[Frontiers in Bioscience 12, 800-815, January 1, 2007]

# Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders

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## 1. ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is the major hematopoietic cytokine involved in the control of neutrophil production and thus serves as a critical regulator of the innate immunity against bacterial infections. G-CSF is applied on a routine basis in the clinic for treatment of congenital and acquired neutropenias, diseases characterized by a critical shortage of neutrophils, leading to severe opportunistic bacterial infections. Very recently, it has become clear that therapeutic application of G-CSF may not be limited to different types of neutropenia, but may extend to non-hematological conditions, in particular cardiac and brain infarctions. G-CSF drives the proliferation, survival and neutrophilic differentiation of myeloid progenitor cells by activation of a receptor of the hematopoietin receptor superfamily, which subsequently triggers multiple signaling mechanisms. These mechanisms exert positive as well as negative effects on the signaling function of the G-CSF receptor. The integrated output of these signaling pathways provide the appropriate balance needed for accurate production of neutrophils under both steady state and "emergency" conditions. Here we review how these mechanisms are thought to act in concert to meet with these demands and how perturbations in the function of the G-CSF receptor are implicated in various types of myeloid disease.

## 2. GRANULOCYTE COLONY-STIMULATING FACTOR(G-CSF)

G-CSF is a member of the cytokine class I superfamily, which is structurally characterized by four antiparallel alpha-helices (1). G-CSF controls proliferation, survival and differentiation of neutrophilic progenitor cells *in vitro* and supports the maintenance of steady-state neutrophil levels *in vivo (*2-6). G-CSF is produced by multiple cell types and activates a single transmembrane receptor ; it exerts a nonredundant role in blood cell development, as is evident from the fact that both G-CSF-deficient (*gcsf*-/-) and G-CSF-receptor-deficient (*gcsfr* -/-) mice are severely neutropenic, with blood neutrophil levels at 15-30% of those in wild type (wt) littermates. The number of myeloid progenitor cells in the bone marrow of these mice is also significantly decreased (5-8). G-CSF signaling is also required for "stress" granulopoiesis in response to bacterial infections (5,7). In addition, G-CSF enhances multiple neutrophil effector functions, such as superoxide anion generation, release of arachidonic acid and production of leukocyte alkaline phosphatase and myeloperoxidase (9,10). In the clinic, G-CSF has been used in treatment of various forms of neutropenia , and leukemia as well as anemia . A potential application for G-CSF is to reduce febrile neutropenia in cancer patients after chemotherapy, but the clinical and cost benefits of this approach have remained controversial and may be limited to restricted groups of

patients (11). G-CSF treatment has also been applied with variable results to improve the response to chemotherapy of acute myeloid leukemia (AML) patients, under the premise that leukemic stem cells and progenitor cells enter the cell cycle upon G-CSF stimulation and thereby become (more) sensitive to cytotoxic agents (12,13). Currently, there are two established niches for the application of G-CSF in the clinic. First and foremost, G-CSF is now routinely administered to patients with severe congenital or chronic neutropenia (SCN), a disease characterized by a myeloid maturation arrest in the bone marrow leading to a drastic reduction in the peripheral neutrophil level and greatly increased susceptibility to fatal opportunistic bacterial infections. G-CSF treatment ameliorates the neutropenia and associated infections in the majority of SCN patients (14-16). A second major application of G-CSF is founded on its ability to induce the release of hematopoietic stem and progenitor cells from the bone marrow into the peripheral blood. This has resulted in the widespread use of G-CSF for the mobilization and isolation of peripheral hematopoietic stem cells for transplantation purposes (17). The mechanism by which G-CSF mobilizes these cells is not yet fully understood but involves multiple effector pathways, including proteolytic enzyme release, activation of chemokine receptors, particularly CXCR4, and modulation of adhesion molecules (18,19). Notably, these effects are not necessarily direct and may involve modulatory effects on accessory cell types. For instance, production of the chemokine CXCL12 by osteoblasts, thought to play a major role in the homing of hematopoietic stem cells through the activation of CXCR4, is inhibited by G-CSF (20). G-CSF also induces mobilization of neutrophils from the bone marrow, probably in part via similar mechanisms (21). Intriguingly, recent studies have assigned a role for G-CSF in myocardial regeneration following cardiac infarction by a direct action on cardiomyocytes (22,23). In addition, it was recently shown that G-CSF inhibits programmed cell death in neuronal cells caused by acute ischemic stroke and stimulated the proliferation of neural progenitor cells, thereby reducing the volume of the brain infarct (24). This study demonstrated that neurons of the central nervous system express both G-CSF and G-CSF-R, suggesting an autocrine protective mechanism. As yet, these findings are limited to experimental rodent models, but may herald important novel therapeutic applications of G-CSF in cardiac and brain infarctions.

## 3. G-CSF RECEPTOR

The G-CSF receptor (G-CSF-R) is a member of the hematopoietin receptor superfamily (25,26), which is structurally characterized by four highly conserved cysteine residues and a tryptophan-serine repeat (WSXWS) in the extracellular domain. Both motifs are located within the so-called cytokine receptor homology (CRH) region. Murine and human G-CSF-receptors are single transmembrane proteins of 812 and 813 amino acid residues, respectively, with 62.5% homology at the amino acid level (27). The extracellular domain of the G-CSF-R contains 603 amino acid residues and includes an immunoglobulin-like module, the CRH domain, and three fibronectin type III (FNIII) modules.

