Cytokine & Growth Factor Reviews 25 (2014) 355–367

Contents lists available at ScienceDirect

Cytokine & Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

Survey

G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent

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ARTICLE INFO

Article history:
Available online 23 July 2014

Keywords:
G-CSF
Stem cell mobilization
Chemotherapy
Stem cell transplantation
Bone marrow microenvironment

ABSTRACT

G-CSF was among the first cytokines to be identified and rapidly transitioned into clinical medicine. Initially used to promote the production of neutrophils in patients with chemotherapy-induced neutropenia it helped to revolutionize the delivery of cancer therapy. Its ability to mobilize hematopoietic stem cells from the bone marrow into the blood was subsequently exploited, changing the face of hematopoietic stem cell transplantation. Today the knowledge gained in unraveling the mechanisms of stem cell mobilization by G-CSF is being explored as a means to increase chemosensitivity in hematological malignancies. This review provides a brief history of G-CSF and then focuses on recent advances in our understanding of G-CSF-induced stem cell mobilization and the potential clinical application of this knowledge in chemo-sensitization.

Contents

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http://dx.doi.org/10.1016/j.cytogfr.2014.07.011
1359-6101/

1. History of G-CSF

Granulocyte colony stimulating factor (G-CSF) was among the first cytokines to be identified and to enter clinical trials. The identification of G-CSF followed the development of an assay measuring the effects of G-CSF and related cytokines by two independent groups in the 1960s: Ray Bradley and Don Metcalf at the University of Melbourne, Australia, and Yasuo Ichikawa and Leo Sachs at the Weizmann Institute, Israel [1,2]. These assays measured the ability of test agents to stimulate colony formation in hematopoietic cells in semi-solid culture and gave many of these cytokines their names, *e.g.* granulocyte colony stimulating factor, granulocyte/macrophage colony stimulating factor *etc.* Although colony forming assays permitted the quantitation of G-CSF, it was more than a decade before Nicos Nicola in Donald Metcalf's laboratory finally isolated the murine cytokine from medium conditioned with the lung tissue obtained from endotoxin treated mice in 1983 [3]. Human G-CSF was purified soon after from the conditioned medium of the bladder carcinoma cell line 5637 by Karl Welte in 1985 [4]. The concurrent development of molecular biology techniques meant that the G-CSF gene was soon cloned by Shigekazu Nagata in Japan and independently by Lawrence Souza from AMGEN in 1986, permitting the large scale production of this cytokine and its subsequent clinical application [5,6].

2. Biology of G-CSF

G-CSF is central to the production of neutrophils in health and diseased states and is responsible for the dramatic increase in neutrophil numbers in response to infection or insults affecting bone marrow function such as anti-cancer cytotoxic chemotherapy. The ability of G-CSF to induce the production of mature neutrophils is evident from the production of neutrophilic granulocyte colonies from bone marrow cells in semi-solid cultures in response to this cytokine [7]. Furthermore mice lacking G-CSF or its receptor, G-CSFR, demonstrate chronic severe neutropenia, although a small number of neutrophils are still detected [8,9].

G-CSF mediates its effects by binding to a single homodimer receptor, G-CSFR [10]. Plasma concentrations of G-CSF are normally low to undetectable, but rise rapidly in response to infection and subsequently decline with recovery [11,12]. Many tissues can produce G-CSF when appropriately stimulated, with inflammatory mediators such as lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, interferon (IFN)-β, vascular endothelial growth factor (VEGF), interleukin (IL)-17 and IL-1 inducing expression in endothelial cells, macrophages, epithelial cells and fibroblasts [13–15]. The release of G-CSF into the bloodstream by tissues stimulates neutrophil production within, and mobilization from, the bone marrow. Furthermore, the locally produced G-CSF within tissues also influences the function of neutrophils at the site of infection. G-CSF can inhibit neutrophil apoptosis [16], increasing survival within infected tissues [17]. Although G-CSF does not directly induce the chemotaxis of neutrophils it has chemokinetic activity on these cells, promoting non-directional motility [18], which increases responses to chemotactic factors such as the bacterial agent N-Formyl-Methionine-Leucine-Phenylalanine (fMLP) [19]. Whether this contributes to the increased accumulation of neutrophils in inflamed tissues is not certain but G-CSF does not promote neutrophil migration through TNF-α activated endothelium [18]. Similarly, while G-CSF does not directly stimulate phagocytosis or superoxide generation, it primes neutrophils, augmenting responses to agents such as fMLP [20]. This suggests that G-CSF may facilitate the activity of neutrophils within an inflammatory environment but has little effect under normal physiological situations, thereby restricting these activities of neutrophils to sites of infection.

2.1. G-CSF in the treatment of neutropenia and bone marrow failure syndromes

The first clinical trials of G-CSF were for the reduction of chemotherapy-induced neutropenia and infections in cancer patients [21,22]. Subsequent randomized multicenter trials from the USA and Europe demonstrated that G-CSF reduced the incidence of febrile neutropenia and culture-confirmed infections. G-CSF also reduced the incidence, duration, and severity of grade IV neutropenia, which translated into reduced intravenous antibiotic use and shorter hospitalization periods [23,24]. The use of G-CSF also increased the likelihood that patients would receive their planned dose of chemotherapy [23]. Today G-CSF is routinely given to patients receiving chemotherapy for a range of malignancies, in particular in situations where the risk of severe neutropenia is estimated to be over 20%, and also to allow for the delivery of "dose-dense" chemotherapy regimens, as outlined in the 2005 ASCO guidelines [25].

G-CSF is also used to treat isolated congenital, cyclic, and idiopathic neutropenia and some cases of myelodysplastic syndrome and acquired aplastic anemia. In patients with isolated neutropenia, G-CSF reduces symptoms with fewer occurrences of mouth ulcers, febrile events, and infections [26]. In aplastic anemia the results are less clear cut with measurable benefits only being seen in the most severe cases and limited to reduced infectious events, but no overall effect on survival [27]. While chronic neutropenias are rare, these patients have an ongoing need for G-CSF and in contrast to patients experiencing chemotherapy-induced neutropenia, require a more refined and personalized dosing regimen. Side effects and adverse events also pose a greater risk and inconvenience to patients with chronic neutropenia. Some of the more acute effects, such as bone pain, can be successfully managed by optimizing the dosing schedule [28]. The longer-term and more concerning risks of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are more difficult to assess as they form part of the natural history of some of these conditions. While patients with congenital neutropenia requiring higher doses of G-CSF are now known to have an increased incidence of MDS and AML [29] it remains impossible to be certain that G-CSF has contributed to the development of these conditions although at this stage this is thought to be unlikely. The use of G-CSF in inherited clonal conditions such as Fanconi anemia is not recommended due to limited benefit and a perceived increased risk of AML.

