Suppressors Of Cytokine Signaling in G-CSF-induced neutrophil development

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Suppressors Of Cytokine Signaling (SOCS) in G-CSF-induced neutrophil development

Suppressors Of Cytokine Signaling (SOCS) in
G-CSF-geïnduceerde ontwikkeling van neutrofiele granulocyten

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Voor mijn ouders

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CHAPTER 1

Introduction

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Granulocyte colony-stimulating factor and its receptor in normal hematopoietic cell development and myeloid disease

Van De Geijn GJ, Aarts LH, Erkeland SJ, Prasher JM, Touw IP

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Hematopoiesis

The formation of blood cells, hematopoiesis, is a tightly regulated, yet flexible, process that ensures appropriate levels of the different blood cell types. In addition, during stress situations such as bleeding or infection, blood cell development rapidly adapts to accomodate the extra demand for cells of specific subtypes. All blood cells are derived from the pluripotent hematopoietic stem cell (HSC), which gives rise to multipotent progenitor cells. The progenitor cell compartiment has a vast proliferative potential that ensures generation of sufficient blood cells out of relatively few and rarely dividing HSCs. Multipotent progenitor cells differentiate into committed progenitors that ultimately give rise to each of the different blood cell lineages: red blood cells, platelets, lymphocytes, macrophages, dendritic cells, natural killer cells and granulocytes.

Granulocytes are essential for a good functioning first line of defence against infections. Granulocytes contain cytoplasmic granules and are classified based on cell morphology and staining of the granules as eosinophilic, basophilic or neutrophilic granulocytes. The latter, also known as neutrophils, are the most common type of granulocytes, and take care of phagocytosis and destruction of invading microorganisms, using amongst others myeloperoxidase, acid hydrolases, collagenase, elastase, lactoferrin and lysozyme, present in their granules. Neutrophils have a life span limited to approximately 4-5 days. They are either destroyed during defensive action or become senescent and die (1). An average adult human produces an estimated 50 million neutrophils per minute, which can be increased drastically during infection (2). This process is, like all hematopoiesis, controlled by signals provided by the stromal environment in the bone marrow and by cytokines and hematopoietic growth factors (HGF).

Hematopoietic growth factors

The cloning and functional characterization of hematopoietic growth factors (HGF) and their cell surface receptors represent milestones in understanding the molecular control of blood cell development (3-9). In addition, these developments have had a profound impact on clinical hematology, most notably through the introduction of HGF-based therapies. HGFs are produced by stromal and endothelial cells, fibroblasts, monocytes and macrophages and specialized cells in organs such as the kidney and the liver. The range of action of HGFs

varies considerably. So called early-acting HGFs act only on pluripotent progenitors whereas late-acting factors stimulate proliferation and terminal differentiation of more committed cells. Moreover, some HGFs are restricted to a specific lineage while others are active throughout the hematopoietic system. The most important HGF that drives formation of neutrophils is granulocyte colony-stimulating factor (G-CSF).

G-CSF

G-CSF is a member of the cytokine class I superfamily, structurally characterized by four antiparallel α-helices (10). G-CSF supports proliferation, survival and differentiation of neutrophilic progenitor cells in vitro and provides nonredundant signals for maintenance of steady-state neutrophil levels in vivo (11-15). Typically, G-CSF-deficient (*gcsf*-/-) or G-CSF-receptor-deficient (*gcsfr* -/-) mice manifest a selective neutropenia, 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 (14-16). Experiments with G-CSF- or G-CSF-R-deficient mice infected with *Listeria monocytogenes* have established that G-CSF signaling is also required for "reactive" or "emergency" granulopoiesis in response to bacterial infections (15, 17). In addition, G-CSF enhances neutrophil effector functions, such as superoxide anion generation, release of arachidonic acid and production of leukocyte alkaline phosphatase and myeloperoxidase by mature neutrophils (18-20).

The clinical application of G-CSF has been particularly beneficial in treatment of various forms of neutropenia. For example, this is the case for severe congenital 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 susceptibility to opportunistic bacterial infections that can be fatal. G-CSF treatment ameliorates the neutropenia and associated infections in a large majority of cases (21-23). Recently, G-CSF-treatment has been shown to improve the response to chemotherapy of standard-risk AML patients (24). Another major and initially unexpected benefit of G-CSF is its ability to induce the release of hematopoietic stem and progenitor cells from the bone marrow into the peripheral blood. This has resulted in utilization of G-CSF in the mobilization and isolation of peripheral hematopoietic stem cells for transplantation purposes (25). The mechanism by which G-CSF mobilizes these cells into the periphery is

not fully understood but is thought to involve multiple effector pathways, including proteolytic enzyme release, activation of chemokine receptors, and modulation of adhesion molecules (26, 27). G-CSF also induces mobilization of neutrophils from the bone marrow, probably via similar mechanisms (28).

G-CSF receptor

The G-CSF receptor (G-CSF-R) is a member of the now well-characterized hematopoietin receptor superfamily (29, 30). This family 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 (31). 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 (Figure 1A).

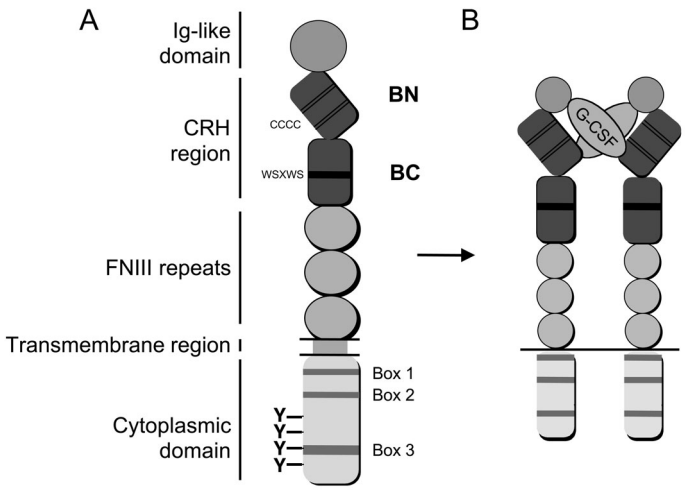

Figure 1: G-CSF receptor structure and ligand binding. A) The extracellular domain of the G-CSF receptor consists of an immunoglobin (Ig)-like domain, the cytokine receptor homology (CRH) domain with its N- and C-terminal barrel (BN and BC), containing four cysteine residues and a WSXWS motif, and three fibronectin type III (FNIII) modules. The cytoplasmic region contains three boxes that are conserved in some members of the hematopoietin receptor superfamily and four tyrosine residues. B) Upon ligand binding, G-CSF and the G-CSF-R form a 2:2 tetrameric complex. G-CSF binds with its type II binding motif to the CRH domain and with its type III motif to the Ig-like domain.

The CRH domain is composed of two "barrel-like" modules, each formed by seven β 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 (32). Crystallography studies of receptor/ligand complexes, epitope mapping with monoclonal antibodies and alanine scanning mutagenesis have provided detailed insight into the composition of these complexes and the contact sites involved in ligand recognition. These data suggest that G-CSF and the G-CSF-R form a 2:2 tetrameric complex (33) (Figure 1B). Although 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, it now appears more likely that G-CSF binds to one site within the CRH domain, via its type II binding motif, and to one site within the Ig-like domain, via type III motif binding (34). This configuration is similar to that found in the IL-6/gp130 complex (32).

The role of the FNIII domains in G-CSF-R function is not clear. 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 (35). 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 limited sequence homology to other hematopoietin receptor superfamily members. However, it does possess two motifs in the membrane-proximal region, called box 1 and box 2, which are also found in the Epo-R, gp130, and in the β chains of the IL-2 and IL-3 receptors (36, 37). This membrane-proximal region is essential for the transduction of proliferation signals (38). The C-terminal (membrane-distal) region of the G-CSF-R contains a third conserved motif (box 3) that is shared only with gp130 (39, 40). This region 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 (41-43). Importantly, as will be discussed later, mutations have been reported in severe congenital neutropenia (SCN) patients that 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 function as docking sites for multiple SH2-containing signaling proteins.

The role of the G-CSF-R tyrosines has been evaluated in different myeloid cell line models but their contributions to G-CSF-induced colony formation and the clonogenic capacity of primary bone marrow progenitors remains unclear. In Chapter 2 of this thesis these questions are addressed and both the collective and individual roles of the G-CSF-R tyrosines were studied.

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 (44-53). G-CSF-R expression has also been detected in nonhematopoietic tissues, for instance at the materno-fetal interface and on vascular endothelial cells and in a wide variety of fetal organ tissues (54-56). The G-CSF-R probably plays minimal or redundant roles in embryonic development, since newborn G-CSF-R deficient mice are normal, without any detectable abnormalities other than severe neutropenia (14, 16). In addition to the WT 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, overexpression of certain isoforms has been reported in cases of acute myeloid leukemia that result in disturbed G-CSF responses in leukemic progenitor cells (7, 31, 57).

Signaling pathways coupled to the G-CSF receptor

In the past decade, the basic principles of hematopoietin receptor signaling have been elucidated. The canonical JAK/STAT pathways are generally seen as the pivotal signaling mechanisms of these receptors. Indeed, studies in mouse knockout models have established specific as well as more general roles for JAKs and STATs in cellular responses to growth factors and cytokines (58-60). The JAK/STAT signaling components activated by G-CSF-R are JAK1, JAK2, Tyk2, STAT1, STAT3, and STAT5 (61-65).

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 2)(16, 66-69). Studies in the chicken B cell system DT40 suggested that activation of PI-3K depends on the presence of p55Lyn. 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 (70-73).

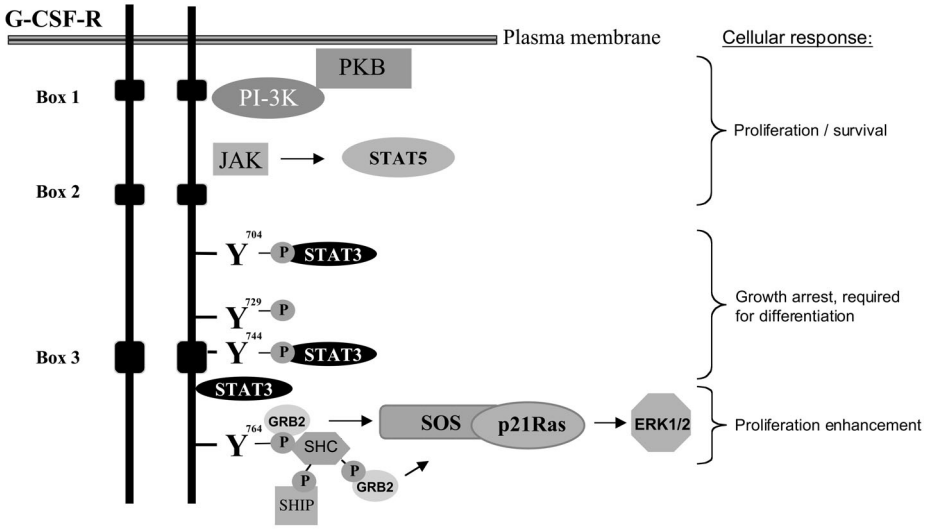

Figure 2: Signaling mechanisms activated by the WT G-CSF receptor. Schematic representation of the intracellular domain of the G-CSF receptor upon ligand binding. The tyrosines of the receptor become phosphorylated and function as docking sites for signaling molecules. In addition, several signaling routes are activated independent of G-CSF-R tyrosines.

JAK/STAT pathways

Although it has been firmly established that G-CSF activates JAK1, JAK2 and Tyk2, the specific roles of these kinases in G-CSF signaling are not clear (61-63). 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 (63). 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 (67). Moreover, JAK1-deficient mice possess normal numbers of neutrophils, which would also argue against a major and non-redundant role of JAK1 in granulopoiesis (74). Clearly, studies in appropriate hematopoietic cell models,

lacking each of the JAK family members activated by the G-CSF-R, are needed to resolve this issue.

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 (75-77). 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 2). At low ligand concentrations, STAT3 activation depends largely on the availability of at least one of these sites (65, 69, 75, 78). In contrast, investigations in Ba/F3 cells, and more recently in 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 (79, 80). Although the exact nature of this tyrosine independent route is still unclear, this observation has led to the idea 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) (79).

The question of how STAT3 contributes to G-CSF-controlled granulopoiesis has been addressed quite extensively 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 (81, 82). 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 (82, 83). 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 (84).

McLemore et al. suggested that STAT3 is not only critical for G-CSF-induced growth arrest and differentiation, but also for proliferation of myeloid

progenitors, which appears partly in conflict with the data obtained in the conditional STAT3 knockout and in the cell line models (85). They based this conclusion 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 almost completely restored by introduction of a constitutively active form of STAT3 (STAT3C). This suggests that STAT3 activation via Y704 plays a major role in proliferative responses. A possible explanation for the phenotypic differences between STAT3-/- and d715F mice is that in the latter model G-CSF signaling is aberrant in more ways than in just its inability to activate STAT3. For instance, internalization of the truncated receptors is severely hampered and signaling abilities and signal duration are quite drastically altered compared to the WT G-CSF-R (see below)(86). Additionally, the constitutively active STAT3 protein is an oncoprotein that may perturb multiple signaling mechanisms and thus synergize with G-CSF in evoking proliferative responses (87). The combination of the truncated G-CSF-R with the constitutively active STAT3 in the study by McLemore et al. might therefore overestimate the role of STAT3 in normal granulopoiesis.

The mechanisms by which STAT3 contributes to cell cycle exit in myeloid progenitor cells remain unclear. The cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$ has been proposed to play a role in this process (82). G-CSF induces expression of $p27^{Kip1}$ in 32D cells. Dominant-negative forms of STAT3 completely block this G-CSF-induced $p27^{Kip1}$ expression. 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. Finally, myeloid progenitors from $p27^{Kip1}$-deficient mice showed significantly increased proliferation and reduced differentiation in response to G-CSF, compared with wild-type controls. Taken together, these findings suggested that STAT3 controls cell cycle arrest of myeloid cells, at least partly, via transcriptional upregulation of $p27^{Kip1}$. It is important to note however that transcription of $p27^{Kip1}$ is also, and arguably more robustly, induced by transcription factors of the Forkhead family, which are negatively controlled by phosphorylation through the PI-3K/PKB pathway (88, 89). Interestingly, recent studies in HepG2 cells indicate that one of these factors, FKHR, acts as a

coactivator of STAT3 in IL-6 induced transcriptional activity (90). Whether this also applies to G-CSF-induced upregulation of $p27^{Kip1}$ remains to be addressed.

STAT5 activation is mediated by the membrane proximal region of the G-CSF-R, independent of tyrosine residues (91). Although the exact mechanism of activation is still unknown, this may involve direct recruitment to JAK kinases (92) (Figure 2). STAT5 has been implicated in proliferation and survival signals provided by the G-CSF-R (91). The role of STAT5 in steady-state granulopoiesis appears limited, as double-knockout mice lacking both the STAT5A and STAT5B isoforms have only moderately reduced numbers of CFU-G and no overt neutropenia (93). Whether STAT5 is involved in G-CSF-driven emergency granulopoiesis has not been established. Irrespective of its role in nonmalignant granulopoiesis, STAT5 may be a crucial player in the pathogenesis of myeloid malignancies. For instance, the transforming abilities of the Tel-JAK2 fusion protein, a hallmark of a specific subset of myeloid leukemia, depend entirely on the presence of STAT5 (94).

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 (95-98). SHP-2 has 2 SH2 domains and a C-terminal phosphatase domain, and needs recruitment to a phosphotyrosine for its activation (99). Despite its apparently crucial role in development of multiple blood cell lineages, the mechanisms of SHP-2 action are poorly understood. 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 (95, 100-106). The mechanisms of SHP-2 recruitment to the G-CSF-R are not clear yet. Whereas Ward et al. reported binding of Y704 and Y764 to the SH2 domains of SHP-2, using Far Western technology, others were unable to detect the latter interaction (69, 107).

The role of SHP-2 in G-CSF-R signaling is still not understood. In the experiments in Chapter 4 we investigated which region of the G-CSF-R is required for SHP-2 recruitment and for the formation of a SHP-2-STAT5 complex that may be involved in downregulation of G-CSF-induced STAT5 activity.

p21Ras/MAP kinase 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 (66, 80). Once phosphorylated, Y764 forms a binding site for the SH2 domains of SHC and GRB2, signaling intermediates of the p21Ras pathway (66, 69, 108, 109). GRB2 can also be recruited via docking to SHC (110-113). Loss of Y764 results in a significant reduction of p21Ras activation, and accelerated neutrophil differentiation (66, 109, 114). Interestingly, SCN-derived G-CSF-R truncation mutants that lack the receptor C-terminus gain the ability of p21Ras activation by an alternative mechanism, probably involving Y704 of the G-CSF-R (66, 69).

A well-known signaling cascade downstream of p21Ras signaling is the Raf-MEK-ERK Mitogen-activated protein kinase (MAP kinase) signaling pathway. MAP kinase pathways are activated upon phosphorylation by MAP kinase-kinases in a cascade like manner in response to stimuli such as growth factors, cytokines or in response to cellular stress. Activation can, depending on the cellular context and stimulus, result in induction of proliferation, differentiation or apoptosis. In addition to ERK, a number of other MAP kinases have been identified, of which p38 and JNK are best characterized to date. A number of studies showed that the ERK MAP kinase cascade is the major effector pathway downstream from p21Ras responsible for proliferative signaling in cell lines as well as in primary myeloid progenitor cells (80, 114-120). 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 still unclear (107, 109, 117).

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.

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 (121, 122), which possess a mutation in the SHP-1 gene

resulting in reduced phosphatase activity (123). These mice exhibit aberrant regulation in several myeloid and lymphoid lineages, including substantial increases in the number of immature granulocytes (124-126). 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 (127). In contrast to the Epo-R or the GM-CSF/IL-3/IL-5-R common β 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 (126-128).

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 (108). This protein was later identified as the SH2-containing inositol phosphatase (SHIP) protein (68). 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, which is due to an elevated number of myeloid progenitors in the bone marrow (129). 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 (130).

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 (131). SOCS1 and SOCS3 have two extra conserved domains in common; the extended SH2 subdomain (ESS) and the kinase inhibitory region (KIR) (132-134). Different SOCS proteins use different

mechanisms for inhibition of signaling (Figure 3). They can compete with positively acting signaling substrates for receptor tyrosine docking, as was demonstrated for inhibition of GH-induced STAT5 signaling by CIS (135). The second mechanism is only used by SOCS1 and SOCS3: they utilize their ESS and SH2 domains for recruitment to activated JAK kinases. Subsequently, the KIR acts as a pseudosubstrate and inhibits kinase activity (132, 133, 136). SOCS1 directly binds to JAK kinases (132, 133, 137, 138) whereas SOCS3 requires recruitment to phosphotyrosines in activated receptors for efficient signal inhibition (139-142). 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 (143-145). This may contribute to ubiquitination and subsequent proteasomal degradation of signaling molecules (143-146). In addition, the SOCS box also regulates the stability of SOCS proteins themselves. However, the exact role of elongin binding to the SOCS box remains to be elucidated since there are conflicting reports on whether the SOCS box contributes to SOCS protein stability or degradation (143, 144, 147-149).

Expression of SOCS proteins is under the direct transcriptional control of STATs (137, 150-152). It is thus conceivable that SOCS proteins are also involved in downmodulation of G-CSF signaling as a direct consequence of activation of STAT5 and the robust and sustained activation of STAT3 by G-CSF. Among the different SOCS family members reported to be upregulated by G-CSF, SOCS3 is most prominently induced (153, 154). 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 (84). SOCS3-deficient mice demonstrated a prominent role for SOCS3 in regulating placental development and signaling by leukemia inhibiting factor (LIF) (155-157).