The CRH domain comprises two "barrel-like" modules, each formed by seven beta strands. Similar to the CRH domains of gp130, the growth hormone (GH) receptor, and the erythropoietin receptor (Epo-R), these barrels are connected by a proline-rich linker that positions them at an approximately perpendicular angle (28). Crystallography studies of receptor/ligand complexes, epitope mapping with monoclonal antibodies and alanine scanning mutagenesis indicated that G-CSF and the G-CSF-R form a 2:2 tetrameric complex (29). It was initially proposed that this involved "pseudo-symmetric" binding of G-CSF to two sites within the CRH domain of the G-CSF-R, but it is now clear that G-CSF binds to a site within the CRH domain, via its type II binding motif, and to another site located within the Ig-like domain, via type III motif binding (30). This configuration is similar to that proposed for the IL-6/gp130 complex (28). The role of the FNIII domains in G-CSF-R function has only been studied to a limited extent. Interestingly, the second FNIII module of the G-CSF-R confers ligand-independent activation to a chimeric G-CSF-R/gp130 receptor in COS cells (31). Although this suggests that the FNIII domain may be involved in the formation of an active receptor complex, the significance of this mechanism for G-CSF-R activation under more physiological conditions remains to be established.

The intracellular domain of the G-CSF-R has only limited sequence homology to other hematopoietin receptor superfamily members. It does contain two motifs in the membrane-proximal region, called box 1 and box 2, which are also found in other members of the family, e.g., the Epo-R, gp130, and in the beta chains of the IL-2 and IL-3 receptors (32,33). Box 1 is proline rich and contains a conserved P-X-P motif. Box 2 is less conserved and comprises a cluster of hydrophobic amino acids, followed by acidic and one or 2 positively charged amino acids. The entire box1/box2 region, in particular also a conserved tryptophan residue (W650) in the in-between amino acid stretch, is essential for the transduction of proliferation signals (34). It is now well established that this region is crucial for the binding of JAK kinases to the receptor chains. The C-terminal (membrane-distal) region of the G-CSF-R has been implicated in the control of G-CSF-induced differentiation of myeloid progenitor cell lines and more recently also in the transduction of phagocytic signals in mature neutrophils (35-37). Importantly, as will be discussed later in this review, mutations have been reported in SCN patients, which result in the truncation of this C-terminal region. The cytoplasmic domain of human G-CSF-R further contains four conserved tyrosine residues, at positions 704, 729, 744 and 764 (equivalent to 703, 728, 743 and 763 in the murine G-CSF-R), which upon phosphorylation function as docking sites for multiple SH2-containing signaling proteins.

G-CSF-R expression has been demonstrated on a variety of hematopoietic cells, including myeloid progenitors, mature neutrophils, monocytes, myeloid and lymphoid leukemia cells and normal B and T lymphocytes (38-47). G-CSF-receptors are also found on nonhematopoietic tissues, for instance at the materno-fetal interface, on vascular endothelial cells, in a wide variety of

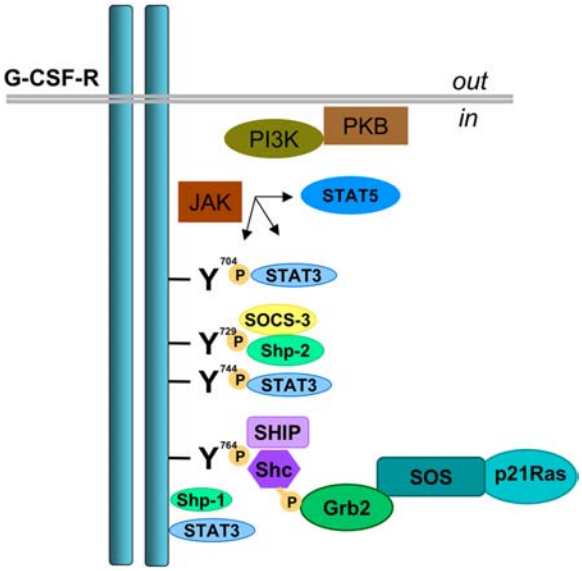



**Figure 1.** Signalling mechanisms activated by the WT G-CSF receptor. Schematic representation of the intracellular domain of the G-CSF receptor upon ligand binding. The tyrosines of the receptor become phosphorylated by Jak kinases and then function as docking sites for several signaling molecules with SH2 domains. In addition, several signaling routes are activated independent of G-CSF-R tyrosines. See main text for functional implications.

fetal organ tissues and on cardiomyocytes and neuronal progenitors (22-24,48-51). However, G-CSF-R probably plays a minimal or redundant role in embryogenesis, since newborn G-CSF-R deficient mice are normal, without any detectable abnormalities other than severe neutropenia (6,8). In addition to the wild type form of the G-CSF-R, at least 6 isoforms have been described, all of which are products of alternative mRNA splicing. The expression levels of these isoforms in bone marrow progenitor cells are low or undetectable compared to the WT G-CSF-R, suggesting that their physiological role in normal myelopoiesis is minimal. However, as will be discussed later in this review, overexpression of certain isoforms has been reported in cases of acute myeloid leukemia that result in disturbed G-CSF responses in leukemic progenitor cells (52-54).

## 4. SIGNALING PATHWAYS COUPLED TO THE G-CSF RECEPTOR

In the past decade, many of the basic principles of hematopoietin receptor signaling have been elucidated. The Jak/STAT pathways are generally seen as the pivotal signaling mechanisms of these receptors. Indeed, studies in mouse knockout models have assigned both specific and more general roles for Jaks and STATs in cellular responses to growth factors and cytokines (55-57). The Jak/STAT signaling components activated by G-CSF-R are Jak1, Jak2, Tyk2, STAT1, STAT3, and STAT5 (58-62). As is the case for most other hematopoietin receptors, the p21Ras and phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (PKB) signaling pathways are activated by the G-CSF-R, and both pathways were found to contribute to G-CSF-induced survival and proliferation (Figure 1) (8,63-66). Studies in the chicken B cell system DT40 suggested that activation of PI-3K depends on the presence of $p55^{Lyn}$. This pathway is thought to involve association of Lyn with c-Cbl, and subsequent docking of the p85 subunit of PI-3K to Y731 of Cbl (67-70). In a recent study, it was demonstrated that activation of this pathway is involved in the induction of reactive oxygen species, leading to increased proliferation signaling by G-CSF-R (71).