3. G-CSF in hematopoietic stem and progenitor cell mobilization

3.1. Origins

Bone marrow transplantation (also now termed hematopoietic cell transplantation (HCT)) is the oldest form of anti-cancer immunotherapy in clinical use, and involves the transplantation of hematopoietic stem and progenitor cells from self (autologous) or histocompatible allogeneic related or unrelated volunteer donors. Autologous HCT is carried out using cryopreserved hematopoietic cells to restore marrow function after the delivery of high dose chemotherapy, predominantly for patients with lymphomas or multiple myeloma [30,31]. Allogeneic HCT is also used to restore hematopoiesis and immune function in patients with bone marrow failure and immunodeficiency syndromes, but is now most commonly used as cellular immune therapy to treat patients with hematopoietic malignancies [32,33].

Traditionally, hematopoietic cells for both autologous and allogeneic transplantation were obtained by collecting large volumes of bone marrow, aspirated from the pelvic crests under general anesthesia. However, pre-clinical data showed that G-CSF could mobilize hematopoietic cells in large numbers from the marrow into the circulation with increased progenitor cells of all lineages detected in the spleens of G-CSF treated mice [34]. The following year Duhrsen et al. confirmed the mobilizing activity of G-CSF in cancer patients [35]. Subsequent clinical trials demonstrated that adequate numbers of these cells could be collected from cancer patients or normal donors to allow successful autologous and allogeneic HCT respectively [36–38]. The use of G-CSF mobilization had the advantage of increasing the number of hematopoietic cells collected, with consequent reductions in the time taken post-transplant to restore neutrophil and platelet numbers to clinically safe levels, and improvements in transplant safety [39]. In addition, despite common side effects of G-CSF such as bone pain, experience in randomized clinical trials was that normal donors preferred donation of hematopoietic cells collected from blood, rather than from pelvic marrow [40]. These clinical trials have led to the widespread use of G-CSF-mobilized hematopoietic cells collected by leucapheresis in the majority of autologous and allogeneic transplants.

3.2. Mechanism of mobilization

The biology underlying the process of HSC mobilization has been extensively studied but our understanding of the process is still incomplete. Perhaps surprisingly G-CSF does not mobilize HSC and progenitors by a direct influence on these cells. This was demonstrated using mice that were chimeric for expression of the G-CSFR on hematopoietic cells. In these animals hematopoietic progenitors lacking the G-CSFR were mobilized with equivalent efficiency as those expressing the receptor [41]. However, mice where all hematopoietic cells lack the G-CSFR completely fail to mobilize. Together this suggests that while the response of hematopoietic cells to G-CSF is essential for HSC mobilization, the effect is indirect and a specific response of individual HSC to G-CSF not required.

3.2.1. Breaking the bond

The notion that HSC reside in a niche within the bone marrow that regulates their growth, survival and differentiation has been long held [42]. For mobilization to occur the bond between the HSC and the niche needs to be broken. A number of retentive factors have been identified over the last two decades with the more prominent being the very late antigen (VLA)-4/VCAM1 adhesive interaction and the CXCL12/CXCR4 chemo-attractive interaction. There is considerable evidence that these interactions are disrupted during G-CSF-induced HSC mobilization [43,44] and that their isolated blockade is sufficient for mobilization [45,46]. However whether the disruption of these pathways is a necessity in all settings remains less certain.

3.2.2. Proteases in mobilization

Initially it was demonstrated, using antibody depletion, that neutrophils were required for HSC mobilization [47]. This led to the hypothesis that neutrophil proteolytic enzymes were required to cleave key microenvironmental retention factors, most notably CXCL12 [44] and VCAM1 [43]. These molecules are cleaved in a manner consistent with the activity of neutrophil proteases following G-CSF administration and inhibition of these enzymes can reduce the extent of mobilization [47]. However, attempts to demonstrate a role for these enzymes using genetically modified animals have been unsuccessful, with mice lacking individual or combinations of these enzymes demonstrating normal HSC mobilization [48]. Cleavage of VCAM1 did not occur in the protease deficient mice, suggesting that cleavage of VCAM1 is not essential for HSC mobilization following G-CSF administration. In contrast, functional CXCL12 and CXCR4 were both decreased in these mice [48]. The only protease that has a demonstrated role in HSC mobilization using genetic models is the amino-dipeptidase, CD26, the deletion of which attenuates but does not completely block mobilization of hematopoietic progenitors [49]. CXCL12 [50], but not CXCR4, is a target of CD26, but CD26 also cleaves a large numbers of other cytokines and chemokines including G-CSF [50,51]. Precisely which CD26 targets are important for G-CSF induced HSC mobilization is not clear. Intriguingly, the lack of CXCL12 cleavage in $CD26^{-/-}$ mice treated with G-CSF has not been demonstrated. It would be interesting to know whether CD26 is responsible for CXCL12 cleavage in response to G-CSF *in vivo*.

3.2.3. The role of lipids

Much is known about the factors that retain HSC in the niche but little attention has been given to mechanisms that may encourage the egress of HSC from the bone marrow. It can be argued that such mechanisms may not exist and that cells leave the bone marrow in a purely stochastic manner following loss of retentive forces. However, recent evidence suggests the bioactive lipid sphingosine 1-phosphate (S1P) may act as an egress factor [52–54]. S1P is a chemo-attractant for hematopoietic cells, including HSC, an activity that is mediated by a series of G-protein coupled receptors $S1P_1$–$S1P_5$, with $S1P_1$ being the principal receptor on HSC [55]. S1P is present at high concentrations in plasma and low concentrations in tissues, including the bone marrow, providing an appropriately directed gradient [56]. Although red blood cells and platelets remain potential sources of plasma S1P, recent studies have demonstrated that non-hematopoietic cells, predominantly endothelial cells, are responsible for the bulk of circulating S1P [57–59]. S1P, and its receptor $S1P_1$, have been repeatedly implicated in HSC mobilization by CXCR4 antagonists, with gene deletion or pharmacological suppression of $S1P_1$ and genetic or pharmacological attenuation of the S1P gradient reducing HSC mobilization [52,53]. The role of $S1P_1$ in G-CSF mediated mobilization is less clear with a minority of studies showing a significant effect [53] while most failed to detect any interaction [52,60]. Clarification of the contribution of $S1P_1$ in G-CSF mediated HSC mobilization awaits further studies.