Which members of the SOCS protein family inhibit G-CSF-induced activation of STAT3, STAT5 and G-CSF-induced colony formation by primary myeloid progenitors is unknown. In addition, which SOCS proteins are expressed during G-CSF-induced neutrophilic differentiation is unclear. The role of the SOCS box in inhibition of G-CSF-R signaling has not been established as well. These questions are addressed in Chapter 3 of this thesis.

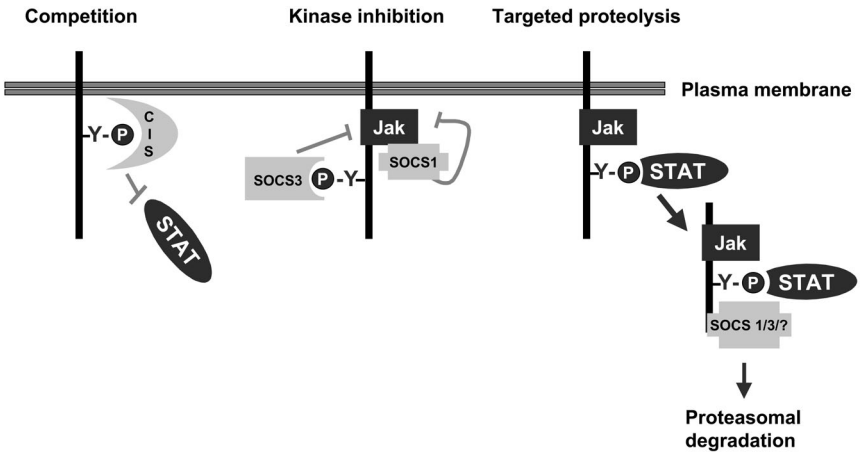

Figure 3: Different mechanisms of SOCS-mediated inhibition of JAK-STAT signaling. Members of the SOCS protein family utilize different mechanisms to inhibit signaling of cytokine receptors as shown in this schematic representation. CIS competes with STATs for recruitment to phosphorylated tyrosine residues in cytokine receptors. SOCS1 and SOCS3 are capable to inhibit JAK kinase activity using their kinase inhibitory region (KIR). SOCS1 is recruited directly to activated JAK whereas SOCS3 has a lower affinity for direct interaction with JAK and therefore is recruited via phosphorylated tyrosines in cytokine receptors. The third mechanism involved targeting of signaling molecules for proteasomal degradation, which is mediated through the C-terminal conserved SOCS box.

G-CSF receptor defects 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. Mutations, rarely found in myelodysplasia (MDS) and de novo AML, occur most frequently in SCN (but not in cyclic or idiopathic neutropenia). Elucidation of the functional consequences of these abnormalities has contributed to our understanding of the role of specific domains of the G-CSF-R in signaling.

Specific mutations in the *GCSFR* gene have thus far not been reported in MDS and AML without a history of SCN. On the other hand, Awaya et al. found an increased occurrence of a novel splice variant of G-CSF-R with an alteration in the juxtamembrane region of the receptor (158). 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 MDS remains uncertain.

In de novo AML, activating mutations in receptor tyrosine kinases FLT3 and c-kit occur in more than 25% of cases and have a significant impact on disease prognosis (159-163). In contrast, mutations in hematopoietin receptors, including G-CSF-R, have only very rarely been detected. A mutation leading to overexpression of a nonfunctional splice variant of G-CSF-R was reported in 1 out of 70 cases analyzed (57). 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 this case so far appears to be unique, altered ratios of Class I(WT)/Class IV 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 (164). 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 (165).

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 NH_2- and COOH-terminal barrels of the CRH domain resulting (166). This was proposed to prevent 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.

The most frequent mutations found in SCN 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 (167, 168). 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 (169). An important question in this context is how G-CSF treatment contributes to the outgrowth of the leukemia. In a recent update from the Severe Chronic Neutropenia International Registry, evolution of SCN to MDS or AML was reported in 35 of 387 patients with congenital neutropenia with a cumulative risk of 13% after 8 years of G-CSF treatment, but there was no apparent relationship to duration or dose of G-CSF treatment (170).

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 *GCSFR* gene by knock-in strategies (G-CSF-R-d715) (171, 172). Although insufficient to cause leukemia themselves, these mutations were recently found to cooperate with additional oncogenic hits, such as loss of the DNA repair protein MSH2, to accelerate tumorigenesis. Interestingly, preliminary results also suggest an association between loss of MSH2 function and G-CSF-R mutations in SCN patients at high risk for AML/MDS progression supporting this murine data (Prasher et al., manuscript in preparation). Mice expressing the truncated G-CSF-R exhibit hyperproliferation of myeloid progenitor cells in reponse to G-CSF (171, 172). Multiple signaling abnormalities have been linked with this hyperproliferation, including defective receptor internalization (173). 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 (174, 175). Mutation of this di-leucine motif reduced receptor endocytosis and delayed the attenuation of signaling (174, 175).

Due to lack of the C-terminus in G-CSF-R-d715, negative feedback by SHP-1 and SHIP is lost as well (68, 128). Furthermore, activation of the PI-3K/PKB pathway is increased and STAT5 activation is drastically prolonged (67, 86). 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 (86).

It is possible is that lack of negative feedback by SOCS proteins contributes to the hyper responsive phenotype of G-CSF-R-d715. Due to the aberrant activation of STAT3 and STAT5 the induction of SOCS expression by G-CSF-R-d715 may be altered as well. In addition SOCS proteins may differentially affect STAT3 and STAT5 activity of G-CSF-R-d715. These issues were examined as described in Chapter 4 of this thesis.

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CHAPTER 2

Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells

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Abstract

Granulocyte colony-stimulating factor (G-CSF) is the major regulator of neutrophil production. Studies in cell lines have established that conserved tyrosines Y704, Y729, Y744 and Y764 within the cytoplasmic domain of G-CSF receptor (G-CSF-R) contribute significantly to G-CSF-induced proliferation, differentiation and cell survival. However, it is unclear whether these tyrosines are equally important under more physiological conditions. Here, we investigated how individual G-CSF-R tyrosines affect G-CSF responses of primary myeloid progenitors. We generated G-CSF-R-deficient mice and transduced their bone marrow cells with tyrosine "null" mutant (mNull), single tyrosine "add back" mutants or wild type (WT) receptors. G-CSF-induced responses were determined in primary colony assays, serial replatings and suspension cultures. We show that removal of all tyrosines had no major influence on primary colony growth. However, adding back Y764 strongly enhanced proliferative responses, which was reverted by inhibition of ERK activitity. Y729, which we found to be associated with the suppressor of cytokine signaling SOCS3, had a negative effect on colony formation. After repetitive replatings, the clonogenic capacities of cells expressing mNull gradually dropped compared to WT. The presence of Y729, but also Y704 and Y744, both involved in activation of STAT3, further reduced replating efficiencies. Conversely, Y764 greatly elevated the clonogenic abilities of myeloid progenitors, resulting in a more than 10^4-fold increase of colony forming cells over mNull after the fifth replating. These findings suggest that tyrosines in the cytoplasmic domain of G-CSF-R, although dispensable for G-CSF-induced colony growth, recruit signaling mechanisms that regulate the maintenance and outgrowth of myeloid progenitor cells.

Introduction

Granulocyte colony stimulating factor (G-CSF) supports the proliferation, survival and differentiation of neutrophilic progenitor cells (1-4). G-CSF deficient mice manifest a selective neutropenia, with blood neutrophil levels at 30% of those in wild type (WT) mice. Blood neutrophil levels in mice lacking G-CSF receptors (*gcsfr* -/-) are also severely reduced, i.e., approximately 15% of WT littermates (5). In addition, the numbers of myeloid progenitor cells in the bone marrow of *gcsfr* -/- mice are significantly decreased (5). These observations have established that the G-CSF-R provides nonredundant signals for maintaining steady-state neutrophil levels (5, 6).

The G-CSF-R belongs to the cytokine receptor superfamily and possesses a single transmembrane region (1). Signaling molecules downstream of the G-CSF-R include JAK1, JAK2 and Tyk2, the signal transducer and activator of transcription (STAT) proteins, STAT1, STAT3 and STAT5 (7-13), the Src kinases p55Lyn and p56/59Hck (14-16), components of the p21Ras/Raf/MAPK pathway (17-19) and the (SH2) domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 (19-21). The cytoplasmic domain of human G-CSF-R contains four conserved tyrosine residues, at positions 704, 729, 744 and 764 (equivalent to 703, 728, 743 and 763 in mouse G-CSF-R). Three of these tyrosines are located in the carboxy terminal region implicated in the control of differentiation (22, 23). Upon receptor activation, these tyrosines are phosphorylated and become docking sites for multiple SH2-containing signaling proteins, e.g., STAT3 (Y704 and Y744), Shc (Y764) and GRB2 (Y764) (21, 24, 25).

We previously constructed a series of tyrosine (Y) to phenylalanine (F) substitution mutants of the G-CSF-R and expressed these in 32D cells to study their involvement in G-CSF signaling (25-27). These studies demonstrated that G-CSF-R substitution mutants lacking just one of the four tyrosines were still fully capable of transmitting differentiation signals in 32D cells (24). Strikingly, cells expressing mutant Y764F showed significantly accelerated differentiation with a concomitant reduction in proliferation, suggesting that Y764 plays an essential role in controlling the balance between proliferation and differentiation. Recently, similar observations have been made in primary bone marrow cells transduced with chimeric EGFR/G-CSF-R Y764F (28). G-CSF-R lacking all tyrosines (mNull) fails to elicit proliferation and differentiation in 32D cells,

although survival signals are still transduced (25). The presence of Y704 or Y744, which serve as major docking sites for STAT3, restored G-CSF-induced proliferation and differentiation to a significant extent (13, 25). Introduction of Y764, involved in p21Ras activation and signaling via ERK and p38MAPK pathways (19, 24, 25, 29), generated strong proliferative signals resulting in exponential growth without neutrophilic differentiation. A specific function and signaling mechanism linked to Y729 did not emerge from these studies.

The mechanisms by which G-CSF signaling is negatively regulated have not been elucidated. In contrast to, for example, the Epo receptor or GM-CSF/IL-3/IL-5 receptor common β chain, G-CSF-R tyrosines do not serve as docking sites for the protein tyrosine phosphatase SHP-1, although negative effects of SHP-1 on G-CSF signaling have been reported (20, 30, 31). Because STAT3 and STAT5 are prominently activated by G-CSF, it is conceivable that suppressor of cytokine signaling (SOCS) proteins, which are under the direct transcriptional control of STATs, are involved in downmodulation of G-CSF responses (32-34). The SH2 domain of SOCS1 has a high affinity for JAK kinases and interferes directly with JAK activity (35). On the other hand, other SOCS proteins, such as SOCS3, are recruited to phosphotyrosines in activated receptors and exert their negative activity either by blocking positively acting signaling substrates docking to the same receptor tyrosine, by inhibiting the activity of receptor associated kinases or by proteosomal targeting of signaling molecules (32-34).

Although myeloid cell lines have provided useful models for studying G-CSF signaling, these cells are transformed and immortalized and therefore do not fully recapitulate the physiological features of normal myeloid progenitor cells. In the present study, we have employed retroviral transduction of G-CSF-R mutants into bone marrow cells of G-CSF-R deficient mice to investigate how signals emanating from the cytoplasmic tyrosine residues in the G-CSF-R contribute to the clonogenic abilities of primary murine myeloid progenitor cells. We show that tyrosines are dispensable for G-CSF-induced colony formation per se, but individually contribute significantly to both G-CSF-induced colony growth and the maintenance of clonogenicity after sequential replatings. Prominent negative regulatory effects on colony growth were projected by Y729, which we found to be associated with recruitment of SOCS3. Conversely, Y764 greatly enhanced proliferative signals through activation of the ERK kinases.

Materials and Methods

Cells and culture

Embryonic stem (ES) cells (ES-E14), a gift from M. Hooper, Edinburgh, U.K., were cultured as described (36). Briefly, cells were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL, Breda, The Netherlands), 50% Buffalo rat liver-conditioned medium, 10% fetal calf serum (FCS, ES-qualified, Gibco-BRL) supplemented with 1% nonessential amino acids (Gibco-BRL), 0.1 mM 2-mercaptoethanol (Sigma Chemical Co, St. Louis, MO), 100 U/ml penicillin, 100µg/ml streptomycin (Gibco BRL) and 1000 U/ml leukemia inhibitory factor (LIF; Gibco BRL) in dishes coated with 0.1% gelatin (Sigma). The cells were passaged every 2 to 3 days.

Targeting construct and probe

The targeting strategy used to inactivate the *gcsfr* gene is shown in Figure 1. Isolation, cloning and sequencing of genomic DNA were done according to standard procedures (37). Genomic DNA was isolated from a mouse 129SV/Cosmid library (Cosmid SC1-6 SuperCos 1, Stratagene cloning systems, La Jolla, California) as described (38). A region of 10 kb including exon 7 to 17 of the *gcsfr* gene was replaced by a *Neo* gene driven by the PGK promoter. To construct the targeting vector a 2.9 kb *Sau*3AI - *Pst*I fragment containing exon 5 and 6, and a 4.5 kb *Xba*I fragment (3-prime of the *gcsfr* gene, including the non-coding region of exon 17) were cloned into pBluescript yielding pEUR11 and pEUR5, respectively. pEUR5 was opened by *Spe*I, and PGK-Neo was inserted in reverse orientation, yielding pEUR17. pEUR17 was opened by *Not*I, blunted, and subsequently cut by *Xho*I. The resulting 6.5 kb *Not*I-*Xho*I fragment containing the 4.5 kb *Xba*I fragment and the PGK-Neo gene was ligated into pEUR11 after *Apa*I opening, blunting and subsequent *Xho*I digestion, resulting in pEUR18. A unique *Not*I site in the vector backbone was used for linearization prior to transfection. A 0.6 kb *Sau*3A fragment 5-prime of exon 4 (probe A) was used to screen for homologous recombination, yielding a 9.6 kb band in germ line configuration, and a 7 kb band after homologous recombination.

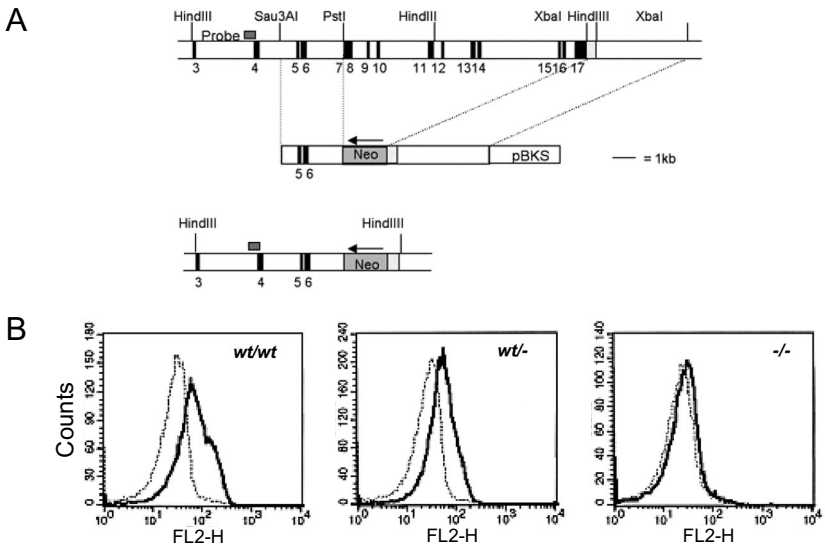

Figure 1: Targeting strategy to inactivate the gcsfr gene. A) Targeting strategy to delete exons 7-16 of the *gcsfr* gene in ES cells. The position of the probe used to screen recombinant colonies is shown. Southern analysis of *Hin*dIII digests of genomic DNA detected an 9.6-kb band from the wild type allele and a 7-kb band from the targeted allele (not shown). B) Specific binding of G-CSF to bone marrow cells from *gcsfr* +/+, *gcsfr* +/-, and *gcsfr* -/- mice analyzed by flow cytometry. Cells were incubated with biotinylated G-CSF in the absence (solid line) or presence (dotted line) of a 100-fold molar excess of nonlabeled G-CSF followed by incubation with PE-conjugated streptavidin.

Disruption of the gcsfr-gene by homologous recombination

E14-ES cells (10^7) were transfected with 25 µg linearized pEUR18 by electroporation using a Progenetor II, PG200 Hoefer Gene pulser set at 350 V/cm, 1200 µF, 10 msec. The next day, cells were transferred to culture medium containing 200 µg/ml G418 (Gibco BRL), with G418 resistant colonies picked on day 8 or 9 after electroporation. Genomic DNA of these colonies was digested with *Hin*dIII, transferred to nylon membranes, and hybridized to probe A and a neomycin probe. Correctly targeted clones were subjected to cytogenetic analysis and clones with a normal karyotype were used for blastocyst injections.

Generation of gcsfr-knock out mice

Two ES cell clones were injected into blastocysts of C57BL/6 mice. The resulting male chimeras were mated to FVB females to generate *gcsfr* +/- F1 mice. Heterozygous *gcsfr* +/- mice were intercrossed to obtain *gcsfr* -/- mice. DNA was isolated from tail segments and analyzed by PCR using primers for

exon 17 (5'-GTATATCCCTGTGTTCAGGAAACC and 5'-GGCAGGGTC-TTCAAGATACAAGG) and primers for the neo gene (5'-TACTCGGATGGAA-GCCGGTC and 5'-AGTCGATGAATCCAGAAAAG).

Flow cytometric analysis of G-CSF-R expression

Expression levels of G-CSF-R on neutrophilic cells were measured by flow cytometry. To this end, G-CSF was biotinylated using D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Biotin-7-NHS; Boehringer, Mannheim, Germany). Free biotin was removed by gel-filtration on Sephadex G-25. Bone marrow cells (10^6) were incubated in 96-well plates for 60 minutes at room temperature in 25 µl PBA (phosphate-buffered saline with 1 % BSA and 0.1 % NaN_3) and 0.2 µg/ml biotinylated G-CSF, either in the absence or the presence of a 100-fold molar excess of non-biotinylated G-CSF. Subsequently, cells were incubated for 30 minutes at 4ºC with phycoerythrin-conjugated streptavidin (SA-PE, Caltag Laboratories, Burlingame, CA). To determine G-CSF-R expression on transduced bone marrow cells, cells were labeled for 30 min at 4ºC with biotinylated anti human G-CSF-R antibody (LMM741, Pharmingen, San Diego, CA) and subsequently with SA-PE. Cells were subjected to flow cytometric analysis on a FACScan (Becton-Dickinson, Sunnyvale, CA).

Construction of G-CSF-R retroviral vectors and virus production

Vectors containing cDNA encoding human G-CSF-R wild-type (WT) and tyrosine substitution mutants have been described previously (13). Inserts were recloned into the retroviral vector pBabe, containing a puromycine resistance gene. Correct insertion was verified by nucleotide sequencing. Phoenix E virus producer cells (a gift from G. Nolan, Stanford, CA) were transfected with these constructs using Promega Profection Mammalian Transfection Systems. Supernatants containing high-titer, helper-free recombinant viruses were harvested after culturing approximately 80% confluent producer cells for 16-20 hours in DMEM medium (with 5% FCS and Penicillin/Streptomycin) and passed through a 45µm filter before use.