### 4.1. Jak/STAT pathways

Although it has been established that G-CSF activates Jak1, Jak2 and Tyk2, the specific roles of these kinases in G-CSF signaling are still not entirely clear (58-60). By employing a Jak-deficient human fibrosarcoma cell model, Shimoda *et al.* showed that Jak1, but not the other activated Jak-family members, is critical for receptor phosphorylation and STAT activation (60). In contrast, co-expression of dominant negative forms of either Jak1, Jak2 or Tyk2 with a wt G-CSF-R in COS cells completely blocked G-CSF-induced STAT5 activation in these cells (64). Moreover, Jak1-deficient mice possess normal numbers of neutrophils, which would also argue against a major and non-redundant role of Jak1 in granulopoiesis (72). Among the different STAT family members, STAT1 is only weakly and transiently activated by G-CSF and studies in STAT1-deficient mice suggest that it is redundant for granulopoiesis (73-75). In contrast, STAT3 is robustly activated by the G-CSF-R. Y704 and Y744 of the G-CSF-R are major docking sites for STAT3 (Figure 1). At low ligand concentrations, STAT3 activation depends largely on the availability of at least one of these sites (62,66,73,76). On the other hand, investigations in Ba/F3 cells and primary bone marrow cultures have established that at saturating G-CSF concentrations STAT3 can also be activated via a tyrosine-independent route. The latter mechanism requires the presence of the membrane-distal region of the G-CSF-R (77,78). Although the exact nature of this tyrosine independent route is still unclear, it was suggested that different mechanisms for STAT3 activation might be involved in the control of steady-state granulopoiesis at a low G-CSF level (mainly tyrosine-dependent) versus "emergency" granulopoiesis initiated by an increased level of G-CSF (tyrosine-independent) (77).

The question of how STAT3 contributes to G-CSF-controlled granulopoiesis has been addressed in both *in vitro* and *in vivo* models. Introduction of dominant negative (DN) forms of STAT3, which either prevent dimerization or DNA binding of STAT3 complexes, in myeloid cell lines resulted in a lack of growth arrest and a block in neutrophilic differentiation (79,80). Importantly, following forced G1 arrest, cells expressing DN-STAT3 fully regained their ability to differentiate, suggesting that STAT3 is required for cell cycle exit, a prerequisite for myeloid differentiation, but not for execution of the differentiation program itself (80,81). Studies in conditional knockout mice with selective deletion of STAT3 in hematopoietic progenitor cells showed that production of functional neutrophils *in vivo* does not require STAT3, thereby confirming the *in vitro* findings that STAT3 is not essential for neutrophil differentiation *per se*. In fact, these

conditional STAT3 knockout mice developed a neutrophilia, which was driven by a hyperproliferative response of bone marrow progenitors to G-CSF (82). STAT3 has been suggested to be not only critical for G-CSF-induced growth arrest and differentiation, but also for proliferation of myeloid progenitors (83). This conclusion was based on a mouse model expressing a truncated G-CSF-R, in which the remaining STAT3 binding site (Y704) is mutated (d715F). The d715F mice demonstrated a complete loss of STAT3 activation in response to G-CSF and were severely neutropenic. G-CSF-driven proliferation of myeloid progenitors from d715F mice in colony cultures was restored by introduction of a constitutively active form of STAT3 (STAT3C), suggesting that STAT3 activation via Y704 plays a major role in proliferative responses. This appears in contradiction with the data obtained in the conditional STAT3 deficient model. However, signaling in the d715F model is aberrant in more ways than in just its inability to activate STAT3. For instance, internalization of the truncated receptors is severely affected, thereby drastically altering both signaling abilities and duration (84). Moreover, the introduced constitutively active STAT3 protein is an oncoprotein that in addition to G-CSF signaling likely perturbs other pathways (85).

The mechanisms by which STAT3 controls cell cycle exit in myeloid progenitor cells are not fully elucidated. It has been suggested that the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ is involved in this process (80). Indeed, G-CSF induced the expression of $p27^{Kip1}$in 32D cells, which was blocked by dominant-negative forms of STAT3. Furthermore, a putative STAT3 binding site was identified in the promoter region of $p27^{Kip1}$ that was functional in both electrophoretic mobility shift assays and in luciferase reporter assays. Supporting the above conclusions, myeloid progenitors from $p27^{Kip1}$-deficient mice showed significantly increased proliferation and reduced differentiation in response to G-CSF, compared with wild-type controls. These findings suggested that STAT3 controls cell cycle arrest of myeloid cells, at least partly, via transcriptional upregulation of $p27^{Kip1}$. However transcription of $p27^{Kip1}$ is also, and more robustly, induced by transcription factors of the Forkhead family, which are negatively controlled by phosphorylation through the PI-3K/PKB pathway (86,87). Arguably, a much more prominent mechanism whereby STAT3 negatively controls G-CSF-induced proliferation is via the upregulation of the suppressor of cytokine signaling (SOCS) protein SOCS3 (see below).

As mentioned above, an entirely novel role for G-CSF in the prevention of cardiac remodeling after myocardial infarction was recently discovered (22,23). Cardiomyocytes were found to express G-CSF-R, and G-CSF activated Jak2 and Stat3 in these cells. G-CSF treatment improved cardiac function after myocardial infarction by inhibiting apoptotic death of cardiomyocytes. In addition, G-CSF reduced apoptosis of endothelial cells and increased the vascularization in the infarcted hearts. These effects were abolished by overexpression of a dominant-negative Stat3 mutant, suggesting an involvement of Stat3 target genes in promoting survival of affected cardiomyocytes (22). In another recent study, it was demonstrated that G-CSF has beneficial long-term effects also on established heart failure, by inducing hypertrophy in cardiomyocytes and by reducing myocardial fibrosis. Also in this context, G-CSF induced activation of Stat3 was implicated, although this could not yet be clearly linked to prevention of apoptosis (23).