A transient increase in S1P plasma concentrations observed in mice has been thought to be involved in HSC mobilization following G-CSF administration. This has been explained by increased release of S1P from red cells due to activation of the complement cascade and the membrane attack complex and increased S1P production by sphingosine kinase 1, combined with

decreased S1P degradation by sphingosine phosphatase 1 [53,54]. However elevated plasma S1P has not been detected in humans mobilized with AMD3100 or G-CSF [52]. The evidence that increased plasma S1P concentrations are required for HSC bone marrow egress requires verification and it appears that baseline S1P plasma concentrations may be sufficient for HSC mobilization.

3.2.4. Microenvironmental disruption

G-CSF has profound effects on the bone marrow including the obvious and readily explained increase in myeloid cell numbers, but also some less easily explained cell losses, particularly cells of mesenchymal origin. Particularly well studied are the effects on the bone endosteal surface specifically the loss of osteoblasts [61], and an increase in osteoclast activity [62]. Acute deletion of osteoblasts, using an elegant double transgenic model permitting the specific deletion of these cells by diphtheria toxin, resulted in HSC mobilization without G-CSF. This demonstrates that osteoblasts are required for the optimal retention of HSC and their loss is sufficient for HSC mobilization [63]. Bone turnover has been associated with G-CSF-induced mobilization in a number of models, including reduced mobilization when osteoclasts are inhibited by calcitonin or maturation blocked by gene deletion of PTPRE (protein tyrosine phosphatase, receptor type, E) or CD45. Osteoclasts could provide enzymes to degrade microenvironmental components in a similar manner to neutrophil enzymes [62,64]. However osteoclasts are not essential for G-CSF-induced HSC mobilization [65] and some reports suggest that the timing of increased osteoclast numbers is not consistent with peak mobilization times [66,67]. More recently loss of other cells including osteomacs, specialized macrophages that blanket osteoblasts [66], and osteocytes, located within the bone [68] has been reported during G-CSF mediated mobilization. In contrast the more vascular associated CXCL12-abundant reticular (CAR) cells and nestin-positive mesenchymal stem cells (N^+MSC) appear to be unchanged in numbers.

3.2.5. The role of phagocytes

More recently phagocytic cells have been implicated in the process of mobilization, with restoration of G-CSF receptor expression on $CD68^+$ monocytes in otherwise G-CSF receptor null mice being sufficient for HSC mobilization by G-CSF [69]. The neutropenic nature of these mice also suggests that neutrophils are not essential for HSC mobilization, although it does not eliminate their involvement in the process in normal animals. G-CSF-induced mobilization in this model was associated with the loss of monocytes from the bone marrow and suppression of osteoblasts. A similar loss of macrophages specifically associated with osteoblasts in the bone marrow, termed osteomacs [66], was also reported following G-CSF administration. Furthermore, depletion of osteomacs [66] or $CD169^+$ macrophages [70] is sufficient to mobilize HSC. While there are clear parallels relating to the involvement of the monocyte/macrophage lineage in G-CSF induced HSC mobilization different underlying mechanisms have been proposed to explain these effects. Osteomacs have been proposed to act by providing protection and support for osteoblasts [66], which once lost leads to the typical suppression of osteoblast numbers and function associated with G-CSF-induced HSC mobilization. In contrast, the $CD169^+$ macrophages were shown to promote CXCL12 production by N^+MSC but not osteoblasts *in vitro* [70], with loss of macrophages *in vivo* reducing CXCL12 gene expression by N^+MSC. Whether $CD169^+$ macrophages are lost following G-CSF administration has not been addressed at this stage. Overall these studies suggest that monocyte derived macrophages play a significant role in G-CSF induced HSC mobilization, apparently resulting from a G-CSF induced loss of these cells, their supportive function for cells of mesenchymal origin and ultimately CXCL12 production. The details regarding precisely which macrophages are key and the identification of mesenchymal cells influences awaits further confirmation by others.

3.2.6. The role of the nervous system

G-CSF-induced mobilization is severely inhibited in mice where the sympathetic nervous system is disrupted by genetic or pharmacological means [71], implicating an active role for the nervous system in G-CSF-mediated HSC mobilization. Both β2 and β3-adrenergic receptors appear to co-operate in this process [72]. G-CSF does not seem to affect the release of norepinephrine from sympathetic neurons but reduces the subsequent uptake, thereby increasing the strength/duration of signals from the sympathetic nervous system [73]. Neurotransmitters could have a direct effect on HSC as human $CD34^+$ cells express β_2-adrenergic and dopamine receptors, the stimulation of which enhances responses to the chemokine CXCL12 [74]. Furthermore, G-CSF increased the expression of β_2-adrenergic and dopamine receptors on these cells. However, the *in vitro* studies found the response of HSC to catecholamines to be modest compared to the potent *in vivo* effects observed, suggesting that the effects of the sympathetic nervous system on the bone marrow microenvironment may be more important.

Many of the G-CSF-induced microenvironmental changes appear to be mediated by the sympathetic nervous system including reductions in osteoblasts and osteocytes [68,71]. The responses of osteoblasts to sympathetic signals are dependent on expression of the vitamin D receptor, which is increased by stimulation through the β_2-adreneric receptor [75]. One of the more surprising findings of these recent papers is that despite the failure to mobilize HSC, CXCL12 expression is still drastically and promptly reduced in the bone marrow in response to G-CSF when osteocytes, the sympathetic nervous system or vitamin D receptor are disrupted [68,71,75]. This finding appears to conflict with the now long held and well supported concept that CXCL12 is critical for the retention of HSC in the bone marrow and prevention of CXCL12 binding its receptor CXCR4 is pivotal to HSC mobilization [44,76]. Each of these three models result in some disruption of the endosteal niche, so it has been suggested that alternate retention factors may compensate for the altered niche in these mice reducing the importance of the CXCL12/CXCR4 axis [68]. However, in each of these models HSC mobilization by the CXCR4 antagonist AMD3100 was unaffected demonstrating that disruption of CXCR4/CXCL12 alone is still able to mobilize HSC and confirming the importance of CXCL12 in HSC retention and its role in mobilization. Another possibility is that despite the overall decrease in CXCL12 concentrations in the extracellular fluid of the bone marrow of these gene-modified mice when G-CSF is administered, CXCL12 concentrations may not be adequately reduced within the HSC niche itself. At least in the osteocyte model, CXCL12 protein expression was found to be high in the bone matrix and was not decreased following osteocyte ablation.