Retroviral infection of hematopoietic progenitor cells

Bone marrow cells were harvested from the femurs and tibiae of 8 to 12 week-old G-CSF-R-deficient mice as described (38). After depletion of adherent cells, the remaining cells were fractionated on a Percoll™ density gradient (Amersham

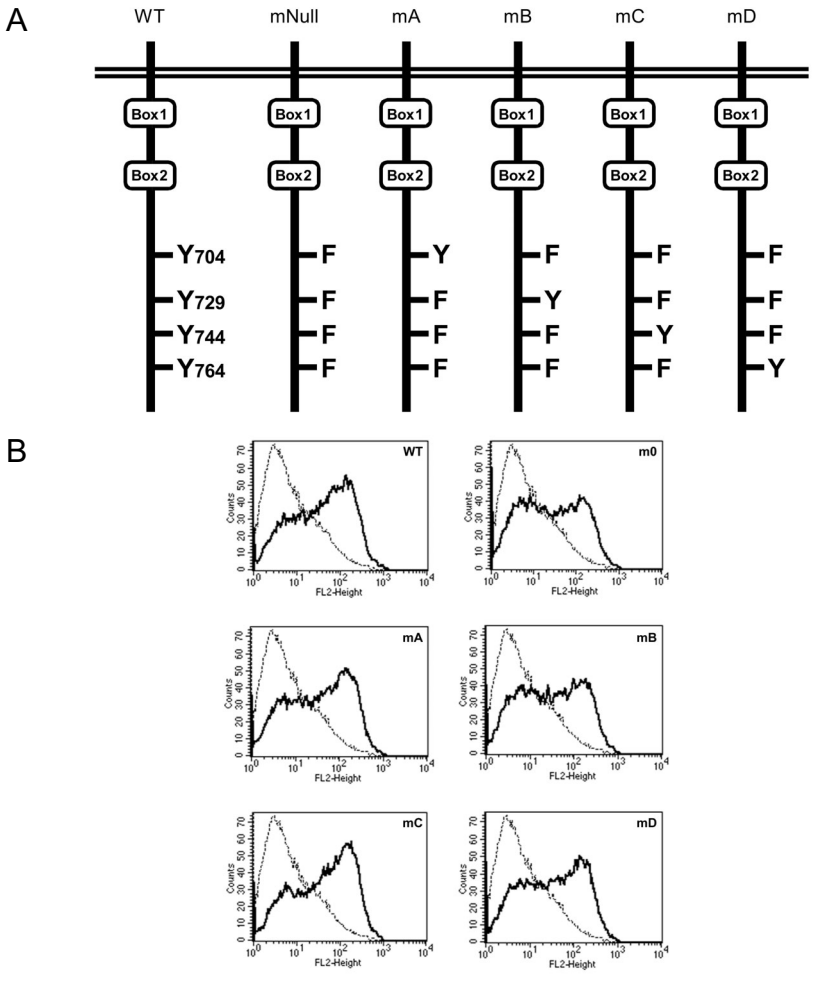

Figure 2: Expression of G-CSF-R mutants. A) Schematic representation of cytoplasmic domains of human G-CSF-R wild-type and tyrosine substitution mutants showing positioning of conserved tyrosines relative to membrane proximal Box1 and Box2. B) Flow cytometric analysis of G-CSF-R expression on *gcsfr* -/- bone marrow cells retrovirally transduced with G-CSF-R WT and tyrosine mutants shown in panel A. Bold histograms indicate cells stained with biotinylated G-CSF-R antibodies and SA-PE; dotted histograms: cells stained with SA-PE only.

Pharmacia Biotech, Uppsala, Sweden) as described (39). Cells were washed twice in HBSS/5% FCS/0.5% BSA, and prestimulated for 2 days in Cell Gro® (Boehringer Ingelheim Bioproducts Partnership Heidelberg, Germany) supplemented with a cytokine cocktail containing mIL-3 (10 ng/ml), hFLT3-ligand, hTpo, mSCF and GM-CSF (all 100 ng/ml) at a final density of $5x10^5$ cells/ml. Cells where then transferred to 35-mm culture dishes (Becton Dickinson, Lincoln Park, NJ) coated with 12 μg/ml recombinant fibronectin

fragment CH-296 (Takara Shuzo, Otsu, Japan) and preincubated with the appropriate virus supernatant for 30 minutes at 37°C. Subsequently, bone marrow cells (10^6 cells/ml) were mixed with fresh virus supernatant in a 1:1 ratio, supplemented with a fresh cytokine cocktail and cultured overnight at 37°C and 5% CO_2. Virus supernatant and cytokine cocktail were once again refreshed the next day and the cells cultured for an additional 24 hrs.

Progenitor cell assays and suspension culture

Bone marrow cells were plated in triplicate at densities of $1x10^5$/mL in methyl cellulose medium supplemented with 30% FBS, 1% BSA, 0.1mM 2-mercaptoethanol, 2mM L-glutamine, and G-CSF (100 ng/ml). To calculate infection efficiencies for the different receptor mutants, cells were also plated in GM-CSF (20 U/ml) containing colony assays, with or without 2.5 μg/ml puromycin (Sigma, Zwijndrecht, The Netherlands). Colonies (30 cells or more) were counted on day 7 of culture. For cytological analysis and replating experiments, colony cells were mass harvested and washed twice in HBSS. Suspension cultures were performed in RPMI (Gibco BRL) supplemented with 10% FCS and 100 ng/ml G-CSF. Every 3- to 4 days, culture medium was renewed and cells were counted on a Casy R-1 cell counter (Scharfe system, Reutlinger, Germany). Cell densities were kept between 0.3 x 10^6 and $1x10^6$/ml. For inhibitor studies, cells were grown as described above, in the presence of either 10 μM SB203580 or U0126 (Calbiochem, San Diego, CA) dissolved in DMSO, or DMSO as a solvent control. Viable cells were counted daily and every second day cells were spun down and resuspended in fresh media with fresh inhibitor. Cell densities of proliferating cells were kept between 0.5 and 1.5 x 10^6 cells/ml. Cell viability was assessed by flow cytometric analysis (FACScan, Becton-Dickinson, Sunnyvale, CA) using 7-amino actinomycin D (7-AAD; Molecular Probes, Eugene Oregon, USA).

Reporter assay for SOCS3 effects on G-CSF-R activity

To determine the effects of SOCS3 on the activity of G-CSF-R and mutants, we employed a STAT5 luciferase assay essentially as described previously (40). In brief, HEK 293 cells, seeded in 24-well dishes at 0.2 x 10^6 cells/well in 1 ml DMEM/10% FCS, were cultured overnight and transfected by means of standard $CaPO_4$ precipitation with a mixture of the following plasmids: pME18S-STAT5 for expression of STAT5, a STAT5 luciferase reporter plasmid consisting of 5 repeats of the β-casein sequence upstream of a SV40 promoter in the pGL-3-

promotor vector (Promega, Madison, WI), a β-galactosidase expression plasmid pRSVLacZ, derived from pCH110 (41), pcDNA3-SOCS3 (42) or empty pcDNA3 (Invitrogen, Breda, The Netherlands) and pBabe with the different G-CSF-receptor mutants. A volume of 100 µl precipitate with a total of 2 µg DNA (400 ng of DNA for each construct) was added to each well. For SOCS3, 12.5 ng SOCS3 supplemented with 387.5 ng of pcDNA3 (empty vector) was added. On day 4, the cells were stimulated for 6 hrs with 100 ng/ml G-CSF and subsequently lysed in 100 ml lysis buffer (25 mM Tris phosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT, 8 mM $MgCl_2$). To measure luciferase activity, cell lysates (25 µl) were transferred to 96-well flat bottom plates (Costar, Corning Inc, Corning, NY) and 25 µl of a 16 mg/ml luciferase substrate-containing buffer (Steady-Glo luciferase assay System, Promega) was added to each well. Emitted light was measured in a TopCount luminometer (Packard, Meriden, CT). To correct luciferase activity levels for variations in transfection efficiencies, 25 µl of cell lysate was incubated in parallel with 75 µl β-galactosidase substrate buffer (100 mM Na-PO4 buffer pH 7.8, 10 mM KCl, 1 mM $MgSO_4$, 2.7 mM DTT) and 0.56 mg/ml o-Nitrophenyl β-D-galactopyranoside (oNPG, Sigma) for 15 minutes at 37°C. Absorption was measured in a microplate reader (Biorad 450, Veenendaal, The Netherlands) at 450 nm. All experiments were performed in duplicate.

Results

Generation of gcsfr-deficient mice

To inactivate the murine *gcsfr* gene, we constructed a targeting vector in which the genomic sequence spanning exon 7-17 was replaced by a pgk-Neo selection cassette (Figure 1A). Two independently isolated ES cell clones were injected into blastocysts and the resulting chimeras were crossed with FVB-mice. Germ line transmission of the knockout allele was achieved for both clones. Flow cytometric analysis of bone marrow neutrophils using biotinylated G-CSF confirmed the absence of G-CSF-R in *gcsfr*-/- mice and a 50% reduced expression in *gcsfr*+/- mice (Figure 1B). In agreement with a previously reported *gcsfr* knockout line (5), peripheral neutrophil counts in this *gcsfr*-/- strain are 15-20% of levels found in WT littermates.

Role of receptor tyrosines in G-CSF-induced colony formation

To study the involvement of receptor tyrosines in G-CSF-R-mediated signaling in primary hematopoietic cells, we introduced single tyrosine add back mutants, mNull, G-CSF-R WT, or pBabe control vector (Figure 2), into *gcsfr*-/- bone marrow cells and determined G-CSF responses in colony assays. As predicted, no colonies were formed by *gcsfr*-/- cells transduced with pBabe vector (Figure 3A). Cells expressing mNull produced colonies at numbers equivalent to cells transduced with G-CSF-R WT. At first glance, these data would suggest that the receptor tyrosines are fully dispensable for G-CSF-controlled colony growth. However, experiments with the add back mutants unveiled a more subtle scenario. Expression of mA (Y704) slightly increased colony formation, while

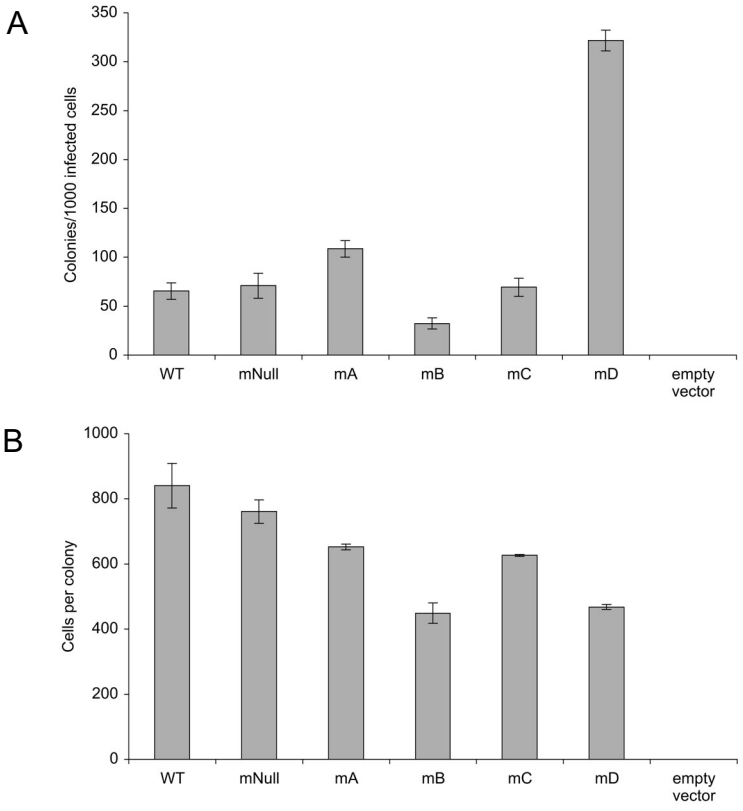

Figure 3: Primary colony formation by *gcsfr*-/- bone marrow progenitors transduced with different G-CSF-R constructs. Colonies were grown in the presence of 100 ng/ml G-CSF. A) Mean colony numbers ± SD per 1000 infected bone marrow cells from triplicate colony dishes; data are representative of 4 independent experiments. Colony numbers were normalized to the numbers of infected cells based on puromycin resistance of CFU-GM (responsive to GM-CSF) to correct for differences due to variations in transduction efficiencies. B) mean numbers of cells per colony ± SD from triplicate dishes.

colony numbers obtained with mC (Y744) were similar to mNull. Colony formation induced by mB (Y729) was reduced by approximately 50%, indicating that Y729 has a negative influence on G-CSF-induced colony growth. The presence of Y764 (mD) resulted in approximately 6-fold increase in cloning efficiency. Assessment of the the mean number of cells per colony did not show a correlation between colony number and size, except for mB (Figure 3B). The latter observation suggests that negative signals emanating from Y729 affect both clonogenicity as well as proliferative potential of the transduced progenitor cells. Morphologic analysis revealed no differences in the composition of colonies induced by the various receptor forms suggesting that the receptor tyrosines are dispensable for G-CSF-induced differentiation (data not shown).

G-CSF-R tyrosines are involved in G-CSF-dependent maintenance of myeloid progenitor cell levels

We next investigated to what extent G-CSF-R tyrosines contribute to the expansion of the progenitor cells in serial replatings. As shown in Figure 4, progenitor cells transduced with WT G-CSF-R maintained recloning abilities at a relatively constant level up to the sixth replating. In contrast, replating abilities of mNull expressing cells gradually declined after the third replating, suggesting that receptor tyrosines are required for sustained G-CSF-dependent maintenance and expansion of myeloid progenitor cells. The presence of Y729 (mB), Y704

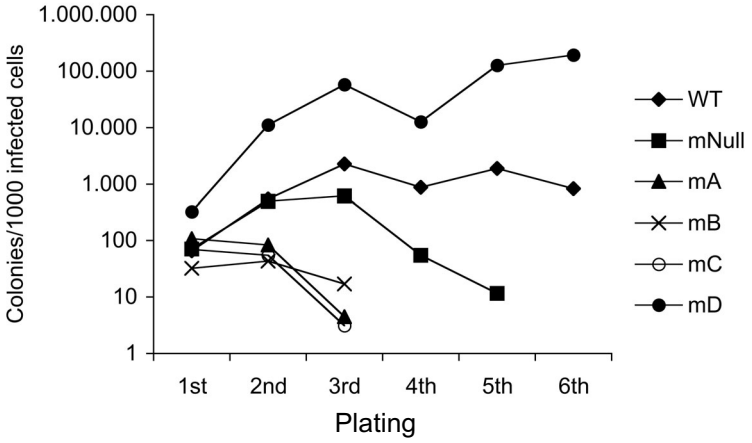

Figure 4: Serial replatings of progenitor cells from gcsfr-/- bone marrow transduced with G-CSF-R expression constructs.

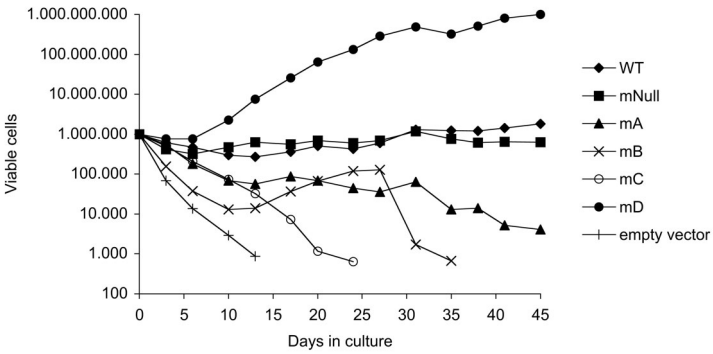

Figure 5: Expansion of *gcsfr-/-* bone marrow cells transduced with G-CSF-R expression constructs in suspension culture. Cells were cultured in the presence of 100 ng/ml G-CSF. Viable cell counts and replenishment of culture media were performed at 3- to 4 days intervals.

(mA), or Y744 (mC) further suppressed the recloning potential of progenitors. In contrast, the presence of Y764 (mD) alone greatly enhanced recloning potential, resulting in colony numbers after the fifth replating that were 100-fold higher than WT and 10,000-fold higher than mNull expressing cells cultured in parallel. The sustained expansion of progenitor cells mediated via Y764 also translated into exponential cell proliferation in long-term suspension culture (Figure 5). These expanded cells did not express mainly features of immature blast cells, but rather represented a mixture of myeloid cell types at various stages of differentiation. Notably, the cells remained fully dependent on G-CSF for proliferation (data not shown). Cell numbers were maintained at relatively stable levels in cultures from cells expressing WT and mNull G-CSF-R, whereas cells expressing mA, mB or mC progressively lost proliferative abilities.

Proliferative signals from Y764 are mediated via ERK

Y764 is a docking site for connector proteins implicated in p21Ras/MAPK signaling and has been shown to play a prominent role in the activation of ERK as well as p38 MAP kinases (17, 24, 29). We therefore studied the effects of the MEK1/2 inhibitor U0126, which blocks activation of ERK1 and ERK2, and the p38 inhibitor SB203580 on G-CSF-induced proliferation of bone marrow cells expressing mD in suspension culture. As shown in Figure 6, addition of U0126 to the cultures inhibited proliferation, while the effects of SB203580 were minimal. These findings establish that ERK kinases are the principle mediators of proliferative signaling via Y764 of the G-CSF-R.

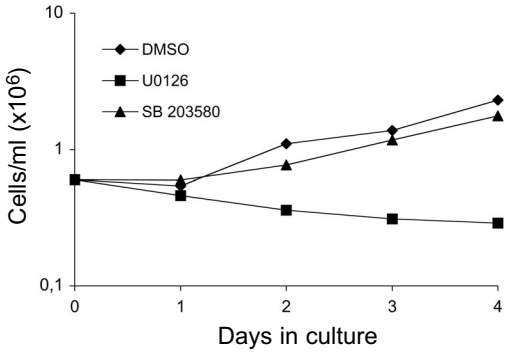

Figure 6: Effects of inhibitors of MEK (U0126) and p38MAPK (SB203580) on proliferation of mD expressing cells.

SOCS3 inhibits G-CSF responses via Y729 of G-CSF-R

Recently, several studies have demonstrated that SOCS3 mediates its inhibitory activity on a variety of cytokine receptors, e.g., leptin receptor and gp130, via binding to receptor tyrosines (42, 43). Based on structural similarities between G-CSF-R and gp130, we performed a G-CSF-R activity assay based on the onset of STAT5-mediated gene expression, which is activated via the membrane proximal region of G-CSF-R (27). Introduction of SOCS3 severely interfered with activity of G-CSF-R WT (Figure 7). In contrast, under similar conditions, activity of mNull was not affected, confirming that one or more of the receptor tyrosines are involved in SOCS3 recruitment. Experiments with single tyrosine add back mutants subsequently showed inhibition only with mB, suggesting that Y729 is the major binding site for SOCS3. We suggest on the basis of these data that the negative effects generated by Y729 on G-CSF-R signaling are mediated by SOCS3.

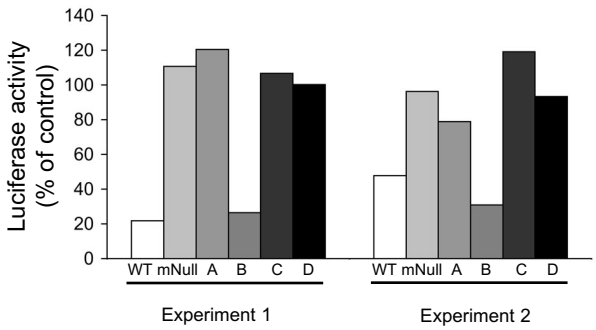

Figure 7: STAT5-luciferase reporter assay showing prominent inhibitory effects of co-transfection of pcDNA-SOCS3 on G-CSF-R WT and mB (Y729). Data are expressed as the percentage of activity measured after cotransfection of pcDNA3 empty vector.

Discussion

The aim of this work was to investigate to what extent signals from G-CSF-R tyrosines contribute to the G-CSF-induced responses of primary myeloid progenitor cells. Previous studies in myeloid factor-dependent cell lines have revealed that these tyrosines contribute to G-CSF-dependent proliferation, differentiation and survival via signaling mechanisms involving the activation of STAT3 and p21Ras (17, 21, 24, 25, 29, 44). Although these models have provided useful information, it has also become clear that major discrepancies in signaling requirements exist between cell lines and primary cells. For instance, STAT3 was shown to induce growth arrest and neutrophilic differentiation in cell lines, whereas a recent study in a transgenic mouse model demonstrated that STAT3 is essential for G-CSF-induced proliferation of primary myeloid progenitors (40, 45, 46). The fact that certain mechanisms underlying growth factor-induced proliferation of primary progenitor cells are bypassed or constitutively activated in cell lines is likely to contribute to such differences.