Activation of STAT5 is mediated by the membrane proximal region of the G-CSF-R, independent of tyrosine residues (88). STAT5 recruitment to the G-CSF-R complex most likely involves direct recruitment to Jak kinases (89) (Figure 1). In contrast to STAT3, which is activated in a sustained fashion, activation of STAT5 is transient, with maximal activation levels 10 to 30 minutes after receptor activation (84,90). STAT5 has been implicated in proliferation and survival signals provided by the G-CSF-R (88). However, double-knockout mice lacking both the STAT5A and STAT5B isoforms have only moderately reduced colony numbers in response to G-CSF- and show no overt neutropenia, indicating that the role of STAT5 in steady-state granulopoiesis is limited (91). Whether STAT5 is involved in G-CSF-driven "emergency" granulopoiesis has not been established.

### 4.2. Role of the protein tyrosine phosphatase SHP-2

Generation of different mouse models demonstrated that SH2 domain-containing phosphatase 2 (SHP-2) is important for the formation of myeloid, erythroid and lymphoid cells (92-95). SHP-2 has 2 SH2 domains and a C-terminal phosphatase domain, and needs recruitment to a phosphotyrosine for its activation (96). SHP-2 has multiple functions; it is able to dephosphorylate STAT5, interacts with Jaks and is also required for efficient activation of the p21 Ras to Erk MAP kinase pathway in response to a number of stimuli (28,92,97-102). The mechanisms of SHP-2 recruitment to the G-CSF-R are not yet fully clear. Although interactions between phospho-Y704 and Y764 with SH2 domains of SHP-2 were demonstrated using Far Western technology, high affinity interaction between phospho-Y764 and SH2-SHP2 could not be detected using a Biocore biosensor (66,103). It now appears that recruitment of SHP2 is predominantly mediated via two distinct mechanisms, one involving phosphorylated Y729 and one involving the distal C-terminus of G-CSF-R (104).

### 4.3. p21Ras/MAP kinase and PI-3K/PKB pathways

Y764 of the G-CSF-R plays a major role in proliferation signaling in cell line models as well as in primary myeloid progenitor cells (8,63,78). Once phosphorylated, Y764 forms a binding site for the SH2 domains of Shc and Grb2, signaling intermediates of the p21Ras/Raf/MAPkinase pathway (Figure 1) (63,66,105,106). Grb2 can also be recruited via docking to Shc (107-110). Loss of Y764 results in a significant reduction of p21Ras activation, and accelerated neutrophil differentiation (63,106,111) and in the ability of primary myeloid progenitors to multiply *in vitro* (78). Conversely, adding back Y764 in a tyrosine "null" receptor background greatly increased the proliferation of myeloid progenitors (8). MAP kinase pathways are activated upon

phosphorylation by MAP kinase-kinases in a cascade-like manner in response to a variety of stimuli such as growth factors, cytokines or cellular stress. Depending on the cell context and type of stimulus, MAP kinase activation contributes to cell proliferation, differentiation or apoptosis. A number of studies showed that the Erk1/2 MAP kinases are the major effectors downstream from p21Ras involved in proliferative signaling in myeloid progenitor cells (78,111-117). Activation of other MAP kinases downstream of p21Ras, i.e., the p38MAP kinase and Jun N-terminal kinase (JNK) is also controlled mainly via Y764, but the role of these kinases in G-CSF signaling is less clear (103,106,114). Finally, Erk5, also known as BMK1, is also activated via the C-terminus of G-CSF-R (118). Erk5 is strongly activated by G-CSF in neuronal cells and has been implicated in promoting neuronal survival, providing a plausible signaling mechanism for the recently described neuroprotective effects of G-CSF (24,51). As is the case for most cytokine receptors, G-CSF-induced activation of the PI-3K/PKB pathway is mainly associated with stimulation of cell survival by inhibiting apoptotic cascades, which provides an additional pathway for the cell protective effects of G-CSF in mature neutrophils as well as neuronal cells (64,119).

## 5. NEGATIVE REGULATION OF G-CSF SIGNALING

The inhibition of cytokine responses is governed by multiple mechanisms including dephosphorylation of signaling molecules by phosphatases, receptor endocytosis, and proteasomal targeting. Mechanisms that have been implicated in downregulation of G-CSF signaling are discussed below.

### 5.1. Protein tyrosine phosphatase SHP-1

The role of the SH2 domain-containing protein tyrosine phosphatase SHP-1 as a negative regulator of granulopoiesis has been established utilizing so-called "moth-eaten" ($me^v$) mice, which possess a mutation in the SHP-1 gene resulting in reduced phosphatase activity (120-122). These mice exhibit aberrant regulation in several myeloid and lymphoid lineages, including substantial increases in the number of immature granulocytes (123-125). SHP-1 protein levels are increased in a post-transcriptional manner during G-CSF-induced differentiation of 32D cells. Ectopic overexpression of SHP-1 in these cells inhibited proliferation and stimulated differentiation, whereas introduction of a phosphatase-dead SHP-1 mutant gave the opposite result (126). In contrast to the Epo-R or the GM-CSF/IL-3/IL-5-R common beta chains, G-CSF-R tyrosines do not serve as docking sites for the SH2 domain of SHP-1, suggesting that intermediate signaling molecules may be involved in the recruitment of SHP-1 into the G-CSF-R complex (125-127).