Evidence for the involvement of the sympathetic nervous system in G-CSF-induced HSC mobilization in humans is limited. A recent study linked low circulating $CD34^+$ cells with increased markers for diabetic neuropathy [77] and although mobilization was not measured in this study, diabetic patients have been shown to mobilize poorly [78]. However, a retrospective examination of allogeneic donors undergoing HSC mobilization failed to reveal better $CD34^+$ cell mobilization in patients who were coincidentally taking β-blockers for other reasons but numbers of patients were small and a larger controlled prospective study would be needed to clarify the importance of this axis in human HSC mobilization [79].

3.2.7. Summary of mobilization mechanisms

Overall at a cellular level it appears that G-CSF triggers a number of potentially parallel events including expansion of neutrophils and their precursors, stimulation of CD169$^+$ bone marrow macrophages, the peripheral sympathetic nervous, osteocytes and osteomacs (Fig. 1). The latter three either directly or indirectly suppress osteoblasts and the production of bone marrow supportive factors, notably CXCL12. The stimulation of CD169$^+$ macrophages, potentially *via* the sympathetic nervous system, suppresses CXCL12 production by N$^+$MSC. The granulocyte expansion provides a proteolytic environment that can degrade retentive factors. Together this results in alteration to the HSC niche making it less attractive for HSC, permitting their egress into the peripheral circulation potentially under the influence of a S1P gradient.

3.3. Inadequate stem cell mobilization

3.3.1. Normal donors

Despite the great success of G-CSF as a mobilizing agent a number of concerns remain. Although the majority of healthy allogeneic donors mobilize adequately, insufficient cells are recovered from a small proportion, particularly when there is a major weight discrepancy between donor and recipient. A few features such as the female donor sex and G-CSF dose scheduling, and in some studies basal platelet count, have been associated with

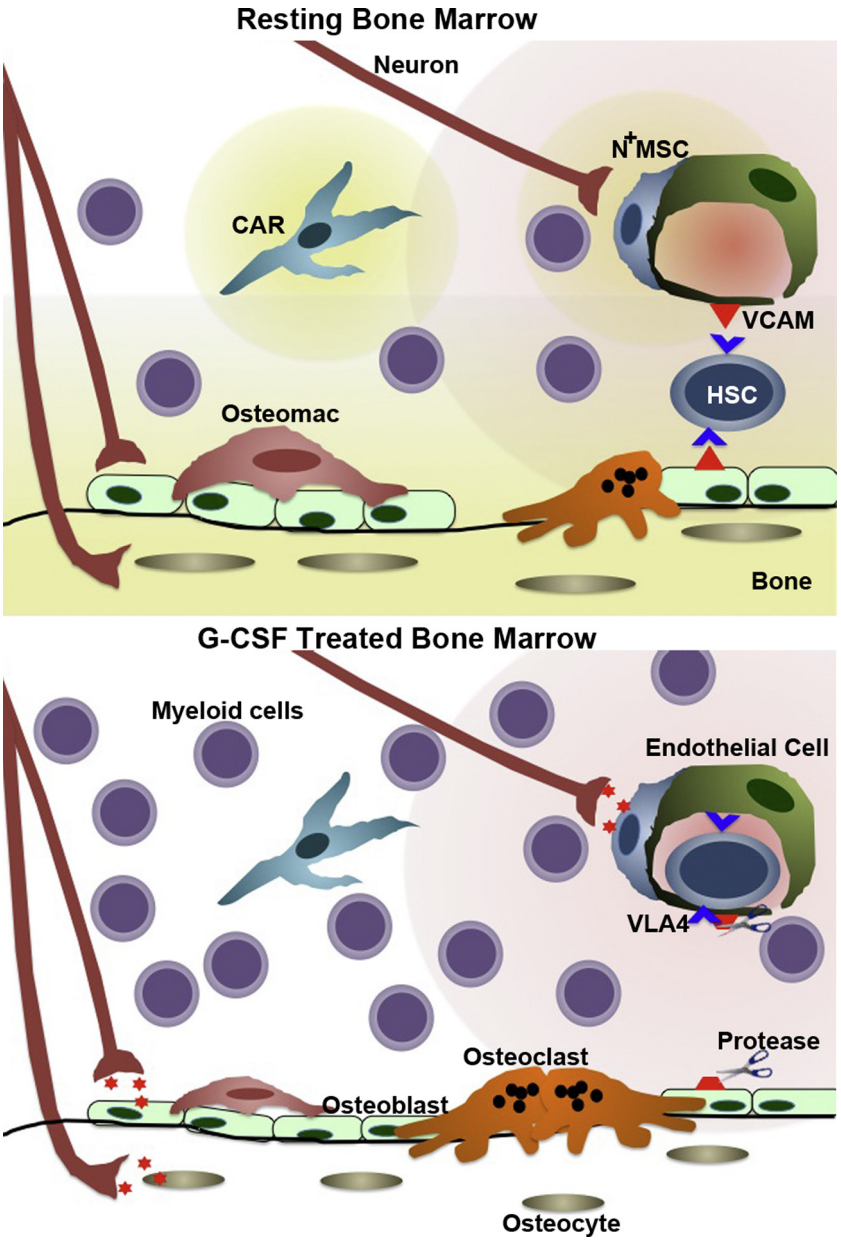


Fig. 1. The upper panel shows resting bone marrow and the lower panel G-CSF treated marrow. The yellow shading indicates CXCL12, the red shading S1P and the red stars catecholamines. Under resting conditions HSC are held within the bone marrow by adhesive interactions and the chemo-attraction provided by CXCL12. G-CSF triggers the expansion of neutrophils and their precursors, creating a proteolytic environment leading to the degradation of adhesive interactions, chemokines and their receptors. G-CSF also stimulates the peripheral sympathetic nervous system, increasing catecholamine concentrations, which in turn suppresses osteocytes and osteoblasts as well as CXCL12 production by Nestin+ MSC *via* effects on CD169$^+$ bone marrow macrophages. Osteomacs are also suppressed facilitating the reduction in osteoblasts and CXCL12 production by these cells. CXCL12 production by CAR cells is also decreased. Together this results in a dramatic decrease in the concentrations of this chemokine within the bone marrow microenvironment. Osteoclast numbers expand, increasing bone turn over. In the absence of CXCL12 and adhesive interactions, HSC are now able to respond to the S1P gradient facilitating the movement of HSC from the niche into the peripheral circulation.