Some of the findings reported here are consistent with certain observations in cell lines. For instance, Y764 confers hyperproliferative responses to G-CSF in both primary progenitor cells and cell lines (25). However mNull, which was unable to transduce proliferation signals in 32D cells, fully supported G-CSF-induced colony formation of primary progenitors at plating efficiencies comparable to G-CSF-R-WT. Thus, coupling of signaling mechanisms to G-CSF-R tyrosines in primary myeloid progenitor cells is redundant for G-CSF-induced colony growth. This might be attributed to either alternative activation of the pathways linked to the tyrosines or to compensatory influences of other signaling pathways.

Although G-CSF-R tyrosines were not required per se for G-CSF-induced colony formation, the experiments with the add back mutants clearly suggested that the individual tyrosines exert regulatory functions. In particular, this applies to the growth inhibitory role of Y729 and the growth promoting role of Y764. No specific inhibitory pathway had previously been assigned to Y729. We have identified Y729 here as the single tyrosine involved in SOCS3-mediated inhibition of G-CSF signaling. In similar experiments we could not functionally link SOCS1 or SOCS2 to Y729 (unpublished results). We hypothesize that SOCS3 binds directly to Y729 via its SH2 domain. Y729 is located in a motif

(VLYGQLLGS) that shows striking homology with the SOCS3-SH2 binding sites within gp130 and the leptin receptor. Characteristics of this motif are the valine at pY-2, a hydrophobic residue at Y+3 and the serine at Y+6 (or Y+5) (42, 43). While this paper was under review, Hörtner et al. published data supporting the notion that the Y729-containing motif of G-CSF-R indeed forms a direct binding site for SOCS3 (47). Notably, G-CSF-R deletion mutants in patients with severe congenital neutropenia that progress to acute myeloid leukemia lack this motif (48-50), which may contribute to the hyperproliferative signaling properties of these receptor forms (51, 52).

The G-CSF-induced colonies grown from the *gcsfr*-/- bone marrow cells transduced with G-CSF-R constructs were of granulocyte, granulocyte-macrophage, macrophage or mast cell origin and contained fully mature cells. We did not observe differences in the composition of the colonies grown from cells transduced with G-CSF-R WT, tyrosine add back or tyrosine null mutants. This argues against a major role of the receptor tyrosines in controlling myeloid differentiation. A similar conclusion was recently drawn by Akbarzadeh et al. (28), who further demonstrated that expression of myeloperoxidase and gelatinase, enzymatic markers of granulocytic differentiation, was not affected by substitution of the tyrosines. Interestingly, these authors observed a slight, but significant, increase in the numbers of macrophage colonies and reduction of granulocyte colonies with mutant Y729F, but not with their Y null mutant. This suggests that Y729, possibly via recruitment of SOCS3, influences the balance between granulocyte and macrophage colony growth only when pathways activated via one or more of the remaining tyrosines remain intact.

Both G-CSF and G-CSF-R deficient mice have reduced numbers of CFU-GM in the bone marrow. Thus, G-CSF not only stimulates myeloid progenitors to proliferate and differentiate towards neutrophils, but also controls the size of the progenitor cell compartment in the bone marrow. The data from the sequential platings suggest that signaling pathways emanating from the G-CSF-R tyrosines contribute significantly to this control. The prominent stimulatory influence of Y764 suggests that activation of the p21Ras/ERK pathway contributes to progenitor cell expansion, whereas the inhibitory signal provided by Y729, most likely involving SOCS3, has the opposite effect. Strikingly, Y704 and Y744, while exerting no inhibitory effect on primary colony growth, suppressed progenitor cell expansion in the replating experiments. The signaling pathways

responsible for this inhibition are not known. Y704 and Y744 both function as direct docking sites for STAT3 (25). STAT3 activation by G-CSF-R can also occur in the absence of receptor tyrosines (13, 28). Still, we think that STAT3 activation via Y704 and Y744 may play an important role because depending on the levels of activation in conjunction with other signaling pathways, STAT3 can exert variable functions in myeloid progenitor cells. The effects of STAT3 on cell proliferation and differentiation are diverse and depend on cell type and stage of differentiation (40, 46, 53). Even within one cell type, unexpected variations in the effects of STAT3, depending on the status of activation of other signaling pathways, have been reported. Based on these findings a model was proposed in which STAT3 orchestrates conflicting signals during G1 to S transition in the cell cycle (54). In view of this more complex role of STAT3 in the regulation of cell growth, we propose that STAT3 mediates stimulatory effects on primary colony growth and neutrophilic differentiation, thereby negatively affecting the expansion of myeloid progenitors controlled by G-CSF. Studies in which STAT3 activation in bone marrow cells can inducibly be inactivated are in progress to unravel the full spectrum of activities of STAT3 in myeloid cell development.

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CHAPTER 3

Distinct activities of suppressor of cytokine signaling (SOCS) proteins and involvement of the SOCS box in controlling G-CSF signaling

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J. Leukocyte Biology, in press

Summary

Granulocyte colony-stimulating factor (G-CSF) induces proliferation of myeloid progenitor cells and controls their differentiation into mature neutrophils. Signal transducer and activator of transcription (STAT) proteins STAT3 and STAT5 are activated by G-CSF and play distinct roles in neutrophil development. Suppressor of cytokine signaling (SOCS) proteins are induced by STATs and inhibit signaling through various negative feedback mechanisms. SOCS proteins can compete with docking of signaling substrates to receptors, interfere with JAK kinase activity and target proteins for proteasomal degradation. The latter process is mediated through the conserved C-terminal SOCS box. We determined the role of various SOCS proteins in controlling G-CSF responses and investigated the involvement of the SOCS box therein. We show that SOCS1 and SOCS3, but not CIS and SOCS2, inhibited G-CSF-induced STAT activation in HEK 293 cells. In myeloid 32D cells, SOCS1 and SOCS3 are both induced by G-CSF. However, relative to IL-3 containing cultures, during G-CSF-induced neutrophilic differentiation SOCS3 expression was further elevated while SOCS1 levels remained constant. SOCS box deletion mutants of SOCS1 and SOCS3 were severely hampered in their abilities to inhibit STAT activation and to efficiently suppress colony formation by primary myeloid progenitors in response to G-CSF. These data demonstrate the importance of the SOCS box for the inhibitory effects of SOCS proteins on G-CSF signaling and show that among the different SOCS family members, SOCS3 is the major negative regulator of G-CSF responses during neutrophilic differentiation.

Introduction

Most hematopoietic growth factors and cytokines exert their action via membrane receptors of the hematopoietin superfamily. Upon ligand binding, these receptors activate JAK tyrosine kinases and subsequently one or more members of the signal transducer and activator of transcription (STAT) protein family (1). The expression of several suppressor of cytokine signaling (SOCS) proteins, i.e., CIS, SOCS1 and SOCS3 is under the direct transcriptional control of STATs (2-7). SOCS proteins, characterized by their SH2 domain and their C-terminal SOCS box (8, 9), act in a classical negative feedback loop to inhibit signaling from a variety of hematopoietic growth factors and cytokines including Epo, IL-2, IL-3 and G-CSF (10-13). SOCS proteins can inhibit signaling via multiple mechanisms. They may out compete other signaling substrates for recruitment to a receptor (14). Alternatively, SOCS1 and SOCS3 can directly inhibit JAK kinase activity utilizing their extended SH2 sub domains (ESS) and their kinase inhibitory regions (KIR), two functional domains lacking in other family members (15-17). SOCS1 has a high affinity for direct binding to JAKs (18), whereas SOCS3 needs recruitment to receptor tyrosines for efficient inhibition (11, 12, 19-21). A third proposed mechanism of inhibition by SOCS proteins involves recruitment to the SOCS box of elongins B and C, which form part of an E3 ubiquitin ligase complex, leading to subsequent proteasomal degradation of signaling substrates (22, 23). The SOCS box may also regulate the stability of the SOCS proteins themselves (23). Indeed, SOCS1, -2 and -3 are highly unstable proteins, suggesting that active degradation is important for the regulation of SOCS protein levels (24). It was shown that the SOCS box is involved in proteasomal targeting of SOCS proteins (23, 25). However, the roles of the SOCS box and elongin binding in SOCS protein degradation remain controversial. Several reports have suggested that these interactions may actually lead to SOCS protein stabilization (22, 26-29).

G-CSF controls survival, proliferation and differentiation of myeloid progenitor cells via multiple signaling mechanisms activated by the G-CSF receptor (G-CSF-R). Among the different STAT family members, STAT3 and STAT5 are most prominently activated by the G-CSF-R. STAT5 contributes to both G-CSF-induced proliferation and survival of G-CSF-R transduced Ba/F3 cells (30). STAT3 has been suggested to play a role in the regulation of the G1 arrest required for neutrophilic differentiation, but not in the execution of the

differentiation process itself (31). Studies in conditional STAT3 knockout mice showed that removal of STAT3 resulted in neutrophilia, supporting the notion that STAT3 is not essential for neutrophilic differentiation in vivo, but is required for maintaining appropriately balanced neutrophil production (7). G-CSF failed to upregulate SOCS3 transcripts in STAT3 deficient bone marrow cells, suggesting that STAT3-induced SOCS3 is a major negative regulator of G-CSF-controlled neutrophil production (7). Recent studies in mice lacking SOCS3 in their hematopoietic cells or neutrophils also pointed towards a role for SOCS3 in controlling G-CSF-induced neutrophil formation (32, 33). However, the mechanism by which SOCS3 inhibits G-CSF-induced granulopoiesis was not addressed in these studies. Generation of mice deficient for different members of the SOCS family has revealed important functions of the SOCS proteins in controlling signaling by multiple cytokines in hematopoietic and non-hematopoietic cells (34-39). In addition to SOCS3, G-CSF induces the expression of SOCS1, SOCS2, and CIS in hematopoietic cells (12, 40). The ability of these additional SOCS proteins to suppress G-CSF-R signaling has not been investigated.

Here, we report that both SOCS1 and SOCS3 inhibit G-CSF-induced STAT3 and STAT5 activation, whereas CIS and SOCS2 do not. Rather, SOCS2 appeared to exert an enhancing effect on activation of STAT3. In addition, we show that transcription of SOCS3 is induced during G-CSF-stimulated neutrophilic differentiation, while SOCS1 remains present at a relatively low and constant level. Finally, using SOCS box deletion mutants, we demonstrate that the SOCS boxes of SOCS1 and SOCS3 are important for efficient inhibition of STAT activation and for inhibition of G-CSF-induced colony formation of primary bone marrow cells. These data suggest for the first time an important role for the SOCS box in SOCS-mediated inhibition of G-CSF-controlled granulopoiesis.

Material and methods

Constructs

Expression constructs of myc-tagged CIS, SOCS1, SOCS2 and SOCS3 in pcDNA3 were provided by A. Yoshimura (18). The SOCS box deletion mutants (SOCSdbox) were made by introduction of a stop codon before the SOCS box in the SOCS1 and SOCS3 constructs using a site directed mutagenesis kit, according to the manufacturer's instructions (Stratagene, La Jolla, CA). Primers

used were for SOCS1: FmutSOCS1: 5'-cccgctgcgctagcgccgcgtg and RmutSOCS1: 5'-cacgcggcgctagcgcagcggg; for SOCS3: FmutSOCS3: 5'-ctactc-cgggggctagaagatccc and Rmut SOCS3: 5'-gggatcttctagcccccggagtag. All SOCS constructs were cloned into the *Bam*HI and *Sna*BI sites of retroviral vector pBabe (41) and correct orientation of inserts was verified by nucleotide sequencing. Human G-CSF-R wild type (WT) in the pBabe vector has been described before (13).

Cells and RNA isolation

Generation and culture of 32D cells stably expressing the G-CSF-R (32D/WT) have been described previously (31). For short-term stimulation experiments, the cells were washed twice with Hanks' balanced saline solution (HBSS) and were starved in RPMI at a concentration of 1 million cells/ml for 4 hrs and stimulated with 100 ng/ml G-CSF for the indicated times. For growth on G-CSF, cells were washed twice with HBSS to remove IL-3 and cultured in the presence of 10 ng/ml G-CSF for multiple days. At the indicated time points, cells were harvested, resuspended in TRIzol® (Invitrogen, Breda, The Netherlands), snap frozen and stored at -80°C. RNA was isolated according to manufacturer's instructions. To remove genomic DNA, 5 µg of RNA was treated with 10 U of DNAse I (Stratagene) in DNAse buffer (40 mM Tris-HCl pH7.5, 6 mM $MgCl_2$, 2 mM $CaCl_2$) for 1 hr at 37ºC.

Quantitative reverse transcriptase-PCR

For generation of cDNA, 1µg RNA was denatured at 65ºC for 5 minutes, followed by 10 minutes on ice. First-strand buffer (250 mM Tris-HCl, pH8.3, 375 mM KCl, 15 mM $MgCl_2$), dNTP's (1 mM final concentration), DTT (1 mM final concentration), 40U Rnasin, 4 µg of random hexamers (Amersham Pharmacia, Uppsala, Sweden) and 200U Superscript II reverse transcriptase (Invitrogen) were added and the reaction was incubated at 42ºC for 2 hrs. Dilutions of cDNA used for PCR amplification for SOCS1, SOCS3 and RNase inhibitor were 1:10, 1:30 and 1:60 respectively. Primers used for amplification of SOCS1 were FTMSOCS1, 5'-tggtagcacgcaaccaggtg, and RTMSOCS1, 5'-tggcgaggacgaagac-gag. For SOCS3, FTMSOCS3, 5'-tcaagaccttcagctccaa and RTMSOCS3, 5'-tcttgacgctcaacgtgaag, were used. Primers for murine RNase inhibitor were: forward, 5'-tccagtgtgagcagctgag, and reverse, 5'-tgcaggcactgaagcacca. Taqman technology (PE Applied Biosystems, Model 7900 sequence detector, Foster City, CA) was used for quantitative real-time PCR. The reactions were performed in a volume of 25 µl of a mixture containing 2 µl of the respective cDNA dilution,

primers at 0.2 μM and 12.5 μl of 2x SYBR green PCR Master mix (PE Applied Biosystems) containing Amplitaq Gold® DNA polymerase, reaction buffer, dNTP mix with UTP, and the double stranded DNA-specific fluorescence dye SYBR green I. The PCR program used was 1 cycle of 2 minutes at 50ºC, 1 cycle of 10 minutes at 95ºC, 45 cycles of denaturation for 15 s at 95ºC, annealing for 30 s at 62ºC and extension for 30 s at 62ºC. Samples were tested in duplicate and the average values of the threshold cycle (Ct) were used for quantification. To quantify the relative expression of SOCS1 and SOCS3, the Ct values were normalized for endogenous reference ($\Delta Ct = Ct_{SOCS} - Ct_{RNase\ inhibitor}$) and compared with a calibrator, using the ΔΔCt method ($Ct = Ct_{Sample} - Ct_{Calibrator}$). As calibrator we used the expression in 32D cells deprived of growth factors and serum for 4 hours (Figure 2A) or the expression in 32D cells grown on IL-3 (Figure 2B).

Luciferase assays

Luciferase assays were performed as described previously (13). In short, HEK 293 cells were transfected by $CaPO_4$ precipitation (42) with a mixture of the following plasmids: pME18S-STAT5 to obtain a robust STAT5 luciferase signal, a β-casein-derived STAT5 luciferase reporter plasmid, pRSVLacZ, different amounts of pcDNA3 with myc-tagged SOCS or empty pcDNA3 (Invitrogen) and pBabe with WT G-CSF-receptor. For STAT3 luciferase experiments pME18S-STAT5 was replaced by empty pcDNA3 and an m67-derived STAT3 luciferase reporter was used (43). Twenty-four hours after transfection the medium was replaced by serum free medium (DMEM + 0.1 % BSA). The next day the cells were stimulated with G-CSF for 6 hrs, lysed and luciferase activity was measured using Steady-Glo reagents (Promega Madison, WI). In parallel, the transfection efficiency was determined using lacZ staining. Luciferase activity levels were corrected for transfection efficiency using β-galactosidase expression levels. All experiments were performed in triplicate.

Western blotting

Lysate from the luciferase assay was used in parallel for expression analysis of myc-tagged SOCS proteins by Western blot. Antibodies used for detection were: mouse anti-myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-actin (Santa Cruz Biotechnology).

Virus production and spot blot analysis

Phoenix E virus producer cells (a gift from G. Nolan, Stanford, CA) were

transfected with pBabe-SOCS constructs by $CaPO_4$ precipitation. Supernatants containing high-titer, helper-free recombinant viruses were harvested from 80% confluent producer cells grown for 16-20 hours in DMEM medium (with 10% FCS and Penicillin/Streptomycin) and passed through a 45μm filter. To determine titers of pBabe-SOCS and pBabe-EGFP viruses, the virus particles were spun down by ultracentrifugation at 30.000 rpm (Beckman, Mijdrecht, The Netherlands), and viral RNA was extracted with phenol (pH = 4.0) and spot-blotted on nitrocellulose filters. This blot was hybridized with a pBabe-specific probe (SV40 fragment, *Bam*HI-*Hin*dIII digest).

Infection of bone marrow progenitors with SOCS constructs

Hematopoietic cells were harvested from the femurs and tibiae of Fvb/N or 129/SV mice (44). Prestimulation and retroviral transduction of the bone marrow cells with pBabe SOCS expression constructs was performed as described before (13). After the transduction procedure, bone marrow cells were plated in duplo at densities of 0.5 and $2.0x10^5$ cells per ml methyl cellulose containing medium supplemented with 30% FBS, 1% BSA, 0.1mM 2-mercaptoethanol, 2mM L-glutamine, with or without 1.5 μg/ml puromycin (Sigma, Zwijndrecht, The Netherlands) and G-CSF (100 ng/ml). Colonies were counted on day 7 of culture. Three independent experiments were performed with fresh virus supernatant in all cases.

Results

SOCS1 and SOCS3 inhibit G-CSF-induced STAT activation

CIS, SOCS1, SOCS2 and SOCS3 are the major SOCS proteins expressed in hematopoietic cells (12, 40). We first assessed which of these proteins affected G-CSF-induced STAT5 and STAT3 activity. SOCS1 and -3 inhibited G-CSF-induced STAT5 luciferase activity in a dose-dependent manner. In contrast, CIS and SOCS2 did not significantly affect STAT5 activity, even at the highest levels of expression (Figure 1A). Similarly, SOCS1 and SOCS3 inhibited STAT3 luciferase reporter activity (Figure 1B), while CIS and SOCS2 had no inhibitory effects. Rather, SOCS2 and to a lesser extent CIS, appeared to stimulate G-CSF-induced STAT3 activation.