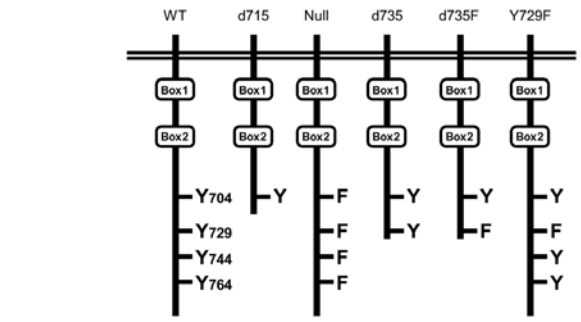
### 5.2. SH2-containing inositol phosphatase SHIP

A 145 kDa phosphorylated protein was detected following G-CSF stimulation in both Shc and in GRB2 immunoprecipitations. The formation of these complexes depended on the presence of Y764 of the G-CSF-R (Figure 1) (105). This protein was later identified as the SH2-containing inositol phosphatase (SHIP) protein (65). Studies in SHIP-deficient mice showed that this phosphatase is important for modulating hematopoietic signaling, particularly in the myeloid lineage. SHIP -/- mice die early, most likely due to the extensive infiltration of myeloid cells observed in the lungs. The numbers of neutrophils and monocytes in these mice are increased, due to an elevated number of myeloid progenitors in the bone marrow (128). Furthermore, survival of neutrophils lacking SHIP is prolonged following apoptosis-inducing stimuli or growth factor withdrawal. Finally, PI(3,4,5)P3 accumulation and PKB activation are both increased and prolonged in SHIP-/- cells. Taken together these data suggest a role for SHIP as a negative regulator of growth factor-mediated PI-3K/PKB activation and survival of myeloid cells (88).

### 5.3. Suppressor of cytokine (SOCS) proteins

Suppressor of cytokine signaling (SOCS) proteins are important mediators of negative feedback in response to many cytokines. To date, the SOCS protein family contains 8 known members: SOCS1-7 and CIS. All SOCS proteins contain an SH2-domain and a C-terminal conserved domain called the SOCS box; for a review, see (129). SOCS1 and SOCS3 have two extra conserved domains in common, the extended SH2 subdomain (ESS) and the kinase inhibitory region (KIR) (130-132). Different SOCS proteins use different mechanisms for inhibition of signaling. They can compete with positively acting signaling substrates for receptor tyrosine docking, as was demonstrated for inhibition of GH-induced STAT5 signaling by CIS (133). The second mechanism is only used by SOCS1 and SOCS3. These proteins utilize their ESS and SH2 domains for recruitment to activated Jak kinases. Subsequently, the KIR acts as a pseudosubstrate and inhibits kinase activity (130,131,134). SOCS1 directly binds to Jak kinases with high affinity (129-131,135). SOCS3 requires recruitment to phosphotyrosines in activated receptors for efficient signal inhibition (136-139), Y729 in the case of G-CSF-R (8,104,140,141). The third mechanism of inhibition of signaling by SOCS proteins involves the C-terminal SOCS box. Elongins B and C bind the SOCS box and although the exact composition of the resulting protein complex is still unclear, it is postulated to have E3 ubiquitin ligase activity (142-144). This may contribute to ubiquitination and subsequent proteasomal degradation of signaling molecules (142-145). In addition, the SOCS box has also been suggested to regulate the stability of SOCS proteins themselves, although conflicting reports exist as to whether the SOCS box contributes to SOCS protein stability or degradation (142,143,146-148).

Expression of SOCS proteins is under the direct transcriptional control of STATs (129,149-151). Among the different SOCS family members reported to be upregulated by G-CSF, SOCS3 is most prominently induced (140,152). G-CSF-induced SOCS3 expression is severely reduced in STAT3-/- mice, indeed suggesting that SOCS3 is the major STAT3 target responsible for inhibition of G-CSF signaling (82). While transcription of SOCS3 is strongly induced during G-CSF-stimulated neutrophilic differentiation, SOCS1 remains present at a relatively low and constant level (153). Thus, although both SOCS1 and SOCS3 are able to attenuate G-CSF signaling




| | | WT | d715 | Null | d735 | d735F | Y729F |
|---|---|---|---|---|---|---|---|
| STAT3 | SOCS1 | +++ | +++ | ND | +++ | | |
| | SOCS3 | +++ | ++ | ++ | +++ | | |
| STAT5 | SOCS1 | +++ | - | +++ | +++ | - | +++ |
| | SOCS3 | +++ | - | + | +++ | - | + |

**Figure 2.** Schematic representation of the intracellular domain of G-CSF receptor wild type and mutants, and their differential sensitivity to SOCS1 and SOCS3-mediated inhibition of STAT3 and STAT5 activation. Boxes 1 and 2 represent sub domains conserved in the hematopoietin superfamily. - : No inhibition; +++ : maximal inhibition; ND: not determined

under experimental conditions, SOCS3 is the most prominent physiological inhibitor (141,153). G-CSF also induces the expression of SOCS2 and CIS in hematopoietic cells, although conflicting data have been reported concerning a role of CIS in G-CSF signaling (140,152). Whereas one report suggested that CIS binds to and inhibits the signaling function of G-CSF-R, inhibitory effects of CIS were not observed in two other studies (154,141,153). Interestingly, in these latter studies, SOCS2 appeared to exert an enhancing effect on G-CSF signaling. A plausible explanation for this is that SOCS2 binds to SOCS1 and SOCS3 and mediates their proteasomal degradation (155).

Conditional knockout mice lacking SOCS3 expression in bone marrow cells show a hyperproliferative response to administration of G-CSF (156,157). In this respect, they closely resemble the knock-in mice expressing a truncated G-CSF-R (gcsfr-delta715) derived from SCN, which provides *in vivo* evidence that loss of SOCS3 recruitment via Y729 of G-CSF-R contributes significantly to the gcsfr-delta715 phenotype. Quite unexpectedly, loss of Y729 alleviated the inhibitory effects of SOCS3 on the activation of STAT5, involved in proliferation and survival signaling from G-CSF-R, but not STAT3, implicated in G-CSF-mediated growth arrest and differentiation (104). Significantly, deletion of the SOCS box largely abrogated the inhibitory action of SOCS3 on G-CSF-induced STAT activation and colony formation by bone marrow progenitors, suggesting that targeting for ubiquitination, rather than abrogation of Jak activity is the dominant inhibitory mechanism (153). How this affects the function and intracellular fate of the G-CSF-R complex is presently unclear.