lower HSC yields. However, the predominant reason for the variation between donors appears to be linked to the genetics of the donor as the same donor mobilized on separate occasions results in a very similar HSC recovery. The CXCL12–3′A allele has been associated with better mobilization in most [80–83], although not all studies [84]. The CC genotype of the CD44 polymorphism rs13347 has been associated with better $CD34^+$ cell yields in two studies, one in healthy and one in autologous donors, although the binning of the heterozygotes differed between the studies [83,85]. These same studies had conflicting outcomes regarding polymorphisms in VCAM1 (rs1041163), CXCR4 (rs2680880), and the G-CSF receptor (rs3917924) with only the study by Martin-Antonio and colleagues finding negative associations with the CC and AA genotypes of VCAM1 and CXCR4 respectively, and total $CD34^+$ yield and the CC and TT genotypes of VCAM1 and the G-CSF receptor respectively and $CD34^+$ cells/μl in the blood prior to collection. Overall it appears that the extent of mobilization is moderated by genetic polymorphisms in the molecules involved in migration and homing of HSC.

3.3.2. Following chemotherapy

In autologous donors mobilization failure is more common and is strongly correlated with prior exposure to myelotoxic agents with DNA damaging agents such as melphalan and fludarabine and the thalidomide derivative lenalidomide [86–88]. This is thought to be partly due to stem cell depletion by chemotherapy exposure, but also partly due to damage to the niche, with a number of reports describing long-term stromal damage resulting from high dose chemotherapy in animals models and patients [89–92]. A recent paper demonstrated in mice that impaired bone marrow function following vincristine and cisplatin exposure resulted from damage to sympathetic neurons. Protection of neurons in this model using 4-methylcatechol, reduced the loss of bone marrow niche cells including N^+MSC and endothelial cells following exposure to neurotoxic chemotherapy, although osteoblasts and macrophages were not affected. Animals with chemotherapy induced sympathetic nervous system damage mobilized poorly with G-CSF and protection of the sympathetic nervous system during chemotherapy could prevent this impaired mobilization [93]. This study suggests that the use of drugs to prevent or minimize nerve damage during induction chemotherapy could minimize mobilization failure during subsequent stem cell transplantation procedures.

3.3.3. Diabetic donors

A retrospective analysis revealed an association between elevated blood sugar and a failure to adequately mobilize HSC in response to G-CSF [63]. This finding was recently confirmed in a prospective clinical trial [78]. The association between diabetes and mobilization failure was replicated using two separate mouse models of diabetes and was shown using transplantation experiments to result from altered microenvironmental conditions. Consistent with this, diabetic mice had reduced osteoblast numbers and although normal in number the N^+MSC expressed less CXCL12 and importantly failed to down-regulate CXCL12 following G-CSF treatment. The failure to down-regulate CXCL12 was due to dysfunction of the sympathetic nervous system in diabetic mice. This is consistent with previous reports of bone marrow neuropathy in diabetic rats [94] and neuropathies in human diabetic patients [95].

3.4. Side-effects

Another issue relating to the use of G-CSF for HSC mobilization, particularly in normal donors, is its side effects. G-CSF can induce a considerable number of short-term side effects, the majority of which are not serious, although undoubtedly uncomfortable, such as malaise, nausea, night sweats, with the most commonly reported being bone pain [96–99]. However a small number of serious events have been reported, with splenic rupture being the most prominent, but interstitial pneumonitis, pulmonary infiltrates, lung fibrosis and respiratory distress syndrome have also been described [100,101]. Improved mobilization strategies are desirable to minimize the risk, particularly to healthy donors, of these adverse events.

3.5. Improving mobilization strategies

The need to improve mobilization protocols, particularly in autologous settings with heavily pretreated patients, is essential to achieve better transplantation outcomes. A number of agents, most commonly cytokines including GM-CSF, FLT3L, IL8 and SCF, have long been known to induce HSC mobilization alone or in combination with G-CSF [102–105] but have not entered routine clinical use for a variety of reasons and these will not be discussed here. Here we will focus on agents or strategies with the potential for clinical application in the future.

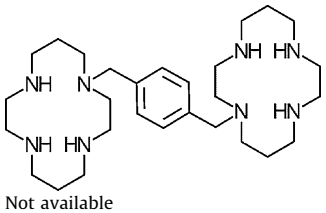
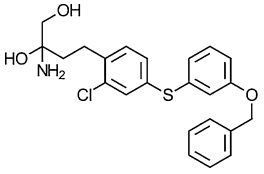
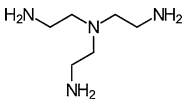
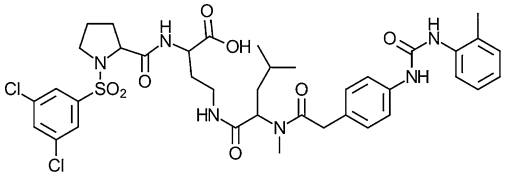
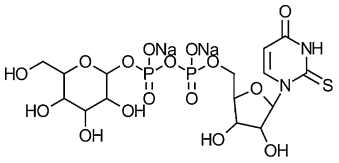
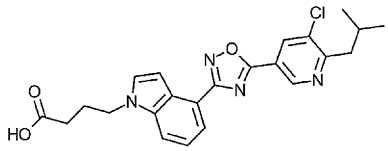
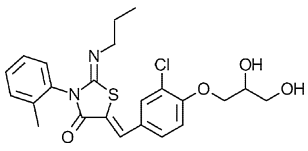
3.5.1. CXCR4 antagonists