G-CSF differentially induces SOCS1 and SOCS3 gene expression

SOCS1 and SOCS3 may be part of a G-CSF-induced negative feedback loop activated during neutrophilic differentiation. To address this, we tested whether and to what extent G-CSF controls the expression of SOCS1 and -3 during both

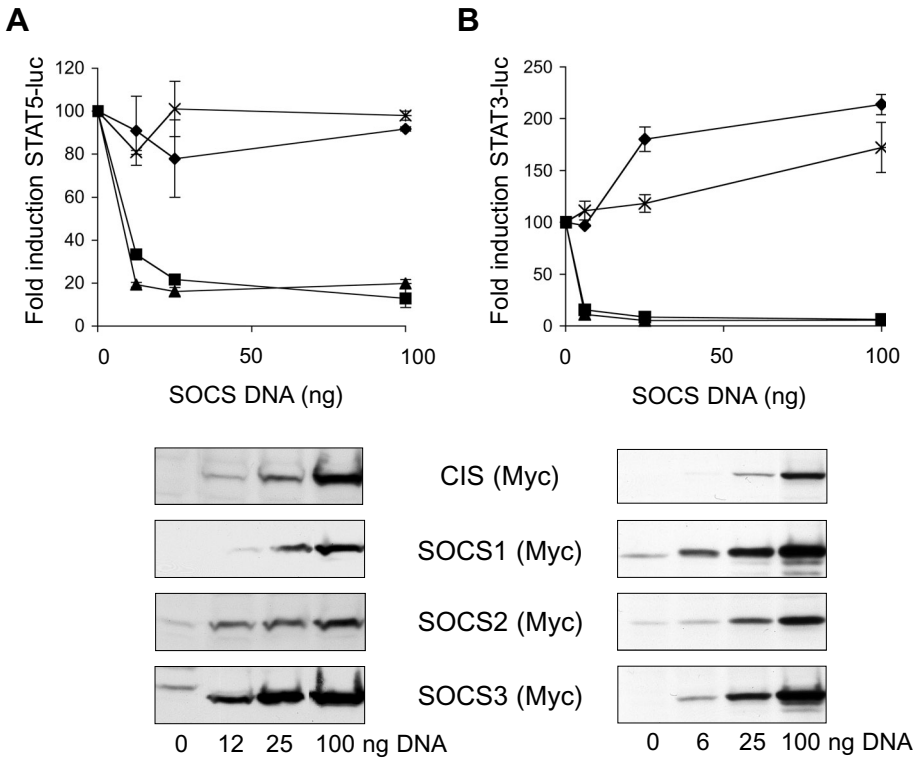

Figure 1: G-CSF-induced activation of STAT5 and STAT3 is inhibited by SOCS1 and SOCS3 but not by CIS and SOCS2. HEK293 cells were transiently transfected with WT-G-CSF-R, STAT5 and lacZ expression constructs in combination with a STAT5 luciferase reporter (A) or WT-G-CSF-R and lacZ expression constructs combined with a STAT3 luciferase reporter (B) and different amounts of CIS (x), SOCS1 (▲), SOCS2 (◆) or SOCS3 (■) DNA. G-CSF-induced luciferase activity was corrected for transfection efficiency using lacZ staining and set at 100% in the absence of SOCS. Data shown are mean ± SEM of at least 2 independent experiments with triplicate measurements. Lysate of the luciferase assay was also used for Western blotting to detect expression of myc-tagged SOCS proteins.

short-term stimulation with G-CSF and upon G-CSF-induced neutrophilic differentiation of 32D/WT cells. Stimulation with G-CSF results in a transient upregulation of SOCS1 mRNA, whereas SOCS3 expression is more robust (Figure 2A). In addition, SOCS3 expression is induced over several days during differentiation on G-CSF, while SOCS1 levels were comparable to values of 32D/WT cells cultured under proliferation conditions in the presence of IL-3 (Figure 2B). These results establish that SOCS3 is the most prominent SOCS member involved in the negative feedback during G-CSF-induced neutrophilic differentiation of myeloid cells.

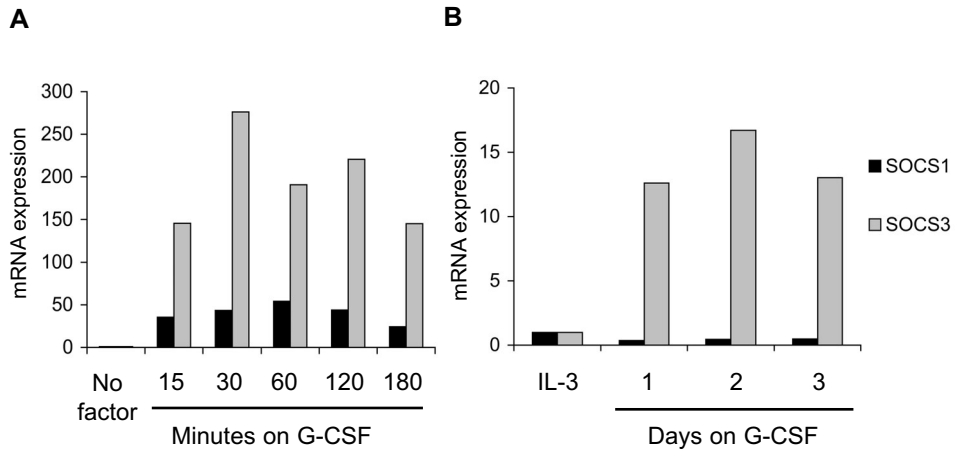

Figure 2: G-CSF stimulation of myeloid cells induces transient SOCS1 mRNA expression whereas SOCS3 is induced during G-CSF-mediated neutrophilic differentiation. 32D cells expressing the WT G-CSF-R were deprived of growth factors for 4 hours and stimulated with G-CSF for the indicated times (A) or cultured in the presence of G-CSF for three days (B). SOCS1 (black bars) and SOCS3 (gray bars) mRNA expression levels were determined by quantitative RT-PCR and were normalized using expression of ribonuclease-inhibitor. SOCS levels were expressed relative to growth factor deprived 32D cells (A) or 32D cells cultured under proliferation conditions in IL-3 containing medium (B).

Role of the SOCS box in suppressing STAT3 and STAT5 activation

SOCS1 and SOCS3 are thought to inhibit cytokine signaling by interfering with the phosphorylation of downstream signaling substrates of JAK kinases via their KIR (16, 17). An alternative mechanism by which SOCS proteins may attenuate signaling is by targeting critical signaling molecules for proteasomal degradation, mediated via the SOCS box. To determine the involvement of the SOCS box in the inhibitory effects of SOCS1 and SOCS3 we performed luciferase reporter assays to test the activity of SOCS mutants lacking the SOCS box (SOCS1dbox and SOCS3dbox) on G-CSF-R-induced activation of STAT5 and STAT3. Loss of the SOCS box of SOCS1 reduced inhibition of STAT5 activation (Figure 3A). In contrast, inhibition of STAT3 activity remained essentially intact upon deletion of the SOCS box (Figure 3B). SOCS3dbox did not inhibit STAT5 reporter activity at all, clearly showing that the SOCS box is essential for the inhibitory effects of SOCS3 on STAT5 (Figure 3D). Suppression of STAT3 activation by SOCS3 was also drastically reduced by deletion of the SOCS box, although the effect was somewhat less pronounced than for STAT5 (Figure 3E). Western blot analysis revealed that upon transfection of comparable

amounts of expression plasmids, WT SOCS protein levels were consistently higher than levels of SOCSdbox mutants (Figure 3, C and F). Upon titrating down the amounts of transfected SOCS plasmids, protein expression of the SOCSdbox mutants decreased more steeply than that of WT SOCS1 and -SOCS3. These latter observations suggest that removal of the SOCS box reduces protein stability of both SOCS1 and SOCS3. However, the amounts of SOCSdbox protein at the highest concentrations tested were comparable with amounts of full length SOCS1 and SOCS3 that already gave complete inhibition of STAT5 signaling. On this basis, we conclude that the reduced inhibition by SOCSdbox mutants is not merely the result of decreased expression levels of the protein but involves an intrinsic loss of its inhibitory capacity as a result of the removal of the SOCS box.

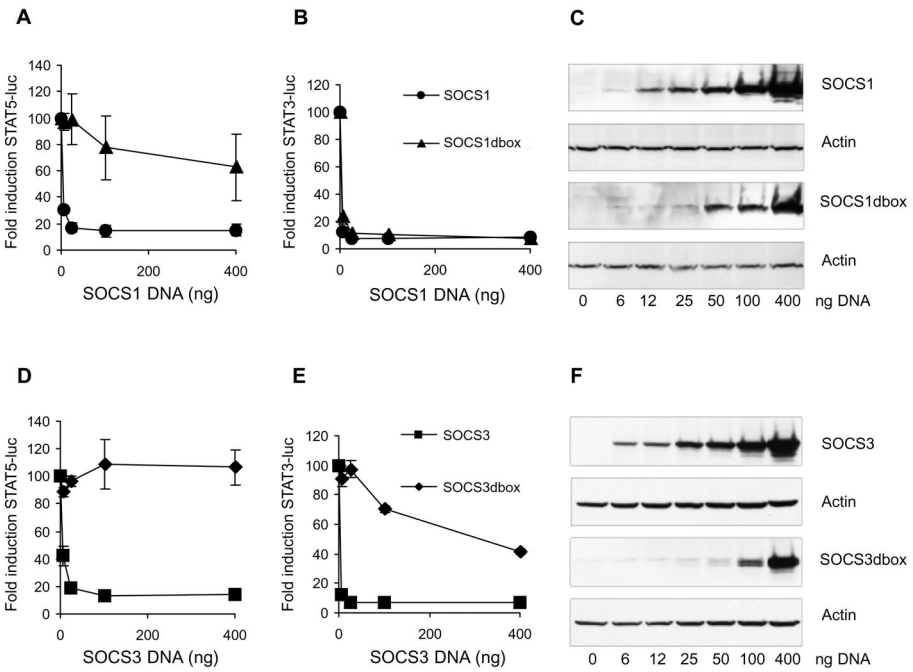

Figure 3: The SOCS boxes of SOCS1 and SOCS3 are important for inhibition of STAT5 activation and to a lesser extent for inhibition of STAT3 activation by G-CSF. STAT5 (A+D) and STAT3 (B+E) luciferase reporter assays with SOCS1 (●), SOCS1dbox (▲), SOCS3 (■) and SOCS3dbox (◆) were performed as described in Figure 1. Average ± SEM out of at least three independent experiments is shown. In parallel Western blot analysis of myc-tagged SOCS proteins and actin as a control for equal loading was performed. Blots from representative experiments are shown in panels C and F.

The SOCS box of both SOCS1 and SOCS3 contributes to inhibition of G-CSF-induced colony formation

To determine the contribution of the SOCS box to the inhibitory effects of SOCS1 and SOCS3 on G-CSF-induced proliferation and differentiation of primary hematopoietic cells, we introduced SOCS1 and SOCS3 and their SOCS box deletion mutants in mouse bone marrow progenitor cells by retroviral gene transfer. To assure that comparable viral titers were used for the various infections, viral RNA was isolated from the supernatants and quantified using spot blot analysis with a vector specific cDNA probe. As shown in Figure 4A, the viral RNA contents of the various supernatants were comparable, indicating that the titers of the different retroviral vectors were similar. Introduction of SOCS1 and SOCS3 dramatically reduced G-CSF-induced colony numbers and size compared with bone marrow cells transduced with EGFP-containing control vector (Figure 4, B and C). The absence of the SOCS box resulted in a significant relief of the inhibitory effects of SOCS1 and SOCS3 on the numbers and size of CFU-G colonies. Thus, the SOCS box is also important for SOCS-mediated suppression of G-CSF responses in primary myeloid progenitor cells.

Discussion

In this study, we investigated the role of the SOCS protein family members CIS, SOCS1, SOCS2 and SOCS3 in G-CSF signaling. We showed that SOCS1 and -3 inhibited G-CSF-induced STAT3 as well as STAT5 activation, and no inhibition by CIS and SOCS2 was detected. Additionally, we have demonstrated for the first time a major role for the SOCS box in the inhibition of G-CSF-R signaling by SOCS1 and SOCS3, in reporter assays and in primary bone marrow progenitors. Upon the introduction of comparable amounts of DNA, the expression of SOCSdbox mutants was consistently lower than that of WT SOCS proteins (Figure 3). This supports a role for the SOCS box in stabilizing SOCS protein levels. Similar conclusions were reported for SOCS1 and SOCS3 in different model systems (26, 29). However, based on protein expression data (Figure 3, C and F), we consider it unlikely that prevention of SOCS degradation is the only contribution of the SOCS box to the inhibition of signaling.

We observed that there are considerable differences between the abilities of SOCS1dbox and SOCS3dbox to inhibit G-CSF-induced activation of STAT3 and STAT5 (Figure 3). Although we currently have no explanation for these findings

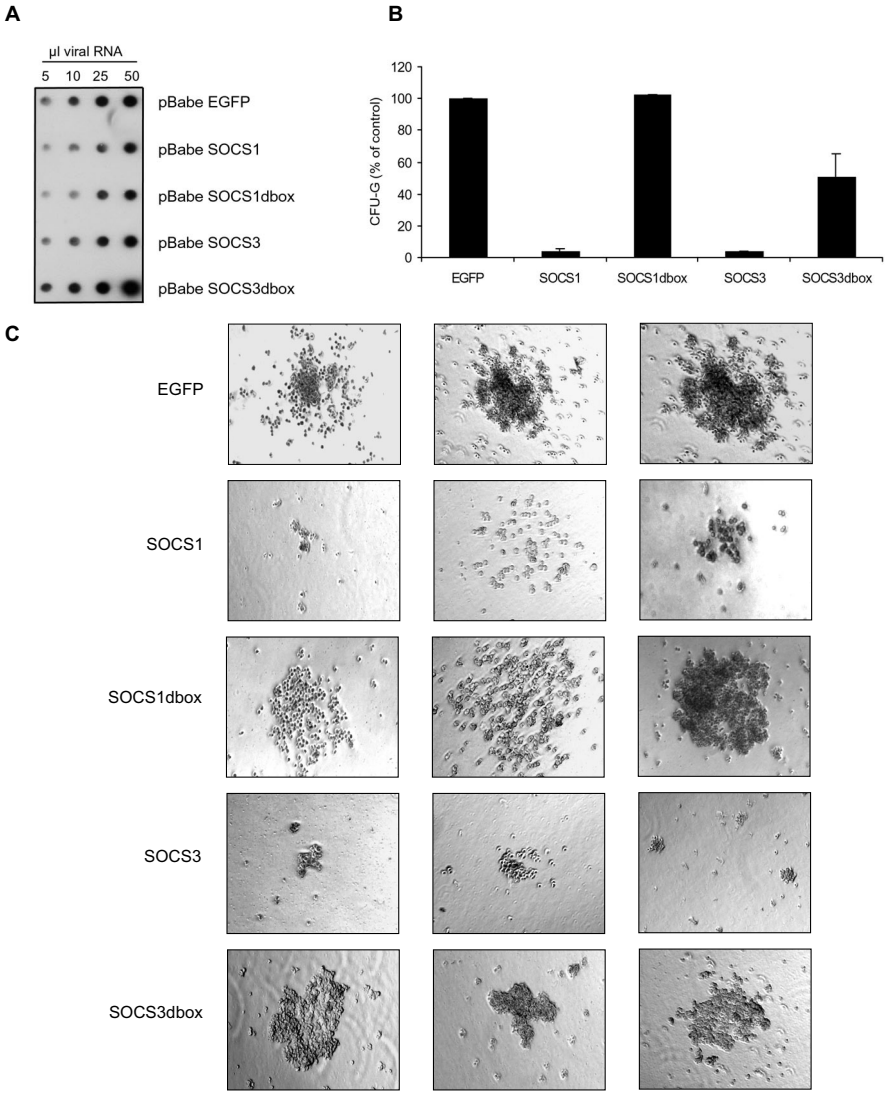

Figure 4: The SOCS box of SOCS1 and SOCS3 are required for efficient inhibition of G-CSF-induced colony formation of bone marrow cells. A) RNA spot blot analysis of supernatants containing pBabe retrovirus, demonstrating that titers used for infection were comparable. B) CFU-G assay of primary bone marrow progenitor cells following infection with pBabe EGFP and SOCS constructs. Cells were plated in methylcellulose medium containing G-CSF (100 ng/ml) and puromycin (1.5 µg/ml). Data shown are mean of at least two independent experiments + SEM. C) Photomicrographs of representative examples of G-CSF colonies of transduced bone marrow cells (magnification 50x), showing reduced colony size of SOCS transduced, but not SOCSdbox transduced cells compared to control (EGFP-transduced) cells.

we anticipate that differences in recruitment mechanisms of SOCS1, SOCS3, STAT3 and STAT5 to the activated G-CSF-R determine the outcome of deleting the SOCS box on G-CSF signaling. It is clear that SOCS1dbox is a more potent inhibitor than SOCS3dbox in both luciferase readouts. Conceivably, because of its high affinity binding to JAKs, SOCS1dbox is able to recruit to JAKs and to suppress signaling with the KIR independently of the SOCS box, even at reduced expression levels. In addition, we showed that STAT3 activation is more sensitive to inhibition by SOCS1dbox and SOCS3dbox than G-CSF-induced STAT5 activation. There are at least two explanations for this. STAT3 activation depends to a significant extent on recruitment to tyrosines 704 and 744 of the G-CSF-R, which first requires tyrosine phosphorylation of the receptor. This multistep process might be more sensitive for inhibition than STAT5 activation, which is mediated through direct recruitment to JAK itself (45). Reports on other cytokine receptors corroborate this by demonstrating that the SOCS box is dispensable for inhibition of STATs that require recruitment to receptor tyrosines for their activation (15, 16). In addition, the difference between the downregulatory effects of SOCS1dbox and SOCS3dbox on STAT3 versus STAT5 may relate to differential involvement of regulatory proteins that are sensitive to proteasomal targeting.

The turnover rate of STAT3 protein is low and phospho-STAT3 protein levels are not affected by proteasomal inhibition, suggesting that proteasomal degradation is not a major downregulatory mechanism for STAT3 (24, 46). Phosphorylated STAT5 protein on the other hand, is stabilized by proteasomal inhibitors. As ubiquitination or degradation of STAT5 protein was not detected, it was proposed that a STAT5 phosphatase is present that is kept inactive by an unidentified protein (46). Upon ubiquitination and proteasomal degradation of this protein, the phosphatase would become active and dephosphorylate STAT5 (46). We have recently shown that G-CSF induces binding of the SH2-containing tyrosine phosphatase-2 (SHP-2) to STAT5, which depends on the presence of Y729 of the G-CSF-R, a major docking site for SHP-2 (47). In view of the data showing that SHP-2 functions as a STAT5 phosphatase (48, 49), these observations may point to a scenario in which recruitment of SHP-2 contributes to STAT5 down-regulation. Although the exact molecular features remain to be elucidated, these findings are consistent with the model proposed by Wang et al. (46), implicating a major role for proteasomal targeting in downregulation of STAT5 by the degradation of a phosphatase inhibitory activity.

It is striking that, although inhibition by SOCS3dbox in the luciferase assays was reduced compared to the effects of SOCS1dbox, SOCS3dbox still inhibited G-CSF-induced colony formation to some extent whereas SOCS1dbox did not. Although the reason for this difference is not clear, this may reflect inhibition of effector mechanisms other than STATs that are involved in colony formation and does not necessarily reflect direct effects of SOCS3dbox specific for G-CSF signaling.

We observed that SOCS2 (and to a lesser extent, CIS) dose dependently stimulated G-CSF-induced STAT3 activation (Figure 1B). We currently have no explanation for this stimulatory effect of SOCS2. A stimulatory role for SOCS2 has also been reported for other cytokine receptors. For instance, SOCS2 stimulated the activation of STAT5 by both the growth hormone receptor and the prolactin receptor (50-52). It is interesting that the murine SOCS2 gene (cish2) was recently identified as a frequent common retrovirus integration site in a screen for novel leukemia genes. The virus integrations occurred 5' of the cish2 gene, which predictively results in the aberrant expression of SOCS2 transcripts (53). In addition, SOCS2 is upregulated in chronic myeloid leukemia (CML) cells in accelerated phase and in cell lines expressing the chimeric oncoprotein Bcr-Abl characteristic of CML (54). These data suggest that SOCS2 can act as a positive regulator of cytokine signaling and therefore might be considered an oncoprotein rather than a negative regulator of growth, at least in hematopoietic cells.

In conclusion we demonstrated in this paper that SOCS1 and SOCS3, but not the other SOCS family members expressed in hematopoietic cells, are capable of inhibiting G-CSF-induced STAT activation. During G-CSF-induced neutrophilic differentiation SOCS3 mRNA is upregulated whereas SOCS1 levels are comparable to levels during proliferation on IL-3, suggesting that SOCS3 is the most important SOCS family member for negative feedback during neutrophil development. Our studies have unveiled a major role for the SOCS box in the inhibition of G-CSF-induced colony formation of primary hematopoietic progenitor cells, suggesting a role for proteasomal degradation mediated via SOCS1 and SOCS3 in the downregulation of G-CSF responses during myeloid cell proliferation and differentiation in a physiological context.

