phenomenon even in classic non-inflammatory brain diseases that are characterized by trauma or degeneration of neuronal structures, such as stroke, Alzheimer disease, or Parkinson disease. The strategy for indirectly protecting neurons and axons partly through immunomodulation may improve the outcome of the patients. Protective autoimmunity is a relatively new concept. It refers to a benign autoimmune response that contributes to the maintenance and protection of injured neurons and the promotion of recovery after traumatic injury to the CNS [103]. Because G-CSF can polarize T cell differentiation from Th1 to Th2 cells and induce Th2 response (or regulatory T cells), an imagination has been proposed that systemic Th2 shift may promote neuroprotection and regeneration [103, 104] (Fig. 4). There are several lines of evidence

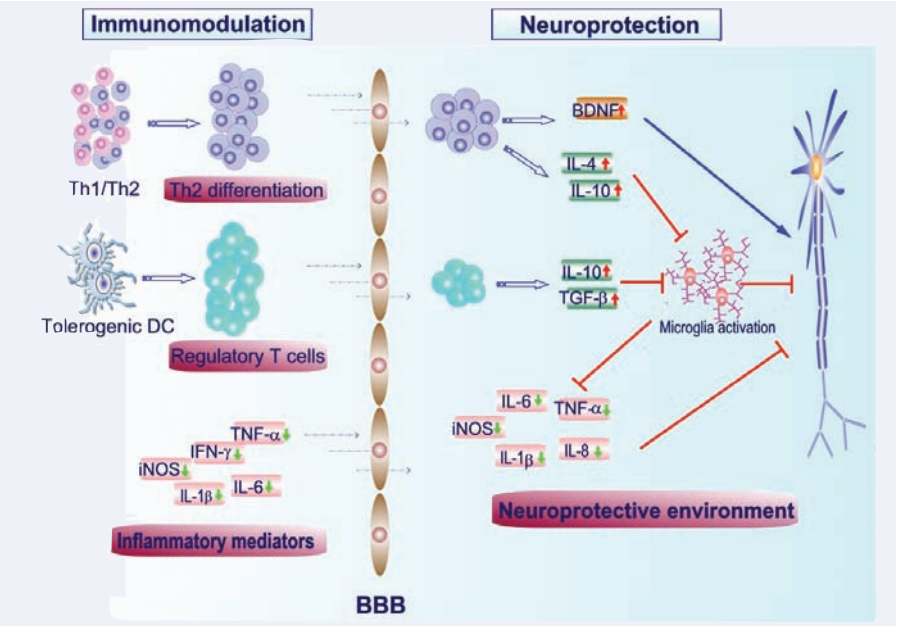

Fig. 4 Possible mechanisms for G-CSF-mediated neuroprotection via immunomodulation. G-CSF polarizes T cell differentiation from Th1 to Th2 cells and induces Th2 responses, or relies on tolerogenic DC to generate regulatory T cells, which can enter into the CNS to contribute to the neuroprotective microenvironment through producing BDNF, IL-4, IL-10 and TGF-b. In addition, G-CSF, as an anti-inflammatory agent, can reduce levels of IFN-γ, IL-1β, IL-6, TNF-α and iNOS production in order to co-construct the neuroprotective microenvironment.

that a Th2 switch is beneficial for the injured CNS: (1) Th2 cells support neuronal survival better than Th1 cells *in vitro*; (2) Th2 cells suppress Th1-induced inflammatory signals in brain slices *in vitro* and (3) Th2-inducing adjuvants such as aluminium hydroxide promote axon regeneration better than the Th1-inducing complete Freund's adjuvant (CFA). Potent inducers of a systemic Th2 switch such as statins support neuroprotection and/or regeneration. A recent review describes that the development of a Th1 response to myelin basic protein (MBP) is associated with worse neurological outcome after stroke while the induction of MBP-specific regulatory T cells is neuroprotective in the setting of stroke [105]. Based on such experiments, it could be that G-CSF plays a neuroprotective role through a Th2 switch or regulatory T cell production. However, extensive studies are still needed to investigate how a therapeutic Th2 switch promotes neuronal survival and axonal regeneration after CNS damage and what potential mechanisms may be involved.

The $CD4^+$ T cells have recently been found to promote facial motoneuron (FMN) survival after nerve injury. To determine whether either the Th1 cytokine (IFN-γ) or the Th2 cytokine (IL-4) is involved in mediating FMN survival, facial nerve axotomy was applied to IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice. A significant decrease in FMN survival after axotomy occurred in IL-$4^{-/-}$ but not in IFN-$\gamma^{-/-}$ mice compared to wild-type mice, indicating that IL-4 is important for FMN survival after nerve injury [106]. In addition, Frenkel *et al.* demonstrated the importance of IL-10-producing $CD4^+$ T cells in the reduction of ischemic infarct volume following MCAO [107]. Adoptive transfer of $CD4^+$ T cells expressing IL-10 reduced ischemic infarct size, while IL-$10^{-/-}$ $CD4^+$ T cells lacked this capacity, further supporting an IL-10-dependent cascade in neuroprotection [107]. Th2 cells also secreted BDNF which promoted remyelination [108]. Furthermore, glial cells within the CNS have also been reported to regulate the ensuing immune response to nerve injury, suggesting that CNS-resident cells may cooperate with Th2 cells to mediate neuronal protection.