A simple but logistically challenging possibility is to make optimal use of the natural circadian rhythm by harvesting donors during the evening [106], taking advantage of normal modulations in CXCL12 and CXCR4 expression in the bone marrow and on HSC respectively. In the last decade pharmacological manipulation of this axis has become available. The leading CXCR4 antagonist AMD3100 was first approved by the Food and Drug Administration in the USA in 2008 for use in combination with G-CSF to mobilize HSC for autologous transplantation in patients with non-Hodgkin lymphoma and multiple myeloma, and is now commonly used world-wide in this setting. Pre-clinical studies in mice also suggest that CXCR4 antagonists such as AMD3100 may be useful in overcoming the reduced mobilization seen in diabetic patients [63]. Unlike G-CSF, where the mechanism of mobilization is complex, CXCR4 antagonists mobilize HSC by blocking the retentive activity of CXCL12. As a result they have a rapid effect, with a peak mobilization at about one hour in mice and nine hours in humans [46,107]. Although inhibition of the CXCL12/CXCR4 axis is considered key to G-CSF-mediated HSC mobilization, CXCR4 antagonists are relatively modest mobilizers when used in isolation but display considerable synergy when combined with G-CSF [107]. This suggests that suppression of CXCL12 in the bone marrow is less than complete following G-CSF treatment, leaving room for further inhibition by a CXCR4 antagonist. However there is an alternative explanation. Peripheral blood HSC numbers are significantly increased by prior splenectomy when mobilizing with G-CSF [108] but this is not observed when AMD3100 is the mobilizing agent [109]. CXCL12 is not only present in the bone marrow but is expressed in many other tissues such as the spleen, lungs and liver [110,111] and so has the potential to retain, at least transiently, circulating HSC in these tissues. Massberg and co-workers elegantly demonstrated the trafficking of HSC through peripheral tissues under basal conditions, although this appears to occur at a low rate [60]. A systemically administered CXCR4 antagonist would be expected not only to mobilize HSC from the bone marrow but also from peripheral tissues, and prevent transient retention in these sites. It is therefore possible that the reduced loss of HSC to peripheral tissues, particularly the spleen, in the presence of CXCR4 antagonists contributes to the increased circulating HSC numbers when CXCR4 antagonists are combined with G-CSF.

Although AMD3100 is currently the only approved CXCR4 antagonist in clinical use, a large number of compounds have been

developed that have varying CXCR4 antagonist activity (reviewed in Debnath et al.) [112]. Only agents with potential for clinical translation for HSC mobilization will be discussed here (Table 1). POL6326 and TG-0054 are the most advanced and currently in clinical trial for HSC mobilization, and both have undisclosed structures. POL6326 is currently in a safety study in normal volunteers (NCT01841476) and a Phase I/II clinical trial for the mobilization of sibling donors (NCT01413568). Results in normal volunteers showed it was well tolerated and effectively mobilized $CD34^+$ cells [113]. Preliminary results from a Phase II study for patients with multiple myeloma undergoing autologous HSC transplantation (NCT01105403) demonstrated adequate mobilization of $CD34^+$ cells in 66% of patients with all of these achieving engraftments. A small number of malignant cells were detected in the POL6326 mobilized product in 3 of 5 patients tested but this was not considered to be greater than expected using G-CSF and chemotherapy [114]. POL6326 has not been tested in combination with G-CSF. The requirement for intravenous administration represents a minor disadvantage to this agent particularly for normal volunteers.

Table 1
Potential mobilizing agents.

Agent	Mechanism	Structure	Developmental stage
AMD3100	CXCR4 antagonist		Approved for use in combination with G-CSF for autologous transplantation.
POL6326	CXCR4 antagonist	Not available	Phase 1 and Phase 2 clinical trials for mobilization in healthy donors and patients with hematological malignancies.
TG-0054	CXCR4 antagonist	Not available	Phase 1 trials in healthy donors completed and phase 2 trials in patients with hematological malignancies, some ongoing.
KRP203	CXCR4 antagonist		Phase 1 trial in patients with hematological malignancies.
Me6TREN	Unknown		Preclinical testing in mice.
BIO5192	VLA-4 antagonist		Preclinical testing in mice
MRS2690	P2RY14 agonist		Preclinical testing in mice.
GSK2018682	$S1P_1$ agonist		Phase 1 trials in healthy volunteers completed.
ACT-128800	$S1P_1$ agonist		Phase 1 trials in healthy volunteers and Phase 2 studies for autoimmune disease.

In mice TG-0054 induced mobilization of HSC with kinetics similar to AMD3100 but achieved a peak HSC mobilization closer to that obtained with G-CSF [115]. TG-0054 has completed safety studies in normal volunteers and patients with multiple myeloma, non-Hodgkin lymphoma and Hodgkin disease, where it was well tolerated and induced the mobilization of $CD34^+$ cells, peaking between 4 and 6 h with acceptable $CD34^+$ cell counts of greater than 20/μl in healthy volunteers [116]. TG-0054 is currently being evaluated in a Phase II trial for autologous transplantation as a single agent and in combination with G-CSF (NCT01458288). A preliminary report showed that 7 of 12 patients with advanced hematological malignancies were adequately mobilized with TG-0054 alone and the remaining 5 patients with a combination of G-CSF and TG-0054. All patient successfully engrafted with the mobilized products [117]. Both POL6326 and TG-0054 have the potential to be used as single agents and therefore could replace G-CSF for HSC mobilization in some settings in the future. In addition to these compounds a new inhibitor ALT-1188 was described at the 2013 American Society for Hematology (ASH) meeting demonstrating superior mobilization in mice than AMD3100 [118]. It is a small molecule inhibitor with an azacarbazole backbone linked *via* a short chain alkyldiamine to a tetrahydroquinoline eliminating the metal-chelating properties of AMD3100, potentially reducing toxicity. The application of this compound in humans remains to be determined.