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CHAPTER 4

G-CSF receptor truncations found in SCN/AML relieve SOCS3-controlled inhibition of STAT5 but leave suppression of STAT3 intact

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Blood, in press

Abstract

Truncated granulocyte colony-stimulating factor receptors (G-CSF-R) are implicated in severe congenital neutropenia (SCN) and consecutive development of AML. Mice expressing G-CSF-R truncation mutants (*gcsfr-d715*) show defective receptor internalization, an increased STAT5/STAT3 activation ratio and hyper-proliferative responses to G-CSF treatment. We determined whether a lack of negative feedback by suppressor of cytokine signaling (SOCS) proteins contributes to the signaling abnormalities of G-CSF-R-d715. Expression of SOCS3 transcripts in bone marrow cells from G-CSF-treated *gcsfr-d715* mice was approximately 60% lower than in wild type (WT) littermates. 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. This was mainly due to loss of the SOCS3 recruitment site Y729, with an additional contribution of the internalization defects of G-CSF-R-d715. Because Y729 is also a docking site for the protein tyrosine phosphatase SHP-2, which binds to and inactivates STAT5, we suggest a model in which reduced SOCS3 expression, combined with 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.

Introduction

Granulocyte colony-stimulating factor receptor (G-CSF) is the major regulator of neutrophil production, both under "steady state" conditions and during stages of bacterial infections (1-3). G-CSF exerts its activity via a receptor (G-CSF-R) of the hematopoietin receptor superfamily (4, 5). Typical of this class of receptors, G-CSF-R has no intrinsic kinase activity but recruits cytoplasmic tyrosine kinases of both the JAK and Src kinase families and activates signal transducer and activator of transcription (STAT) proteins (6-11). G-CSF activates STATs 1, 3 and 5 (12-14). Whereas the contribution of STAT1 to G-CSF responses remains unclear, STAT3 has been implicated in G-CSF-mediated growth arrest preceding differentiation, while activation of STAT5 has been linked to proliferation and survival signaling (15-17). Four tyrosine residues (Y704, Y729, Y744 and Y764) in the G-CSF-R carboxy-terminus are involved in the recruitment of signaling molecules, such as the adapter molecules GRB2 and SHC of the p21Ras-MAPkinase pathway, and the protein tyrosine phosphatase SHP-2 (18-20). In addition, activation of STAT3 depends on its recruitment to the G-CSF-R via tyrosines 704 or 744 (20-22). At higher G-CSF-concentrations STAT3 can also be activated in a tyrosine independent way via the G-CSF-R C-terminus (22, 23). In contrast, activation of STAT1 and STAT5 is achieved via the membrane-proximal region of G-CSF-R and does not require receptor tyrosine residues (13, 17).

In approximately 20% of patients suffering from severe congenital neutropenia (SCN), G-CSF-R mutations are found that result in the expression of a G-CSF-R with a truncated C-terminus (24, 25). These patients have an increased risk to develop AML (25, 26). Activation of a G-CSF-R mutant truncated at amino acid 715 (G-CSF-R-d715) causes a hyperproliferative response in 32D cells, without induction of neutrophilic differentiation (27). Mice with a targeted G-CSF-R-d715 mutation show various degrees of neutropenia and their myeloid precursors react to G-CSF administration with hyperproliferation, resulting in a sustained neutrophilia (28, 29). Interestingly, transgenic (tg) mice overexpressing G-CSF-R mutants truncated at amino acids (aa) 718 and 731, demonstrated increased susceptibility to infection with *Staphylococcus aureus*, suggesting that production of functional neutrophils is compromised in these animals (30). Indeed, these tg mice had only one third of the peripheral neutrophil levels of wild-type controls and their bone marrow showed increased percentages of immature myeloid cells.

Functional analysis of truncated receptors revealed that a number of properties are altered compared to WT G-CSF-R. Ligand-induced internalization of G-CSF-R-d715 is severely affected due to the loss of 2 distinct motifs in the receptor C-terminus that are important for internalization (27, 31, 32). G-CSF-R-d715 also has a somewhat reduced ability to activate STAT3, possibly due to the loss of the STAT3 recruitment site Y744 and the receptor C-terminus (22, 31). In contrast, activation of STAT5 is strongly increased and is sustained after removal of G-CSF, suggesting a prominent role for the C-terminus in mediating negative feedback on STAT5 activation (17, 27, 31). Although the defective internalization properties of truncated G-CSF-R forms contributed significantly to their sustained signaling function, it was also clear that this did not fully explain these findings (27, 32). In particular, the differential effects of receptor truncations on the kinetics of STAT3 versus STAT5 activation remained unclear.

Suppressor of cytokine signaling (SOCS) proteins are involved in the down regulation of signaling from a number of hematopoietic growth factor receptors, including G-CSF-R (33-36). A conserved SH2 domain and a C-terminal SOCS box are characteristic for the SOCS family (reviewed in (37-39)). The expression of most SOCS genes is controlled by STAT transcription factors (40-45). SOCS proteins therefore act in a classical negative feedback loop to suppress cytokine signaling. Three distinct inhibitory mechanisms have been linked to SOCS proteins. CIS, founding member of the family, inhibits activation of STAT5 by competing for STAT5 recruitment to phosphotyrosine motifs in e.g., the growth hormone receptor (GH-R) and the erythropoietin receptor (Epo-R)(46, 47). SOCS1 and SOCS3, on the other hand, directly suppress JAK kinase activity by means of a kinase inhibitory region (KIR)(48-50). Upon recruitment to the signaling complex via the SH2 domain of SOCS, the KIR mediates inhibition by blocking access of both ATP and substrate to their binding sites in the catalytic groove of JAK2 (49-53). Finally, SOCS proteins are also thought to down regulate signaling via SOCS box-mediated targeting of signaling proteins for proteasomal degradation (54, 55). An important difference between SOCS1 and -3 relates to how they are recruited into activated receptor complexes. Whereas the SH2 domain of SOCS1 has a high affinity for, e.g., phosphorylated Y1007 in the JH1 domain of JAK2, the affinity of the SH2 domain of SOCS3 for this residue is much lower (49, 52, 56). Instead, SOCS3 is recruited with high affinity to phosphotyrosine-based motifs in certain receptors and then subsequently inhibits JAK activity via its KIR (34, 35, 48, 57-59). Importantly, for a number

of cytokine receptors it has been established that the protein tyrosine phosphatase SHP-2 and SOCS3 dock to identical tyrosine-based motifs with comparable affinities (57-60).

Studies in SOCS deficient mice have demonstrated major physiological roles for SOCS proteins in controlling the levels of cytokine signaling in both non-hematopoietic and hematopoietic cells (61-72). G-CSF induces the expression of SOCS1, SOCS2, SOCS3 and CIS in hematopoietic cells (35, 73), but only SOCS1 and SOCS3 appeared to inhibit G-CSF-induced STAT activation (74). In two independent studies, Y729 of G-CSF-R was identified as the major recruitment-site for SOCS3 (35, 36). Recently, it was reported that SOCS3 is a key negative regulator of G-CSF-induced neutrophil production in vivo (75).

In the present study, we investigated to what extent and by which mechanism(s) truncation of the G-CSF-R C-terminus, as found in SCN, affects the negative feedback regulation of G-CSF signaling by SOCS3. We show that truncation of the G-CSF-R completely relieved the inhibitory effects of SOCS3 on activation of STAT5. In striking contrast, the suppressive effects of SOCS3 on G-CSF-R-d715-induced activation of STAT3 were hardly affected by the truncation. These findings provide a new mechanistic explanation for the increased ratio of STAT5/STAT3 activation in *gcsfr-d715* mice, which has previously been linked to the shift in the proliferation/differentiation balance in the myeloid progenitor cell compartment found in these animals (31).

Material and methods

Expression constructs

The constructs of human G-CSF-R WT, d715, Y729F, d735, d749-769, d715-735, and the GFP-G-CSF-R fusions in the pBabe vector (76) have been described before (18, 20, 32, 36) (see Figure 1A). The d735F and d749-769F mutants were generated from mutant Y729F, by introduction of a stop codon or by deletion of region 749-769 respectively, with a site-directed mutagenesis kit according to the manufacturers instructions (Stratagene, La Jolla, CA). Expression constructs of myc-tagged SOCS1 and SOCS3 in pcDNA3 were a gift from A. Yoshimura (56). For expression of SHP-2, HA-tagged human SHP-2 was cloned into the *Eco*RI - *Xho*I sites of expression vector pSG5 (77). For expression of STAT5, pME18S-STAT5B was used (78).

Bone marrow cells and isolation of RNA

WT and *gcsfr-d715* mice (28) were stimulated daily for 4 days with G-CSF or received solvent only. Each experimental group contains 2 mice of each genotype. Bone marrow cells were isolated, resuspended in TRIzol® RNA extraction reagent (Invitrogen, Breda, The Netherlands), snap frozen and stored at -80ºC (28). For the in vitro stimulation, bone marrow cells were harvested from two WT and two *gcsfr-d715* mice that had not been treated with G-CSF. Cells were cultured for 1 hr in HBSS (Invitrogen) with 5% FCS, non-adherent cells were taken and starved for 4 hrs in RPMI (Invitrogen) + 0.5% BSA. Cells were stimulated for the indicated periods with G-CSF (100 ng/ml) and resuspended in Trizol. RNA was isolated according to manufacturer's instructions and subsequently treated with DNAse to remove genomic DNA. DNAse treatment of 5 µg of RNA was performed in DNAse buffer (40 mM Tris-HCl pH7.5, 6 mM $MgCl_2$, 2 mM $CaCl_2$) with 10 U of DNAse I (Stratagene) for 1 hr at 37ºC.

Quantitative RT-PCR

To generate cDNA, 1µg RNA was denatured at 65ºC for 5 minutes followed by 10 minutes on ice. After addition of first-strand buffer (250 mM Tris-HCl, pH8.3, 375 mM KCl, 15 mM $MgCl_2$), with dNTP's (1 mM final concentration), DTT (1 mM final concentration), 4 µg random hexamers (Amersham Pharmacia, Uppsala, Sweden), 40U RNasin and 200U Superscript II reverse transcriptase (Invitrogen), the reaction was incubated at 42ºC for 2 hrs. The cDNA was diluted 1:10, 1:30 and 1:60 for SOCS1, SOCS3 and RNase inhibitor respectively before PCR amplification. Primers used for amplification of SOCS1 were FTMSOCS1: 5'-tggtagcacgcaaccaggtg and RTMSOCS1: 5'-tggcgaggacgaagacgag, and for SOCS3: FTMSOCS3: 5'-tcaagaccttcagctccaa and RTMSOCS3: 5'-tcttgac-gctcaacgtgaag. Primers for murine RNase inhibitor were forward 5'-tccag-tgtgagcagctgag, and reverse 5'-tgcaggcactgaagcacca. For the quantitative real-time PCR, Taqman technology was used (PE Applied Biosystems, Model 7900 sequence detector, Foster City, CA). The reactions were performed in a volume of 25 µl of a mixture containing 2 µl of the respective cDNA dilution, primers at 0.2 µM and 12.5 µl of 2x SYBR green PCR Master mix (PE Applied Biosystems) containing Amplitaq Gold® DNA polymerase, reaction buffer, dNTP mix with UTP, and the double stranded DNA-specific fluorescence dye SYBR green I. The PCR program used was 1 cycle of 2 minutes at 50ºC, 1 cycle of 10 minutes at 95ºC, 45 cycles of denaturation for 15s at 95ºC, annealing for 30 s at 62ºC and

extension for 30 s at 62ºC. To determine the expression levels, samples were tested in duplicate and the average values of the threshold cycle (Ct) were used for quantification. To quantify the relative expression of SOCS1 and SOCS3, the Ct values were normalized for endogenous reference ($\Delta Ct = Ct_{SOCS} - Ct_{RNase\ inhibitor}$) and compared with a calibrator, using the $\Delta\Delta Ct$ method ($Ct = Ct_{Sample} - Ct_{Calibrator}$). As calibrator for G-CSF stimulation in vivo we used the expression in WT bone marrow of unstimulated mice. As calibrator for the in vitro stimulation we used expression after 4 hours of starvation.

Luciferase assays

Luciferase assays were performed as described previously (36). In short, HEK 293 cells, grown in 24 well plates, were transfected by the calcium phosphate precipitation method with a mixture of the following plasmids. For STAT5-luciferase experiments: expression vector pME18S-STAT5, a β-casein-derived STAT5 luciferase reporter plasmid, a β-galactosidase expression plasmid pRSVLacZ, a pBabe construct with WT or mutant G-CSF-R (Figure 1A) and different amounts of pCDNA3 with myc-tagged SOCS or empty pcDNA3 (Invitrogen). For the STAT3-luciferase experiments pME18S STAT5 was replaced by pcDNA3 vector and an m67-derived STAT3 luciferase reporter was added instead of the STAT5 reporter. A volume of 100 µl calcium phosphate precipitate with in total 2 µg DNA was added to each well. With the exception of SOCS3, 400 ng of DNA for each construct was added per well. Different amounts of pcDNA3-SOCS3 were added, supplemented with empty pcDNA3 vector up to 400 ng. After 24 hours the cells were starved overnight in DMEM + pen/strep + 0.1% BSA. The next day, the cells were stimulated with 250 ng/ml G-CSF for 6 hrs, lysed and assayed for luciferase activity using Steady-Glo reagents (Promega, Madison, WI). In parallel, the transfection efficiency was determined using lacZ staining. Luciferase activity levels were corrected for transfection efficiency using β-galactosidase expression levels. All experiments were performed in triplicate. Fold induction by G-CSF was calculated and set at 100% in the absence of SOCS. For inhibitor studies, the Src inhibitor PP-2, the JAK inhibitor WHI-P154 (Calbiochem, San Diego, CA) or DMSO as a solvent control was added to the cells 1 hr prior to G-CSF-stimulation. Unless stated otherwise, data were analyzed by means of ANOVA. To compare G-CSF-R expression levels, transfected cells were stained with biotinylated mouse anti human G-CSF-R antibody (Pharmingen, San Diego, CA) followed by phycoerythrin-conjugated streptavidin (SA-PE; Caltag laboratories,

Burlingame, CA) and analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, Sunnyvale, CA). As shown in Figure 1B, G-CSF-R expression is detectable with increased (less than 1 log unit) expression of the internalization-defective mutants (G-CSF-R-d715, d749-769 and d749-769F). As previously reported, this difference is mainly due to decreased spontaneous internalization of these mutants in the absence of G-CSF in non-myeloid cells (32).

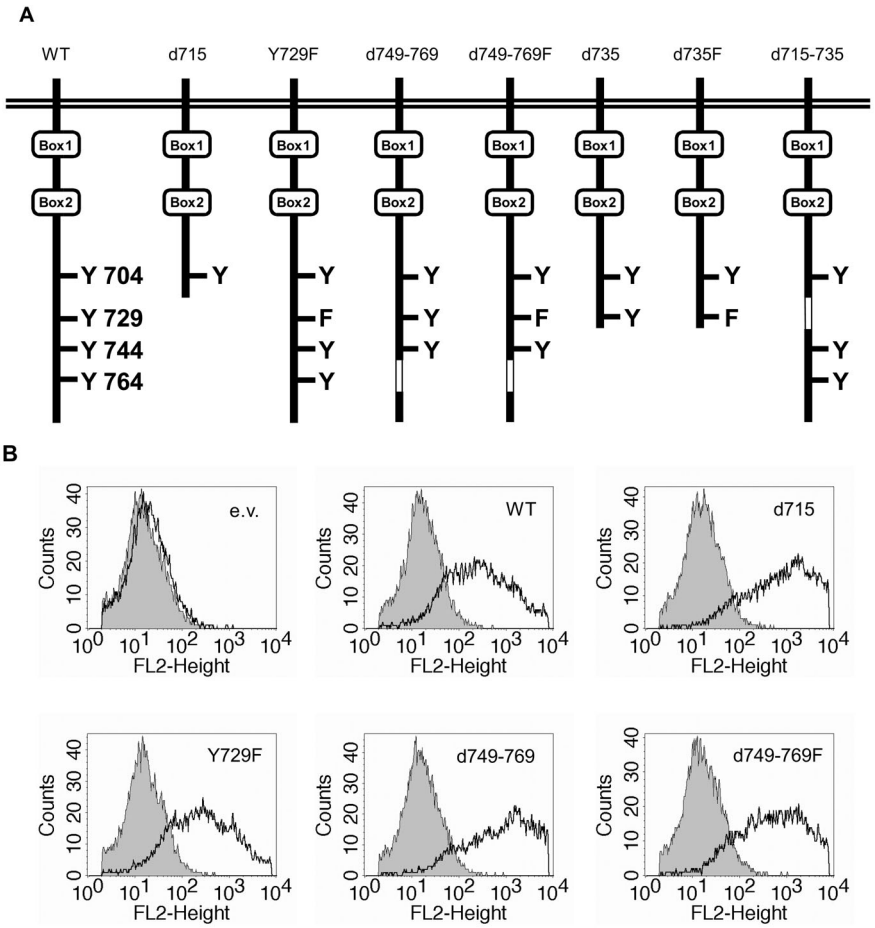

Figure 1: Expression of WT and mutant G-CSF receptors. A) Schematic representation of the intracellular domain of the G-CSF receptor and mutants. Boxes 1 and 2 represent subdomains conserved in the hematopoietin receptor superfamily. The open box indicates the deleted region in G-CSF-R-d749-769 and G-CSF-R-d715-735. B) Flow cytometric analysis of the expression levels of the different G-CSF-R forms used in luciferase reporter assays. Bold histograms indicate cells stained with biotinylated G-CSF-R antibodies and SA-PE; shaded histograms: cells stained with SA-PE only; e.v.: cells transfected with empty vector.

Immunoprecipitations and Western blotting

Phoenix E cells (a gift from G. Nolan, Stanford, CA) were transfected with G-CSF-R, SHP-2 and in the case of the SHP-2-STAT5 co-immunoprecipitations, as well with the STAT5 expression construct. After 24h the media was replaced by DMEM + 0.1% BSA. The next day, cells were stimulated for 10 minutes with G-CSF, washed twice with cold PBS and lysed in lysisbuffer containing 20 mM Tris-HCl pH8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP40, 10% glycerol, 2 mM Na_3VO_4 and 1 mM Pefablock SC, 50 µg/m1 aprotinin, 50 µg/ml leupeptin, 50 µg/ml bacitracin and 50 µg/ml iodoacetamide as protease inhibitors. Immunoprecipitations with anti-HA antibody and protein G-sepharose beads (Sigma, Zwijndrecht, The Netherlands) and subsequent Western blotting were performed as described previously (7). Antibodies used were mouse anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-SHP-2 (Santa Cruz Biotechnology), mouse anti-green fluorescent protein (Roche, Almere, The Netherlands) and rabbit anti-STAT5B (Santa Cruz Biotechnology).