Although SOCS1 and SOCS3 are prominent inhibitors of Jak/Stat signaling, they may also affect other pathways. For instance, SOCS1 inhibits c-Kit and Flt3 receptors and has been shown to associate with the tyrosine kinases Tec, Pyk2 and FGF receptor, the docking molecule Grb2, and the hematopoietic signaling protein Vav (158-161). SOCS3 has been shown to bind to the Src-like tyrosine kinase Lck, FGF receptor and Pyk2 and more recently Cacalano and colleagues reported that SOCS3 contributes to degradation of the negative regulator of Ras signaling RasGap, thereby enhancing Erk activation in response to cytokines and growth factors (135,162). How these alternative mechanisms affect G-CSF responses of myeloid progenitor cells is unknown.

### 5.4. SHP-2 versus SOCS3-mediated inhibition of G-CSF responses

G-CSF-R-Y729 is an important recruitment site for SOCS3 as well as for SHP-2 (104)(Figure 1). This is not unique for the G-CSF-R. For instance the leptin receptor and gp130, the signal transducing subunit of Oncostatin M (OSM), Leukemia inhibitory factor (LIF), IL-6 and IL-11 receptors, have combined docking sites for SOCS3 and SHP-2 (136-138,163). A number of studies have addressed the roles of negative feedback by SOCS3 and SHP-2 in gp130 and leptin signaling. Both SHP-2 and SOCS3 inhibit gp130 signaling in response to LIF and IL-6 (148,164). In contrast, OSM signaling is inhibited via Y759 of gp130, even in the absence of SOCS3 and SHP-2, implicating involvement of another yet unknown inhibitor acting either directly or indirectly via this tyrosine (165). A direct comparison of the data from these different receptors with those reported on the G-CSF-R is complicated for two reasons. Firstly, Y759 of gp130 is also involved in the activation of the Erk route via SHP-2, whereas such a role for Y729 of the G-CSF-R could not be demonstrated (166,106). Secondly, most of the cytokines that were tested in these reports do not activate STAT5 and therefore, a possible role of SHP-2 as a STAT5 phosphatase (see below) could not be tested in these models (167,168).

### 5.5. Differential effects of SOCS inhibition on G-CSF-induced STAT activation

A surprising finding was that the mechanisms by which SOCS proteins suppress G-CSF-induced activation of STAT3 and STAT5 are discrepant. This became evident by studying the effects of SOCS1 and SOCS3 on a panel of G-CSF-R mutants in luciferase reporter assays (104, van de Geijn: unpublished data) (Figure 2). The first prominent difference is seen when WT G-CSF-R and G-CSF-R-d715 are compared. Whereas the inhibitory effects of SOCS1 and SOCS3 on activation of STAT3 by G-CSF-R-d715 are preserved, inhibition of both SOCS proteins on activation of STAT5 is completely lost. This clearly indicates that, in contrast to inhibition of STAT3, SOCS-induced inhibition of STAT5 requires additional mechanisms controlled by the G-CSF-R C-terminus. Comparison of the action of

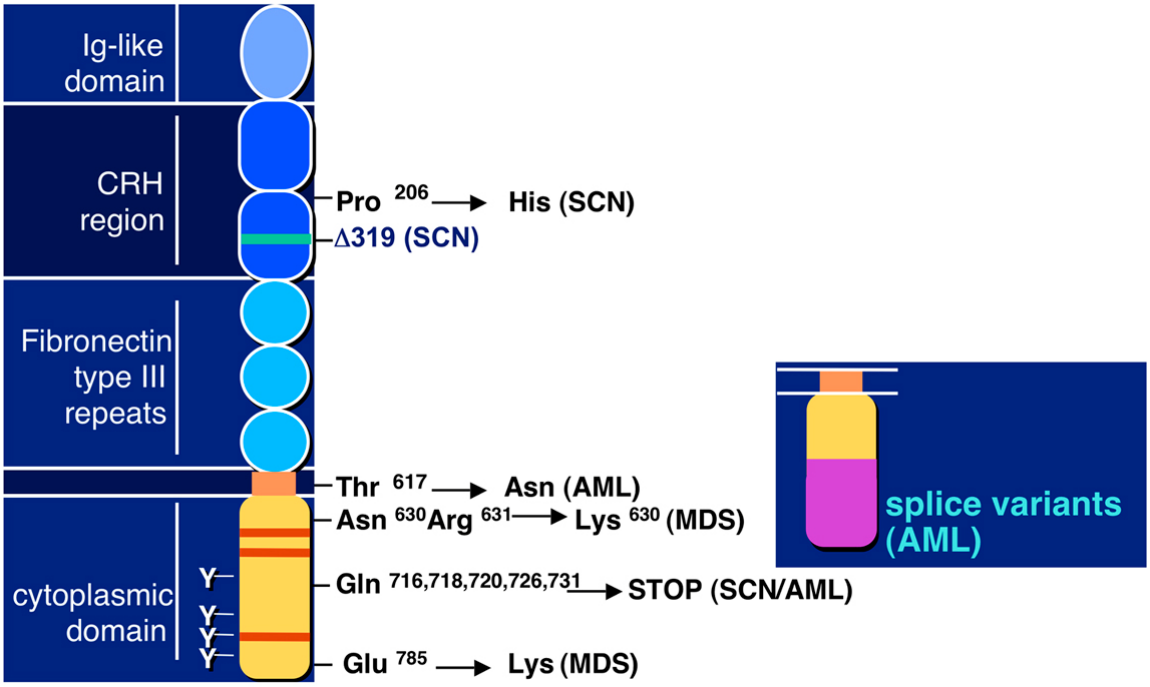



**Figure 3.** Mutations and polymorphisms in G-CSF receptor identified in myeloid disorders. Different domains within the G-CSF-R extracellular and intracellular domains are indicated. In red, conserved stretches in the intracellular domain (boxes 1-3) are indicated. AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; SCN: severe chronic neutropenia.