3.5.2. VLA-4 inhibitors

VLA-4 binding to VCAM1 and to a lesser extent fibronectin and osteopontin provides a major adhesive interaction for HSC within the bone marrow microenvironment, the disruption of which leads to HSC mobilization [109,119]. As with CXCL12/CXCR4 interactions, G-CSF treatment disrupts VLA-4/VCAM1-mediated bone marrow retention [43], and inhibition of VLA-4/VCAM1 binding enhances the efficacy of G-CSF [119]. A small molecule inhibitor of VLA-4 binding, BIO5192, has been developed and as anticipated, increases the degree of mobilization induced by G-CSF in mice [109]. Whether this or a next generation VLA-4 antagonist will find a place in clinical mobilization strategies remains to be determined.

3.5.3. UDP-glucose

Recently the nucleotide sugar UDP-glucose was shown to mobilize HSC in mice. In contrast to most mobilizing agents described to date, UDP-glucose preferentially mobilized the most primitive cells and did not result in a general leukocytosis [120]. The mobilized stem cells were more quiescent than those mobilized by G-CSF and demonstrated greater long-term repopulating activity with a distinct skewing toward the lymphoid lineages. The effect was independent of the G-CSF receptor but dependent on the generation of reactive oxygen species and apparently through the induction of RANK ligand and osteoclast activity. While UDP-glucose synergized with G-CSF the mechanism of action appeared to differ from when it was use in isolation, perhaps questioning whether the proposed mechanism is correct. The receptor for UDP-glucose is thought to be P2RY14 [121], although this is a subject of debate [122,123] and hence was not directly examined. The authors proposed that the small molecule agonist of P2RY14, MRS 2690, could be a useful agent, however safety data for this compound are not yet available. In mice the administration of UDP-glucose did not induce any measurable toxicities. The very primitive nature of the HSC mobilized resulted in delayed engraftment, suggesting that it may not be useful as a single agent but better combined with G-CSF.

3.5.4. Agonists of $S1P_1$

Amplifying the S1P gradient between the blood and bone marrow provides a potential mechanism to increase HSC trafficking into the peripheral blood. While the S1P receptor agonist SEW2871 had no significant effect on circulating HPC numbers when used alone or in combination with G-CSF in mice, it significantly enhanced mobilization induced by AMD3100 in both good and poor mobilizers and the combination of AMD3100 and G-CSF in good mobilizers only [52]. This suggests that $S1P_1$ agonists may be used in combination with CXCR4 antagonists, eliminating the need for G-CSF in some settings. A potential advantage of the use of $S1P_1$ agonists is that the increased mobilization was not associated with increased total peripheral white blood counts. Considering that agonists of $S1P_1$ that induce receptor internalization result in the retention of lymphoid cells in secondary lymphoid tissues such as spleen and lymph nodes, it is likely that these agents will change the immune make up of the harvested graft, with a likely skewing away from naive to memory T cells. This has the potential to reduce graft *versus* host disease.

There are an increasing number of S1P receptor agonists at various stages of development, with those that have entered clinical trial being examined for the known immunosuppressive activity of these agents in conditions such as multiple sclerosis (GSK2018682, ACT-128800 and FTY720) psoriasis (ACT-128800), sub-acute cutaneous lupus erythematosus and ulcerative colitis (KRP203), Rett's syndrome and asthma (FTY720) and the prevention of graft *vs* host disease (KRP203). Indeed FTY720 is currently approved for the treatment of multiple sclerosis. All of these agents mediate their effects by inducing internalization and loss of function of S1P receptors. Only SEW2871 is known to activate $S1P_1$ without inducing internalization, a feature desirable for use as a mobilizing agent [124]. However, SEW2871 has not been tested in humans and due in part to its poor solubility is unlikely to be useful clinically. Perhaps the biggest problem facing the development of $S1P_1$ agonists in HSC mobilization are the cardiac effects of $S1P_1$ agonists with these agents producing pronounced transient bradycardia [125]. Considering acute high doses will be needed for mobilization strategies the development of an ideal agent represents a significant challenge.

3.5.5. Me6TREN

Me6TREN is a novel small molecule identified from a chemical screen that induces the mobilization of HSC as a single agent and enhances HSC mobilization when combined with G-CSF or AMD3100 [126]. The mechanism of action of Me6TREN involves the induction of MMP9 expression *via* phosphoinositide-3 kinase and p38 mitogen-activated protein kinase pathway signaling. Although Me6TREN inhibits CXCL12 induced chemotaxis direct evidence of receptor binding is lacking. The agent demonstrated safety and efficacy in mice but remains to be tested in humans.

4. Mobilization of malignant cells

The bone marrow microenvironment provides a supportive environment not only for normal hematopoietic stem and progenitor cells but also malignant hematopoietic cells including leukemias [127–129]. This environment also provides protection from chemotherapeutic agents [130,131], potentially facilitating the survival of small numbers of residual cells that can ultimately lead to disease relapse. This has led to the concept that disruption of leukemic cell interactions with the bone marrow microenvironment could be used to therapeutic advantage. In AML the stimulatory effects of G-CSF on

leukemic cell proliferation, which increase sensitivity to cell cycle dependent chemotherapeutic agents *in vitro* [132], could theoretically augment any effects of G-CSF on stromal mediated support deprivation through disruption of the bone marrow niche (Fig. 2). Disappointingly, several randomized clinical trials incorporating G-CSF with induction chemotherapy for AML failed to show a significant benefit in leukemia-free survival [133–136], although two studies have shown improvements in subpopulations of patients [137], most recently in patients receiving dose escalation of cytarabine [138]. However agents such as inhibitors of CXCR4 or VLA4, that mobilize AML cells from the bone marrow without having direct proliferative effects have demonstrated benefit in pre-clinical settings [131,139]. Clinical data have suggested that mobilization of AML blasts can be achieved safely in patients [140] but the benefit, if any, waits the outcome of current clinical trials. Results in chronic myeloid leukemia have been less promising with conflicting data from two studies using CXCR4 antagonists with one reporting increased CNS involvement following treatment with Plerixafor [141,142].