Results

Reduced G-CSF-induced SOCS3 expression in gcsfr-d715 mice

SOCS proteins are under the transcriptional control of STATs (41, 43, 44, 79). Because G-CSF-R-d715 shows altered activation of STAT3 and STAT5 compared to WT G-CSF-R (27, 31), we first investigated expression of SOCS1 and -3 in WT versus *gcsfr-d715* mice under steady state conditions and after daily treatment of the animals with G-CSF for 4 days. As shown in Figure 2A, steady state levels of SOCS1 in *gcsfr-d715* mice and their WT littermates were approximately similar. Also after G-CSF treatment, SOCS1 transcript levels in WT and *gcsfr-d715* mice had not changed dramatically. A slight (2.5-fold) increase in SOCS1 mRNA levels in *gcsfr-d715* mice compared to WT animals was noted (Figure 2A). In contrast, SOCS3 expression was strongly (>12-fold) induced by G-CSF in WT mice, while expression levels in *gcsfr-d715* mice reached only about 30% of these levels both after G-CSF stimulation as well as in steady state (Figure 2B). In addition we isolated bone marrow cells from untreated WT and *gcsfr-d715* animals and stimulated them with G-CSF in vitro. As shown in Figure 2C, stimulation with G-CSF gives a strong induction of SOCS3 mRNA. Again, SOCS3 transcript levels are reduced in G-CSF-R-d715 cells upon stimulation with G-CSF. This demonstrates that the reduced upregulation of SOCS3 mRNA also occurs outside the bone marrow compartment and is due to

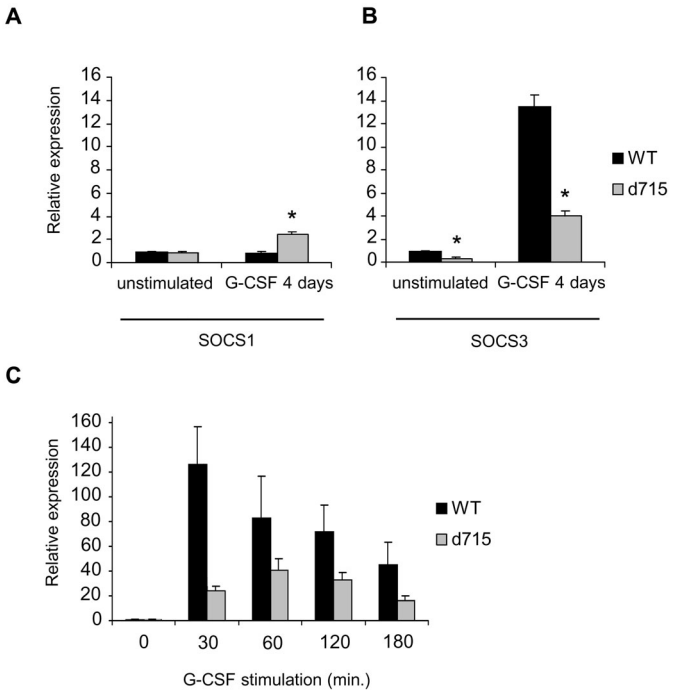

Figure 2: Reduced SOCS3 but not SOCS1 expression in *gcsfr-d715* mice. WT mice and *gcsfr-d715* littermates were injected with vehicle or G-CSF for four consecutive days. RNA was isolated and SOCS1 (A) and SOCS3 (B) transcript levels were measured by quantitative RT-PCR. Expression of RNase inhibitor was used for normalization of the data. SOCS levels were expressed relative to untreated WT mice. Data shown are mean + SEM of three experiments (*: Difference between WT and *gcsfr-d715* animals is significant, Students' t-test: $p < 0.05$). C) SOCS3 expression in isolated bone marrow cells, growth factor-deprived for 4 hrs and then stimulated with G-CSF in vitro for the indicated times. SOCS3 levels were expressed relative to growth factor-deprived cells. Data shown are mean + SEM of four experiments, difference between WT and G-CSF-R-d715 is significant (Students' t-test: $p<0.05$).

altered signaling in the G-CSF-R-d715 cells. These results establish that SOCS3 is the principle SOCS protein induced by G-CSF and that C-terminal truncation of the G-CSF-R results in a significantly reduced ability of the receptor to induce SOCS3.

G-CSF-R truncation relieves the suppressive effects of SOCS3 on G-CSF-induced STAT5 but not STAT3 activation

We next studied the consequences of SOCS3 expression on G-CSF-induced activation of STAT5- and STAT3-luciferase reporter constructs in HEK293 cell transfectants expressing either WT G-CSF-R or G-CSF-R-d715. G-CSF-induced

STAT5 activity by WT G-CSF-R was dose dependently inhibited by SOCS3 (Figure 3A). In contrast, STAT5 activity induced by G-CSF-R-d715 was not affected, even at the highest expression levels of SOCS3. This finding can be reconciled with recent reports showing that Y729 in G-CSF-R is the major docking site for SOCS3, a residue that is lacking in G-CSF-R-d715 (35, 36). Surprisingly, STAT3 activation by G-CSF-R-d715 remained highly sensitive to inhibition by SOCS3 (Figure 3B). These data establish that Y729, while essential for downregulation of STAT5, is dispensable for the inhibitory effects of SOCS3 on G-CSF-induced STAT3 activation.

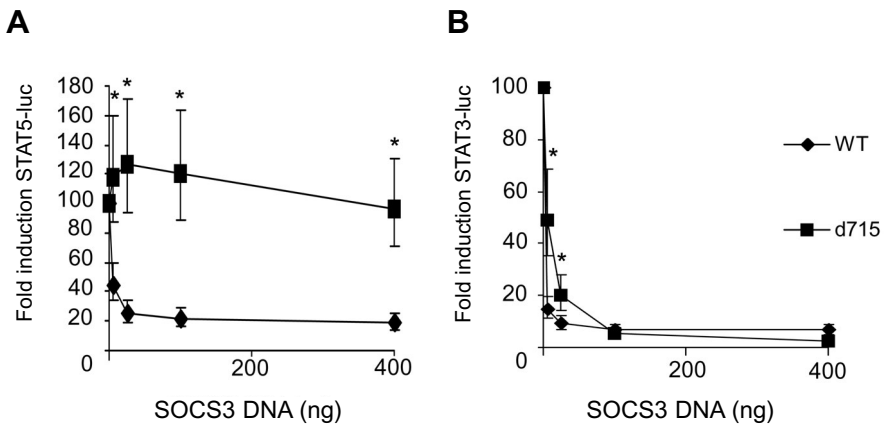

Figure 3: STAT5 activation by the G-CSF-R-d715 is insensitive for inhibition by SOCS3 whereas STAT3 activation is not. HEK 293 cells transfected with STAT5 or STAT3 luciferase reporter constructs were stimulated with G-CSF for 6 hours and assayed for luciferase activity. G-CSF-induced STAT5 (A) or STAT3 (B) luciferase reporter activity in the absence of SOCS3 was set at 100%. Data are expressed as mean + 95% confidence interval of four independent experiments. (*: differences between WT and G-CSF-R-d715 are significant; $p<0.01$).

Differential effects of SOCS3 on G-CSF-R-d715-mediated STAT3 and STAT5 activation are not due to distinct involvement of upstream tyrosine kinases

G-CSF-R activates the Janus kinases JAK1, JAK2 and Tyk2, but also the Src kinases Lyn and HCK (6-8, 10, 11). Both the JAK and Src kinases can phosphorylate STAT proteins in hematopoietic cells (80, 81). Importantly, SOCS proteins differentially affect these kinases: in contrast to JAK kinases, the Src kinase Lyn is insensitive to SOCS-mediated inhibition (82). We considered a possible scenario in which JAK and Src kinases are differentially involved in the activation of STAT3 and STAT5. In that hypothetical context, G-CSF-induced STAT5 activation by G-CSF-R-d715 would become SOCS-insensitive because,

as a result of the truncation of the receptor C-terminus, involvement of Src activity in the activation of STAT5 might become prevalent. To investigate this possibility, we performed the STAT reporter experiments in the presence of the JAK inhibitor WHI-P154 (83) or the Src inhibitor PP-2 (84). Both STAT3- and STAT5-induced luciferase activity was inhibited by WHI-P154, but not by PP-2, indicating that JAK, but not Src-kinase activity is essential for G-CSF-induced activation of both STAT3 and STAT5 by the WT G-CSF-R (Figure 4). Notably, this remained essentially unchanged when activation was induced via G-CSF-R-d715. Although PP-2 slightly reduced STAT5 activation, G-CSF-R-d715 signaling was still completely dependent on JAK activity.

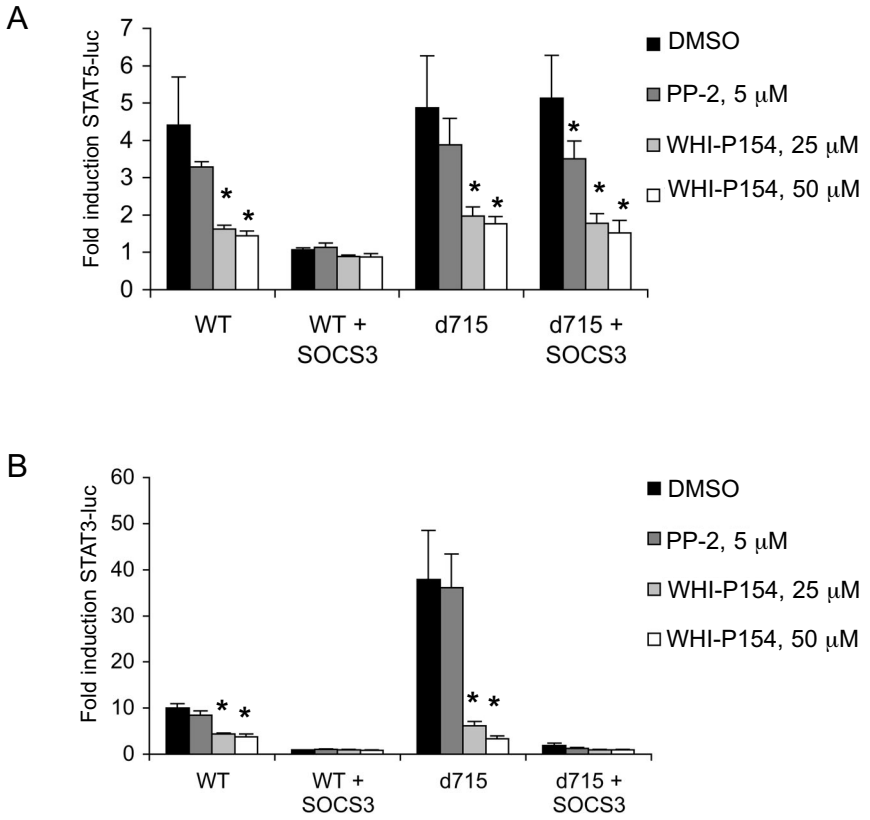

Figure 4: Effects of JAK and Src inhibitors on STAT5 and STAT3 activation by WT G-CSF-R and G-CSF-R-d715. Luciferase assays were performed as in Figure 3. One hour before initiation of STAT5 (A) and STAT3 (B) luciferase reporter assays, the Src inhibitor PP-2 or the JAK inhibitor WHI-P154, dissolved in DMSO, were added to the cells at concentrations indicated. Solvent control cells were treated with DMSO only. Data are expressed as mean + SEM of three independent experiments (*: difference with DMSO-treated control of same group is significant, $p<0.05$).

These results thus exclude the possibility that, in the case of G-CSF-R-d715, the loss of SOCS-mediated inhibition of STAT5 signaling is caused by altered involvement of tyrosine kinases (e.g., Lyn instead of JAK) as a consequence of the receptor truncation. We have previously published data from EMSA assays showing that activation of STAT3 by G-CSF-R-d715 is decreased compared to the WT G-CSF-R when measured between 0-60 minutes after stimulation. This is due to the partial lack of STAT3 recruitment mechanisms (31). We observed that activation of STAT3 by G-CSF-R-d715 in the luciferase reporter assay was increased relative to the WT G-CSF-R (Figure 4B). This could be directly linked to the defective receptor internalization of G-CSF-R-d715 (data not shown). Apparently, the internalization defect of G-CSF-R-d715 causes reduced off-switch of signaling, which results in increased accumulation of luciferase activity during the 6 hr time period of the experiment. However, despite this increased STAT3 activation this signal remains fully sensitive for inhibition by SOCS3 (Figure 3B and 4B).

Defective G-CSF-R internalization reduces sensitivity to SOCS3 only when combined with the loss of Y729

G-CSF-R-d715-induced STAT5 activation was considerably more resistant to the suppressive effects of SOCS3 than was the case for a full length G-CSF-R mutant lacking the SOCS3 docking site Y729, e.g., G-CSF-R-Y729F (Figure 5). This could be suggestive of a second, tyrosine independent mechanism of SOCS3 recruitment via the G-CSF-R C-terminus or relate to defective ligand-induced internalization of G-CSF-R-d715 (27, 31, 85). To directly address this issue, we compared the SOCS3 sensitivity of WT G-CSF-R and G-CSF-R-d715 with that of G-CSF-R-d749-769 (Figure 1A). This mutant is as defective in internalization as G-CSF-R-d715, due to the lack of two internalization domains (32). As shown in Figure 5, SOCS3 inhibits G-CSF-R-d749-769 as effectively as WT G-CSF-R, indicating that the loss of receptor internalization per se does not alleviate STAT5 inhibition by SOCS3. However, mutation of the SOCS3 recruitment site Y729 in this internalization defective mutant (G-CSF-R-d749-769F) resulted in a complete loss of SOCS3-mediated STAT5 inhibition. These results provide evidence for two distinct mechanisms of SOCS3 recruitment to the G-CSF-R, one that is independent of internalization (via Y729) and one that requires internalization. Possibly this latter mechanism involves direct interaction of SOCS3 to JAKs. Both of these mechanisms are disrupted in G-CSF-R-d715 (Figure 5).

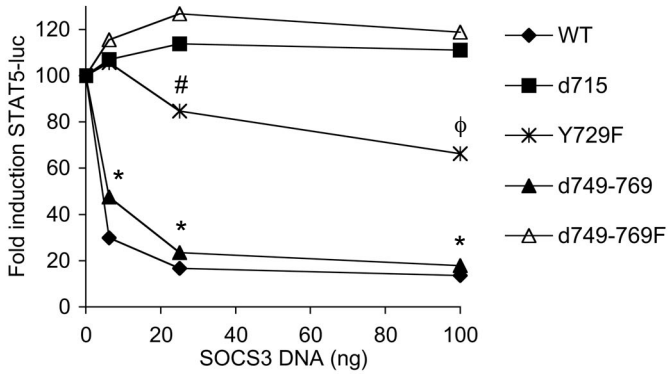

Figure 5: Reduced internalization of truncated G-CSF-R mutants alleviates inhibition by SOCS3 only in the absence of Y729. Comparison of G-CSF-R mutants with normal (WT and Y729F) and defective (d715, d749-769 and d749-769F) internalization kinetics for sensitivity to SOCS3-mediated inhibition of STAT5 luciferase reporter activity. STAT5 luciferase reporter assay was performed as described in Figure 3. Data are expressed as mean of at least two independent experiments with triplicate measurements. (*: difference between WT and d749-769 versus all other G-CSF-R mutants is significant, $p < 0.01$. #: difference between Y729F and d749-769F: $p = 0.07$. ϕ: difference between Y729F versus d715 and d749-769F is significant $p<0.02$).

Y729 is a combined recruitment site for SOCS3 and SHP-2

For a number of receptors it has been demonstrated that SOCS3 recruitment sites are also SHP-2 docking sites (57-60). By combined immunoprecipitation (IP) and Western-blotting (WB), we tested whether SHP-2 binds to Y729 of G-CSF-R. We performed these experiments with GFP-tagged receptor constructs and used anti-GFP antibodies for immune detection. The GFP-tagged receptors were shown to behave identically as untagged receptors with respect to proliferation, differentiation and activation of STAT3 and STAT5 (32). Stimulation with G-CSF induced co-immunoprecipitation of SHP-2 with the WT-G-CSF-receptor (Figure 6A, lanes 1 and 2). Truncation of the receptor at aa 715 resulted in a complete loss of the interaction with SHP-2 whereas truncation at aa 735 did not affect the SHP-2-G-CSF-R interaction (Figure 6, lanes 3 and 4). This result strongly supports the notion that Y729, the only tyrosine present in this region, is a binding site for SHP-2. To determine the relative contribution of Y729 to SHP-2 recruitment to full length G-CSF-R, we also tested G-CSF-R mutant d715-735 (Figure 1) in co-immunoprecipitations. Although this mutant demonstrated a significantly reduced SHP-2 binding compared to WT G-CSF-R, it clearly bound

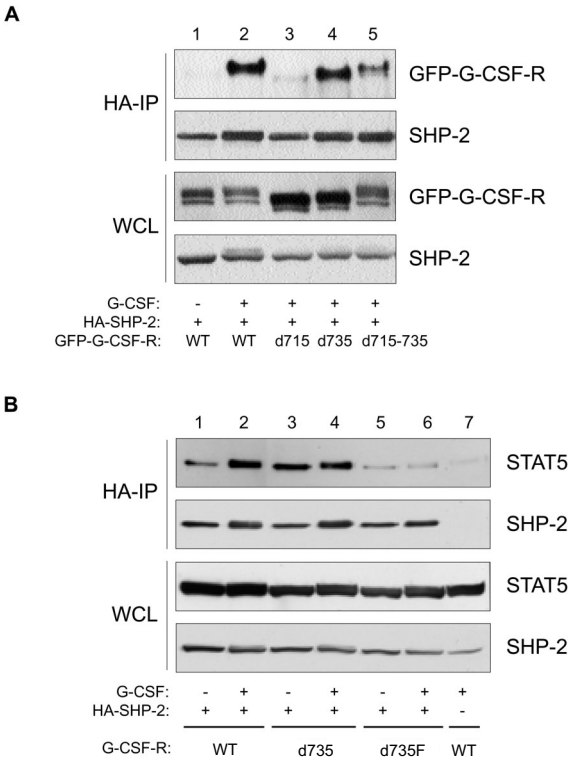

Figure 6: SHP-2 associates with distinct regions of the G-CSF-R and Y729 is required for the formation of a SHP-2-STAT5 complex. A) HA-SHP-2 immunoprecipitations (IP) of Phoenix E cells expressing GFP-tagged G-CSF-R mutants. Cells were starved overnight (-) and stimulated for 10 minutes with G-CSF. As a control, expression of GFP-G-CSF-R and SHP-2 in whole cell lysate (WCL) is shown in the lower two panels. B) HA-SHP-2 IP's of Phoenix E cells expressing different G-CSF-R mutants and STAT5 were performed as described in Figure 6A. Lane 7 is a HA-IP in the absence of HA-SHP-2 demonstrating the specificity of the IP.

more SHP-2 than did G-CSF-R-d715 (Figure 6, lane 5). Taken together, these results support the notion that Y729 of G-CSF-R forms a combined SOCS3 and SHP-2 recruitment site and show that an alternative mechanism of SHP-2 binding to the C-terminal region (aa 736-813) of G-CSF-R exists. The latter mechanism possibly involves recruitment of SHP-2 via Y764 (20).

Y729 of the G-CSF-R is required for the formation of a SHP-2-STAT5 complex

It was recently shown that SHP-2 can interact with STAT5, resulting in dephosphorylation and inactivation of STAT5 (86, 87). Given the requirement of Y729 for recruitment of SHP-2 to the G-CSF-R, we investigated if the formation of a SHP-2-STAT5 complex would be dependent on the presence of Y729 of the

G-CSF-R as well. As shown in Figure 6B, stimulation of the WT and G-CSF-R-d735 indeed results in co-immunoprecipitation of STAT5 with SHP-2 (lanes 1 to 4). However, in the absence of Y729 formation of this complex is disrupted (lane 5 and 6), demonstrating the importance of Y729 of the G-CSF-R for the formation of a SHP-2-STAT5 complex.