OCS1 and SOCS3 on more subtle G-CSF-R mutants provided some leads about the possible nature of these mechanisms.

STAT5 activation by G-CSF-R-d715 is resistant to inhibition by both SOCS1 and SOCS3. This result was expected for SOCS3, which requires recruitment to Y729 of G-CSF-R, but not for SOCS1. SOCS1 directly interacts with JAK kinases, which bind to the membrane proximal region of G-CSF-R and therefore does not involve recruitment to G-CSF-R tyrosines (34,129,135). Indeed, this is corroborated by the observation that STAT5 activation by the G-CSF-R null mutant is fully sensitive to inhibition by SOCS1. Strikingly however, Y729 becomes crucial for the effects of SOCS1 on STAT5 activation in G-CSF-R deletion mutant d735 (Figure 2). Thus even though Y729 is dispensable for SOCS1 function in the context of the full length G-CSF-R, its presence is essential for inhibition of STAT5 activity of truncated G-CSF-receptors. Although the reason for this differential requirement of Y729 for inhibition of STAT5 by SOCS1 is currently unknown, these results imply that SOCS1 itself is unable to abrogate STAT5 signaling by G-CSF-R-d735, unless combined with another mechanism projected by Y729 and/or the G-CSF-R C-terminus. STAT5 dephosphorylation by SHP-2 (which is recruited to Y729) is a candidate mechanism that could explain the requirement of Y729 in the truncated receptors (104,167,168). The finding that Y729 is not critical for the effects of SOCS1 on the null mutant could imply that the G-CSF-R C-terminus is also able to recruit SHP-2. Indeed, we were able to show that mutant d715-735, which lacks Y729 but retains the receptor C-terminus, can still bind a significant amount of SHP-2 (104).

### 5.6. Constitutive and ligand-induced internalization of G-CSF-R

The intracellular distribution and internalization kinetics of G-CSF-R in living cells was studied using fusion constructs of wild type or mutant G-CSF-R and enhanced green fluorescent protein (EGFP) (169). Under steady-state conditions the G-CSF-R localized predominantly to the Golgi apparatus, late endosomes, and lysosomes, with only low expression on the plasma membrane. Endosomal and lysosomal localization was due to slow, spontaneous internalization. Internalization of the G-CSF-R was significantly accelerated by addition of G-CSF. This ligand-induced switch from slow to rapid internalization required the presence of G-CSF-R residue Trp650, shown to be essential for JAK binding and activation (34). Both spontaneous and ligand-induced internalization depended on 2 distinct amino acid stretches in the G-CSF-R COOH-terminus, amino acids 749-755, containing a dileucine internalization motif, and amino acids 756-769. Ser749 at position –4 of the dileucine motif appeared to play a major role in the switch to rapid internalization, suggesting a possible involvement of an as yet unidentified serine/threonine kinase in this process.

## 6. G-CSF-R IN MYELOID DISORDERS

A number of mutations or rare polymorphisms in the *GCSFR* gene have been reported in myeloid disorders and these were found to perturb signaling functions of the

receptor **(**Figure 3**)**. Elucidation of the functional consequences of these abnormalities has contributed significantly to our understanding of the role of specific domains of the G-CSF-R in signaling.

G-CSF-R mutations are regularly found in severe congenital neutropenia (SCN). The most frequent mutations are nonsense mutations in a critical glutamine-rich stretch, which result in C-terminal truncation of the G-CSF-R. Clones harboring such acquired mutations are detected in the neutropenic phase of the disease in approximately 20% of patients (170,171). In some cases, affected myeloid cells arise from minority clones, originally making up only 1 to 2 % of the myeloid progenitor cell compartment. However, clones with G-CSF-R mutations become overt in more than 80% of the SCN cases upon progression to MDS and AML, suggesting that G-CSF-R truncations represent a critical step in the expansion of the (pre-) leukemic clones (172). An important question in this context is how G-CSF treatment contributes to the outgrowth of the leukemia. The Severe Chronic Neutropenia International Registry reported evolution of SCN to MDS or AML in 35 of 387 patients, with a cumulative risk of 13% after 8 years of G-CSF treatment. However, there was no apparent relationship to duration or dose of G-CSF treatment (14). The role of these truncation mutations in leukemic transformation has been analyzed in further detail in mouse models in which the nonsense mutation was introduced in the G-CSF-R gene by knock-in strategies (*gcsfr-delta715*) (173,174). Mice expressing the truncated G-CSF-R exhibit hyperproliferation of myeloid progenitor cells in reponse to G-CSF but do not develop leukemia. However, recent studies have shown that G-CSF-induced ROS production is significantly increased in cells derived from of *gcsfr-delta715* mice, a feature that was associated with increased DNA damage and leukemogenesis (71). Moreover, in a mismatch repair deficient (*Msh2-/-*) background, *gcsfr-delta715* mice developed tumors with a significantly shorter latency than *gcsfr-wt* mice (Prasher *et al.*, manuscript submitted). These findings support the role of G-CSF-R truncations in leukemic progression of SCN. Multiple signaling abnormalities have been linked with G-CSF-R truncations, including defective receptor internalization (175). This is in part due to the loss of a serine type di-leucine motif in box 3 (amino acids 749-755) and the immediate downstream sequence stretch of amino acids 756 to 769 (90,169). Mutation of this di-leucine motif results in reduced receptor endocytosis and delayed attenuation of signaling (90,169).