In contrast, B cell malignancies only rarely express the G-CSF receptor [143] and so are unable to respond directly to this cytokine. Preclinical studies targeting CXCR4 and VLA-4 have produced promising results in *in vivo* models of acute lymphoblastic leukemia (ALL) suggesting that microenvironmental disruption may be of therapeutic benefit [144,145]. While G-CSF has been given to many patients with lymphoid malignancies to facilitate chemotherapy delivery and support hematopoietic stem cell transplantation, it has not been administered prior to chemotherapy with the intention of disrupting the microenvironment for the purpose of enhancing chemo-sensitivity. A clinical trial (NCT01331590) examining the potential of priming the bone marrow with G-CSF is currently being conducted with preliminary data presented at the ASH meeting in 2013 [146]. Although the baseline bone marrow microenvironment was highly disrupted in relapsed and refractory ALL patients, CXCL12, although not IL-7 or osteocalcin mRNA was reduced in the majority of patients after 4 days of G-CSF. While there was no measurable effect on ALL blast proliferation, apoptosis was increased in response to G-CSF priming in the 3 patients tested. A pre-clinical study of human ALL in NOD/SCID gamma/$c^{-/-}$ mice presented at the previous meeting suggested that this strategy may not be beneficial for all patients, with 2 of 6 xenografts tested demonstrating increased tumor burden following 10 days of G-CSF compared to control treated animals bearing the same xenograft [147]. Whether G-CSF priming will lead to measurable increases in chemo-sensitivity awaits the outcome of clinical trials.

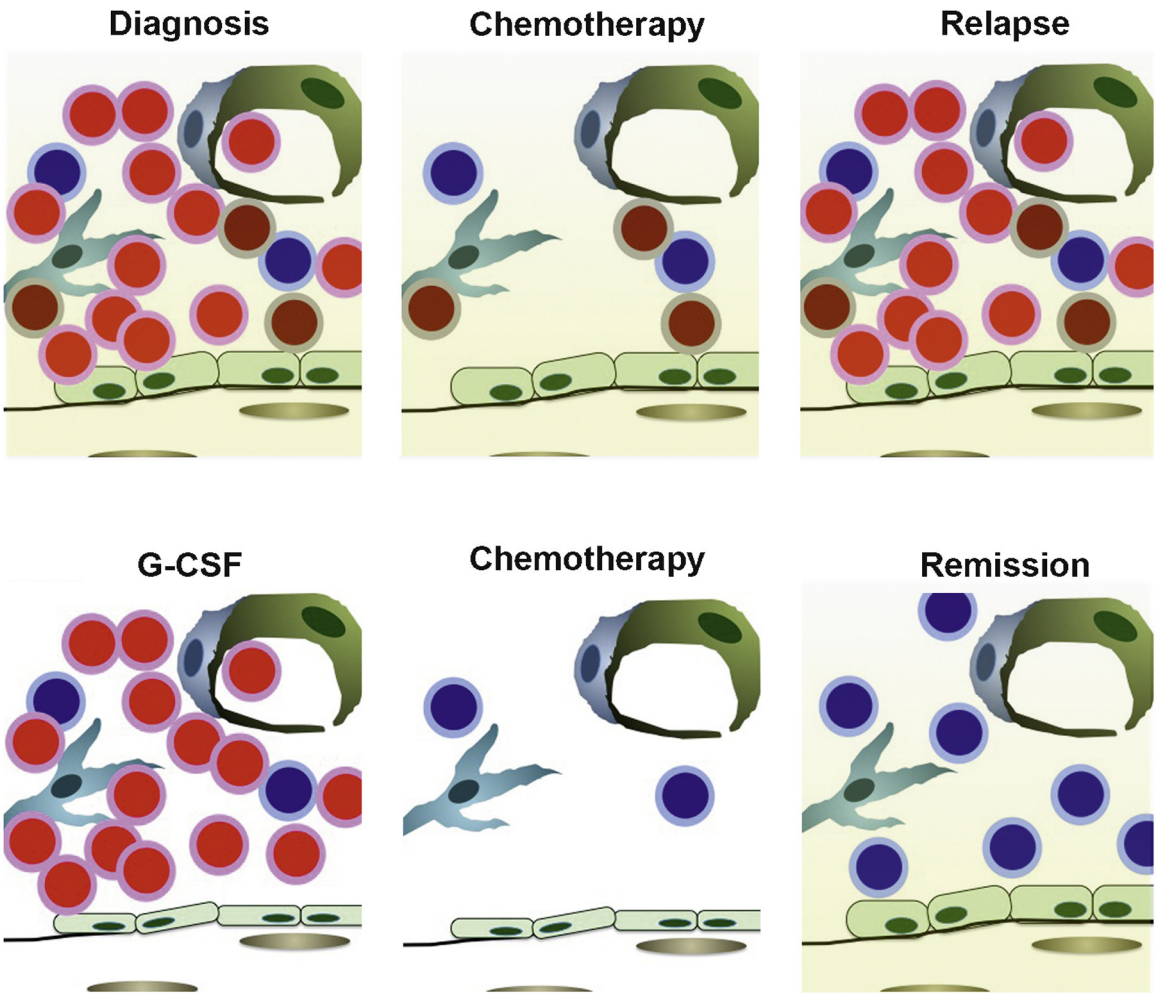


Fig. 2. The upper panel shows the response of the bone marrow chemotherapy, with proliferating AML cells (red) dying while quiescent AML cells (maroon) are resistant resulting in disease relapse. In the lower panel G-CSF reduces the niche and induces proliferation in quiescent AML cells facilitating killing by chemotherapy. Normal hematopoietic cells (purple) can then expand once chemotherapy is completed.

5. Conclusions

Today G-CSF is an established therapeutic agent routinely used for the management of neutropenia, reducing morbidity, particularly in cancer patients undergoing chemotherapy. G-CSF has also been fundamental to the transition from bone marrow to peripheral blood as the source of hematopoietic stem cells for transplantation, increasing the safety, efficiency and broadening the applicability of the procedure. While the use of G-CSF in stem cell mobilization is well established, there is scope for improvement of this procedure and new agents are emerging, hopeful of bridging the shortcomings and possibly, at least in some settings, replacing G-CSF. Looking to the future, elucidation of the mechanisms of action of G-CSF as a stem cell mobilization agent have delineated a number of potential pathways whereby disruption of the bone marrow stem cell niche may be exploited to enhance the efficacy of current treatment protocols for the leukemias. While G-CSF is clearly a well established therapeutic, its use continues to evolve, becoming more sophisticated as its application expands into new areas.

Acknowledgements

L.B. is supported by and NHMRC Senior Research Fellowship (1042305) and a Cancer Institute NSW Fellowship (12/CDF/2-03).

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