Although clinical trials of immunomodulatory therapy after stroke have not yet proven successful, it is clear that an inflammatory response occurs within the brain after stroke, and modulation of this inflammatory response improves outcome in experimental models of cerebral ischemia. The lack of clinical success does not necessarily mean that the immune response does not contribute to post-ischemic brain injury, but it could imply that our approach to controlling this immune response may be flawed. G-CSF has been used as an anti-inflammatory agent. Thus, a therapeutic approach that reduces inflammation may protect against cerebral ischemic injury. G-CSF protected against cell death in a non-septic model of ischemia/reperfusion injury and concluded that such

beneficial effect is the consequence of either reduction of TNF-α or inhibition of inducible nitric oxide synthase (iNOS) activity [109, 110]. Other studies reported that G-CSF decreased the levels of inflammatory IL-1β, IL-6 and IL-8 under several conditions [111]. Analyses of iNOS Western blot and immunohistochemistry have clearly indicated that G-CSF significantly reduced iNOS levels and decreased the activation of microglia expressing iNOS [76]. Our results demonstrate that G-CSF reduced NO production from cultured astrocytes. Based on these findings, one neuroprotective mechanism of G-CSF is at least partially mediated *via* anti-inflammation. Further studies are required to determine the effect of G-CSF on immunomodulation after neurological damage and the importance of this pathway in G-CSF–induced neuroprotection.

Weighing G-CSF in immunomodulation and neuroprotection

Despite the fact that our understanding of the role of G-CSF in adaptive immunity is rapidly increasing, numerous questions remain: (1) activated $CD4^+$ T cells can potentially exert neurodegenerative action, as demonstrated by the demyelination in MS patients or neuroprotective action resulting in neuron survival after injury [107]. These discordant outcomes may result from varying environmental milieus, including different APC that regulate the $CD4^+$ T cell response. The activity state of T cells will determine the outcome of many pathological conditions. (2) neutrophils are an essential component of the immune response and a major contributor to inflammation. Recent studies have begun to elucidate the mechanisms by which G-CSF induces neutrophil release from the bone marrow, leading to novel strategies to modulate neutrophil responses in host defence and inflammation [112]. Though it has generally been considered safe, there are increasing observations that G-CSF administration can exacerbate underlying inflammatory diseases in humans and mice. G-CSF deficiency is profoundly protective against CIA in mice. In MS patients, G-CSF treatment caused disease exacerbation probably by enhancing the adhesion of encephalitogenic T cells to extracellular matrix components [46]. G-CSF–induced E-selectin ligand expression on immature (and mature) myeloid cells may thus prime these circulating cells to adhere to inflamed or ischemic endothelium [113], consistent with emerging clinical experiences and reports raising warnings for the use of G-CSF. These findings implicate G-CSF as an important anti-inflammatory agent [52].

For neuroprotection, a small pilot trial constitutes the first clinical study reporting on the safety and feasibility of G-CSF therapy in stroke patients. Despite these successes, it should be noted that this was a preliminary study and, because of the small number of participating patients, any observations are tentative. Efficacy and further confirmatory safety data will need to come from additional phase I studies and from larger phase II studies that are randomized and blinded. A variety of unresolved questions remain to be answered. Does G-CSF play its role through the mobilization of CD34 or act directly on G-CSF receptors on glial cells or neurons? How to elucidate the consequence of granulopoiesis or neovascularization induced by G-CSF after long-term application? Thus, critical analyses, well-designed preclinical studies and limited clinical trials of the safety, toxicity, optimal drug dosage, route and timing of delivery post-stroke will ultimately determine whether or not we are ready to advance G-CSF therapy into definitive large-scale clinical application for stroke, making G-CSF an ideal drug candidate for expansion of the therapeutic time window in patients with cerebral ischemia.

In the immunomodulation-neuroprotection of G-CSF, purposeful T cell-mediated 'protective autoimmunity' with a beneficial outcome is a relatively new concept. T-cell-dependent plasticity and neurogenesis are also associated with resident microglia and their local dialogue with T cells. According to this view, the difference between beneficial autoimmunity (neuroprotection) and potentially destructive autoimmunity (autoimmune disease) derives not from the nature of the autoimmune T cells but from their regulation, which fulfils its protective potential.

Conclusion and perspectives

Recent studies have demonstrated that G-CSF administration achieves a significant immunomodulation

in adaptive immunity or neuroprotection after cerebral ischemia and neurodegeneration through different mechanisms. The successful experimental models and the small number of clinical trial in human stroke suggest that G-CSF is a potential new agent for immunomodulation and neuroprotection, but should be used cautiously in patients, especially in Th2-mediated disorders.

Besides further randomized, double-blinded and placebo-controlled trials, a potential problem in the use of G-CSF for clinical application will be the undesirable side effect of granulopoiesis, particularly following multiple doses. The identification and separation of the structural determinants of G-CSF molecule might provide alternative ways to minimize side effects. The strategy to develop derivatives of G-CSF lacking activity of granulopoiesis, but keeping the immunoregulatory and/or neuroprotective potential allows for long-term usage of G-CSF in autoimmune diseases and neurologic disorders. So far, we have no indications to assume that it is possible to construct derivatives of G-CSF that lack systemic effects, as has been done for erythropoietin.

Much additional work is needed to better understand the microenvironment of G-CSF production within the CNS, including cell source, production inducer and autocrine/paracrine pathways. Once these questions become clear, one can use other agents to induce an endogenous G-CSF mobilization within the CNS. Based on rather low concentration in the periphery, the endogenous G-CSF within the CNS does not stimulate granulopoiesis, and limit the undesirable side effect.

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