Due to lack of the C-terminus in G-CSF-R-d715, negative feedback by SHP-1 and SHIP is lost as well (65,127). Furthermore, activation of the PI-3K/PKB pathway is increased and STAT5 activation is drastically prolonged (64,84). Although the exact underlying molecular mechanisms remain to be elucidated, the increased STAT5/STAT3 activation ratio of the G-CSF-R-d715 is implicated in prolonged survival and proliferation of G-CSF-R-d715 cells (84). SOCS3 efficiently suppressed STAT3 and STAT5 activation by WT G-CSF-R in luciferase reporter assays. In contrast, while SOCS3 still inhibited STAT3 activation by G-CSF-R-d715, STAT5 activation was no longer affected (104) (Figure 2). This was largely due to the loss of the SOCS3 recruitment site Tyr729, with an additional minor contribution of the internalization defects of G-CSF-R-d715. Because Tyr729 is also a docking site for the protein tyrosine phosphatase SHP-2, which binds to and inactivates STAT5, a model is suggested in which the loss of recruitment of both SOCS3 and SHP-2 to the activated receptor complex determine the increased STAT5/STAT3 activation ratio and the resulting signaling abnormalities projected by truncated G-CSF-R mutants (104).

Mutations affecting the extracellular domain of G-CSF-R have only very rarely been reported in SCN (176-178). Although such anecdotal cases do not unveil a more general disease mechanism, they have given new insights in G-CSF-R function and intracellular routing. For instance, in an SCN patient who failed to respond to G-CSF treatment, a mutation in the extracellular domain of the G-CSF-R mutation was found that changed a conserved proline residue in the "hinge" motif located between the NH2- and COOH-terminal barrels of the CRH domain resulting (176). This mutation prevents the formation of 2:2 ligand/receptor complexes. Contrary to the C-terminal truncations, this mutant receptor showed drastically reduced activation of STAT5 and was severely hampered in proliferation and cell survival signaling in 32D cells, while differentiation-inducing properties were retained.

In *de novo* AML, activating mutations in receptor tyrosine kinases FLT3 and c-KIT occur in more than 25% of cases. These mutations result in ligand independent activation of the receptors and have a significant impact on disease prognosis (179). In contrast, such activating mutations have only rarely been reported for G-CSF-R. An activating mutation in the transmembrane domain of G-CSF-R was identified in 2/555 AML patients (180). This mutation confers growth factor independence on Ba/F3 cells and results in the constitutive phosphorylation of signaling substrates (Jak2, Stat3, ERK1, ERK2) as well as the receptor itself. A mutation leading to overexpression of a nonfunctional splice variant of G-CSF-R was reported in 1 out of 70 cases analyzed (54). This variant receptor has the alternative C-terminal 34 amino acids of the class IV G-CSF-R (alternatively known as D-7), linked to amino acid 682, which is just C-terminal of box-2. It thus lacks most of the functional domains, including all the tyrosine based docking motifs, which explains why it lacks most of its signaling abilities. Although so far this case appears to be unique, altered ratios of Class I (wild type)/ClassIV G-CSF-R levels have been reported in more than 50% of AML samples, which could be suggestive of a more general role for abnormal G-CSF-R function in AML (72). Significantly, even at relatively low levels of expression, the Class IV variant was reported to interfere with differentiation induction mediated via the wt G-CSF-R in 32Dcl3 cells (181).

Awaya *et al.* reported an increased occurrence in MDS of a novel splice variant of G-CSF-R with an alteration in the juxtamembrane region of the receptor

(182). Via an as yet unknown mechanism, this variant conferred increased proliferative signals in response to G-CSF compared to the wild type G-CSF-R. However, because this receptor variant is also found at low frequencies (2%) in normal bone marrow cells and is still only detectable in less than 8% of the myeloid progenitor cells in MDS, its role in the pathogenesis of this disease remains uncertain. A second polymorphism was recently reported to be associated with the development of high risk MDS (183). This single nucleotide polymorphism results in a substitution of glutamic acid (Glu) by lysine (Lys) at amino acid position 785 (G-CSF-R_785Lys). Glu785 is located in a conserved amino acid stretch in the most distal part of G-CSF-R C-terminus (Gln-Glu-Asp-Asp-Cys-Val-Phe-Gly-Pro), which has not previously been implicated in signaling or in receptor internalization. Intriguingly, contrary to the G-CSF-R truncation mutants in SCN/AML, G-CSF-R_785Lys shows reduced instead of increased proliferation signaling in primary hematopoietic progenitor cells (183). How the glutamic acid to lysine substitution affects the signaling function and intracellular fate of the G-CSF-R is unknown. Conceivably, Lys785 serves as a target for protein modification by ubiquitination and thus could be involved in lysosomal or proteasomal routing of G-CSF-R. Alternatively, ubiquitinated Lys785 may form a binding site for proteins with a modular ubiquitin-binding domains and recruit alternative signaling mechanisms to the G-CSF-R (184).

## 7. PERSPECTIVE

During the last one and a half decade, G-CSF has become an established therapeutic for patients suffering from neutropenia. In addition, G-CSF is now routinely used to mobilize hematopoietic stem cells into the peripheral blood of healthy donors, so that the stem cells can be harvested conveniently by apheresis for transplantation purposes. Moreover, recent studies suggest that G-CSF may have therapeutic applications that go beyond the hematopoietic system and may e.g., perhaps be beneficial for patients suffering from stroke or heart attack. Finally, abnormal responses to G-CSF due to G-CSF-R dysfunction are implicated in (pre-)malignant myeloid diseases. These major clinical aspects will undoubtedly maintain a lively interest in the signaling properties of the G-CSF-R. Important questions that still need to be addressed relate, e.g., to how the kinetics of receptor routing to early and late endosomes affect signaling, the role of receptor ubiquitylation herein, and how this influences the balanced output underlying the appropriate stimulation of proliferation and differentiation. Another major challenge is to investigate the entire physical complexity of the G-CSF-R complex and possible heterologous interactions with other receptors, e.g., via scaffolding structures. This will not only be of interest from a biological/biochemical point of view, but may also lead to further advances in therapy development of hematological and perhaps other types of disease.

## 8. ACKNOWLEDGMENT

Supported by the Dutch Cancer Society “KWF kankerbestrijding”.

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**Key Words:** Granulocyte Colony Stimulating Factor, G-CSF, Granulopoiesis, Signal Transduction, Neutropenia, Review

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