Discussion

We investigated whether and by which mechanism(s) altered susceptibility to the inhibitory effects of SOCS proteins contributes to the hyperproliferative signaling of G-CSF-R C-terminal truncation mutants, which are frequently found in SCN patients with disease progression towards AML (24-26). We first concentrated on the regulation of expression of SOCS1 and SOCS3, which represent the SOCS family members with the most prominent negative effects on G-CSF signaling (74). Both SOCS1 and SOCS3 promoters contain STAT binding sites (41, 43, 44, 79) and dominant negative STAT3 blocks SOCS1 as well as SOCS3 mRNA expression, indicating that both SOCS genes are transcriptional targets of STAT3 (41). STAT1 also binds to the SOCS3 promoter (44), but STAT3 is the major STAT protein involved in SOCS3 induction by G-CSF. This was demonstrated most clearly in conditional STAT3 knock-out mice which also lacked G-CSF-induced upregulation of SOCS3 (45). Because the loss of the G-CSF-R C-terminus affects both intensity and duration of the activation of STATs, we investigated SOCS1 and SOCS3 transcript levels in WT and *gcsfr-d715* mice, both before and after 4 days of G-CSF treatment of the mice. G-CSF treatment of WT animals did not affect SOCS1 transcript levels. Receptor truncation resulted in a modest increase in SOCS1 expression after G-CSF stimulation, possibly due to the increased STAT5 activation by G-CSF-R-d715 (31). This is in agreement with data showing that IL-3-induced SOCS1 expression is almost completely abrogated in the presence of dominant negative STAT5 (88). SOCS3 mRNA levels on the other hand, increased more than 12-fold in response to G-CSF. SOCS3 levels in *gcsfr-d715* mice were clearly lower than in their WT littermates during "steady state" and upon stimulation with G-CSF in vivo or in vitro. These results establish that distinct regulatory mechanisms for upregulation of SOCS1 and SOCS3 expression exist in the context of the G-CSF-R. Recent studies with conditional STAT3 and SOCS3 knockout strains demonstrated that the levels of neutrophilia in these mice in response to G-CSF treatment are similar to those observed in *gcsfr-d715* mice

(28, 29, 45, 75). This would support the hypothesis that the loss of the negative feedback loop involving STAT3-controlled induction of SOCS3 might suffice to explain the *gcsfr-d715* phenotype. However, in view of previous data showing that STAT3 activation in *gcsfr-d715* mice is only moderately and temporarily reduced (31), it is conceivable that factors other than STAT3 also contribute to G-CSF-induced SOCS3 expression.

In addition to the reduced expression of SOCS3 in the *gcsfr-d715* mice, the truncated G-CSF-R lacks Y729, which is the major recruitment site for SOCS3 (35, 36). While this provided an obvious second mechanism by which the truncated G-CSF-R forms bypass the suppressive effects of SOCS3, this applied to the activation of STAT5 but not STAT3, which still remained SOCS3 sensitive (Figure 3). This result provides a likely explanation for the increased ratio of STAT5/STAT3 activation by G-CSF-R-d715, which has been linked to shifting the proliferation/differentiation balance towards proliferation and extended cell survival (31). Two potential explanations, i.e., differential involvement of upstream tyrosine kinases and differential sensitivity due to altered internalization kinetics were shown to play respectively no or only a limited role in this study. For efficient activation of STAT3, first G-CSF-R tyrosine phosphorylation is required, which creates STAT3 recruitment sites. This indicates that activation of STAT3 is a multistep process. It is conceivable that inhibition of STAT3 is still achieved when recruitment of SOCS3 is suboptimal due to the lack of Y729. In contrast, inhibition of STAT5, which is activated through direct interaction with JAK kinases (89), may require optimal SOCS3 recruitment for complete inhibition. STAT5 activation by G-CSF-R mutant Y729F was inhibited to a considerable extent at the highest levels of SOCS3, which is probably mediated through low affinity interaction of SOCS3 and JAKs.

Moreover, we think that the loss of recruitment of SHP-2 activity to Y729 also plays a major role in the sustained STAT5 activation by G-CSF-R-d715. It was shown for a number of cytokine receptors, e.g., the leptin receptor and the shared cytokine receptor subunit gp130, that SOCS3 and SHP-2 dock to the same phosphotyrosine-based motif (57-60, 90, 91). Our data on the G-CSF-R (Figure 6) corroborate this and implicate Y729 as a common recruitment site for SOCS3 and SHP-2. Because SHP-2 was recently identified as a STAT5 phosphatase, a model can be envisaged in which impaired recruitment of SHP-2 is key to the loss of negative control of STAT5 activation (86, 87). In this scenario, reduced

recruitment of SHP-2 activity to the G-CSF-R would thus result in increased levels of active STAT5 complexes. Indeed, as shown in Figure 6B, G-CSF-R-Y729 is essential for formation of a SHP-2-STAT5 complex which strongly argues for a scenario in which loss of Y729 not only affects SOCS3-mediated inhibition of STAT5 activity but also disrupts formation of a SHP-2-STAT5 complex that has been reported to contribute to STAT5 dephosphorylation.

In conclusion, our study has unveiled a new mechanism by which C-terminal truncation mutants of G-CSF-R, associated with leukemic progression of SCN, attain altered signaling abilities. While originally being attributed mainly to their defective internalization properties (27, 31, 85), the loss of negative feedback projected by SOCS3 and possibly SHP-2 via binding to Y729 of G-CSF-R, have now been identified as additional signaling defects of truncated G-CSF-R. Our results fit into a model in which the combined loss of regulation by SOCS3 and SHP-2 contributes to the perturbed signaling by G-CSF-R-d715 resulting in an increased STAT5/STAT3 activation ratio.

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CHAPTER 5

General discussion

5.1 G-CSF signaling

Elucidation of the signaling pathways that are activated by G-CSF is important for understanding regulation of normal granulopoiesis and will also shed light on regulatory defects that underlie diseases characterized by disturbed granulopoiesis, such as severe congenital neutropenia (SCN) and acute myeloid leukemia (AML). As introduced in Chapter 1 of this thesis, major signaling mechanisms activated by G-CSF, such as recruitment of STAT3 and intermediates of the Ras pathway, depend on recruitment via one or more of the four conserved tyrosines in the intracellular domain of the G-CSF-R. In this thesis, the role of receptor tyrosine-mediated signaling mechanisms in controlling G-CSF responses was further addressed. In Chapter 2, the individual contribution of the G-CSF-R tyrosines in primary myeloid progenitors was examined by transducing G-CSF-R knockout bone marrow cells with a G-CSF-R tyrosine null mutant and single tyrosine add-back mutants. These studies established that Y764 mediated strong proliferative and self-renewal signals in colony assays and suspension cultures, which could be linked to activation of the ERK pathway. In contrast, Y729 projected major inhibitory effects on G-CSF-induced colony formation and maintenance of myeloid progenitor cells in suspension culture, which was found to be associated with recruitment of the suppressor of cytokine signaling SOCS3. In experiments described in Chapter 3, the effects of different SOCS proteins on G-CSF signaling were investigated in further detail, while in Chapter 4 the consequences of C-terminal truncations of the G-CSF-R found in patients with SCN/AML on the suppressive action of SOCS3 are reported. These studies have left a number of issues that require further investigation, some of which will be discussed in this chapter.

Although SOCS1 and SOCS3 are prominent inhibitors of Jak/STAT signaling, they may also affect other pathways. For instance, SOCS1 inhibits c-kit and the FLT3 receptor, 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 (1-4). SOCS3 has been shown to bind to the Src-like tyrosine kinase Lck, FGF receptor and Pyk2 (4). More recently Cacalano et al. 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 (5). How these alternative mechanisms affect G-CSF responses of myeloid progenitor cells is unknown.

5.2 SHP-2 versus SOCS3 mediated inhibition via G-CSF-R-Y729

The fact that G-CSF-R-Y729 is an important recruitment site for both SOCS3 and SHP-2 (Chapters 2 and 4), 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 (6-9). A mouse strain with gp130 lacking the critical residue for SOCS3 and SHP-2 binding (Y757 or 759) resulted in arthritis-like joint disease, with hyperresponsive T-cells and autoantibody production (10). Although this model demonstrated that the combined recruitment site for SHP-2 and SOCS3 is crucial for balanced signaling, the individual roles of SHP-2 and SOCS3 remained unclear (10).

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 (11, 12). 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 (13). Others used HEK 293 cells transfected with SOCS3 or SHP-2, and mice expressing truncated gp130 that retained Y759 but had severely reduced SOCS3 expression, to demonstrate that SOCS3 but not SHP-2 inhibited leptin and gp130 signaling (7, 14, 15). Fairlie et al. addressed this issue by generation of a gp130 mutant that recruited SHP-2 but not SOCS3 by changing the amino acid context around Y757. This receptor showed enhanced responses to cytokine stimulation, indicating that recruitment of SHP-2 is not involved in downregulation of gp130 signaling (16). More recently, Fischer et al. also suggested that SOCS3 mediates desensitization of gp130 signaling in HEPG2 cells. They postulated that SOCS3 has to be induced first and subsequently inhibits IL-6 signaling at a later stage (17).

A direct comparison of the data from these different models with those reported in this thesis on the G-CSF-R is complicated for two reasons. First, Y759 of gp130 is also involved in the activation of the ERK route via SHP-2 (18) whereas such a role for Y729 of the G-CSF-R could not be demonstrated (19). Second, 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 could not be tested in these models. Future studies, in which e.g., siRNA-mediated knockdown

technology is used, should give a better insight in the individual and combined contribution of SOCS3 and SHP-2 in G-CSF-controlled granulopoiesis.

5.3 Contribution of SOCS1 to lineage choice?

In the experiments described in Chapter 3, it was shown that introduction of SOCS1 or SOCS3 in primary bone marrow cells drastically inhibits G-CSF-induced colony formation, establishing their role as suppressors of proliferation in myeloid progenitor cells. This was recently confirmed by others, who generated mice that lack SOCS3 in their hematopoietic cells. These animals demonstrated increased size of G-CSF-dependent colonies and developed neutrophilia or even inflammatory neutrophil infiltration into multiple tissues, illustrating the importance of SOCS3 in controlling G-CSF-induced granulopoiesis (20, 21). In addition we noted that introduction of SOCS1 resulted in a partial shift in the composition of the G-CSF-colonies towards the monocytic/macrophage-lineage. This applied particularly to SOCS1 and was not observed for SOCS3 (Figure 1, A and B). These findings might suggest that expression of SOCS1 in myeloid progenitors at a certain level favors macrophage development at the expense of differentiation towards neutrophils in response to G-CSF. Interestingly, two studies, of which one was performed with primary

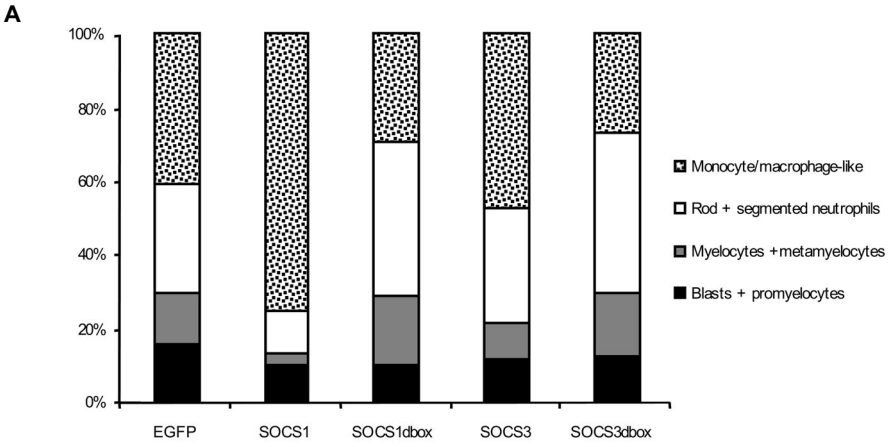


Figure 1: Introduction of SOCS1 stimulates macrophage-like differentiation of bone marrow cells grown on G-CSF, depending on the presence of the SOCS box. A) Differential counts of May Grünwald-Giemsa stained cells harvested from colony cultures of primary bone marrow cells transduced with pBabe constructs with EGFP (control), SOCS1, SOCS1dbox, SOCS3 or SOCS3dbox. Data are the mean of at least two independent experiments.

bone marrow as well, have shown that increased G-CSF-induced differentiation into the macrophage lineage in myeloid cells is also observed when cells express a G-CSF-R mutant in which Y729 is mutated (Y729F) (22, 23). Because this mutant remains sensitive for inhibition by SOCS1 (Figure 2), but lacks sensitivity to SOCS3 and SHP-2, SOCS1 is the most prominent negative feedback mechanism that is still intact in these cells. Possibly, feedback mediated by SOCS1, when not accompanied by signals of SOCS3 or SHP-2, facilitates a differentiation shift from neutrophilic towards macrophage-like differentiation. This hypothesis is corroborated by recent observations in mice lacking SOCS3 in their hematopoietic progenitors. These animals demonstrated an increase in the number and proportion of macrophages in G-CSF-induced colonies (21). Taken together this suggests that SOCS proteins are not merely suppressors of signaling, but also contribute to the control of lineage choices during hematopoietic development. Recently, similar roles for SOCS-mediated negative feedback in balancing T-cell differentiation (24, 25) and balancing IL-6 signaling as pro- or anti-inflammatory response have been proposed (26-28).

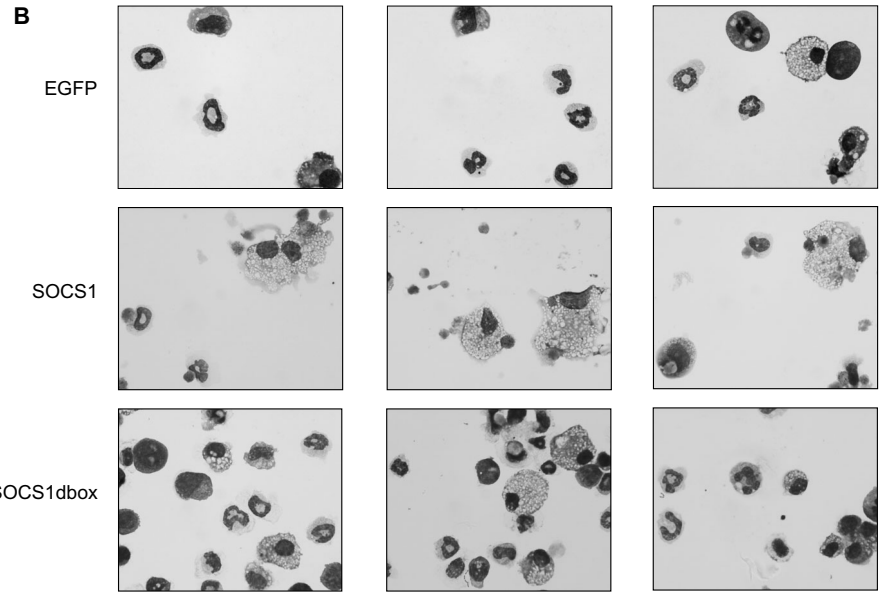


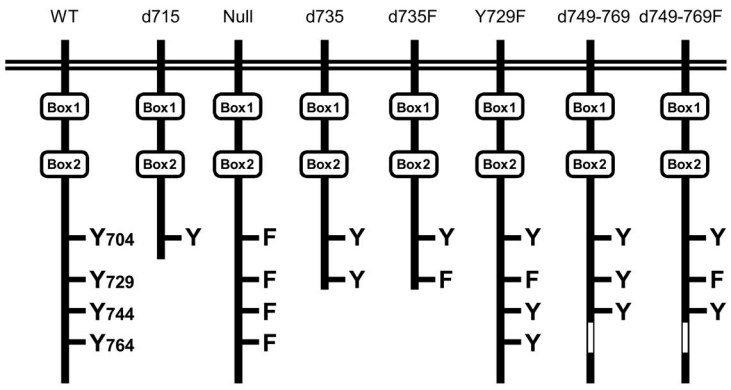
Figure 1: Introduction of SOCS1 stimulates macrophage-like differentiation of bone marrow cells grown on G-CSF, depending on the presence of the SOCS box. B) Photomicrographs of May Grünwald-Giemsa stained cells harvested from colony cultures of primary bone marrow cells transduced with pBabe constructs with EGFP (control), SOCS1 or SOCS1dbox. Magnification: 1000x.

5.4 Differential effects of SOCS inhibition on G-CSF-induced STAT activation revealed by mutation of critical domains in the G-CSF receptor

A surprising finding that emerged from the work presented in this thesis is that the mechanisms by which SOCS1 and SOCS3 suppress G-CSF-induced activation of different STAT proteins are markedly distinct. This became evident by studying the effects of SOCS1 and SOCS3 on a panel of G-CSF-R mutants. The first prominent difference is seen when WT G-CSF-R and mutant 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 by G-CSF-R-d715 is completely lost (Figure 2). 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 SOCS1 and SOCS3 on more subtle G-CSF-R mutants provided some leads about the possible nature of these mechanisms. Data of these experiments are summarized in Figure 2.

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 (4, 29, 30). 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, 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 not able to abrogate STAT5 signaling by G-CSF-R-d735 unless combined with another mechanism mediated via Y729 or the G-CSF-R C-terminus. As discussed in chapter 4, 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. The finding that Y729 is not critical for SOCS1 effects 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 (Chapter 4).



		WT	d715	Null	d735	d735F	Y729F	d749-769	d749-769F
STAT3	SOCS1	+++	+++	ND	+++				
	SOCS3	+++	++	++	+++				
STAT5	SOCS1	+++	-	+++	+++	-	+++	+++	++
	SOCS3	+++	-	+	+++	-	+	+++	-

Figure 2: Schematic representation of the intracellular domain of G-CSF mutants and their sensitivity to SOCS1- and SOCS3-mediated inhibition of STAT3 and STAT5 signaling. Boxes 1 and 2 represent sub-domains conserved in the hematopoietin superfamily. The white region in G-CSF-R-d749-769 and -d749-769F represents the deleted receptor internalization region. - = No inhibition; +++ = maximal inhibition; ND = not determined.

5.5 G-CSF receptor internalization and SOCS-mediated inhibition

Previous studies have demonstrated that G-CSF-R-d715 is severely hampered in both steady state and ligand-induced receptor internalization (31, 33). As reported in Chapter 4, lack of G-CSF-R-d715 internalization contributes to resistance to SOCS3, although this effect is seen only when Y729 is lacking. We therefore considered the possibility that defective internalization combined with the loss of

Y729, contributed to loss of SOCS1-mediated inhibition of STAT5 activation by G-CSF-R-d715. To address this question, we tested the effects of SOCS1 on G-CSF-R-d749-769, which retains Y729 and most of the receptor C-terminus, but is equally hampered in its internalization as G-CSF-R-d715 (30). We found that SOCS1 still completely inhibits STAT5 activation by the G-CSF-R-d749-769 and that loss of Y729 (G-CSF-R d749-769F) in combination with defective internalization only marginally reduces inhibition by SOCS1 (Figure 2). This supports the notion that reduced receptor internalization does not contribute to the lack of sensitivity of G-CSF-R-d715 for SOCS1.

The experiments described in this thesis have thus identified Y729 as being important for the actions of SOCS1, and SOCS3, and also SHP-2, on G-CSF responses. Because a major part of the data was obtained in a non-hematopoietic cell model (HEK293), it is relevant to ask whether these conclusions hold for myeloid cells. To this end, studies in the myeloid 32D cell system to address the role of Y729 in downregulation of STAT3 and STAT5 signaling have been performed in our lab. Electrophoretic mobility shift assays demonstrated that inhibition of STAT3 activation was marginally affected by the lack of Y729, whereas downregulation of STAT5 signaling was significantly alleviated by mutation of Y729 (Aarts et al., manuscript in preparation). These experiments confirm the data described in this thesis and argue for a major role for Y729 in the downregulation of G-CSF-induced STAT5 activity also in a myeloid cell context.

5.6 Current model of aberrant signaling of G-CSF-R-d715

The results presented in this thesis have shed further light on aberrant signaling by truncated G-CSF-R found in SCN/AML patients (G-CSF-R-d715). In addition to the previously reported defects in internalization, altered intracellular distribution (31, 32, 33), and the loss of STAT3 recruitment sites (Y744 and C-terminus)(22, 34) it is clear that altered negative feedback by SOCS1, SOCS3, and SHP-2 also plays a major role in the aberrant signaling properties of G-CSF-R-d715. This rather complex model will be explained step-by-step in Figure 3. Since SOCS1 expression is not induced during G-CSF-induced neutrophilic differentiation, only the roles of SHP-2 and SOCS3 are presented in the model.

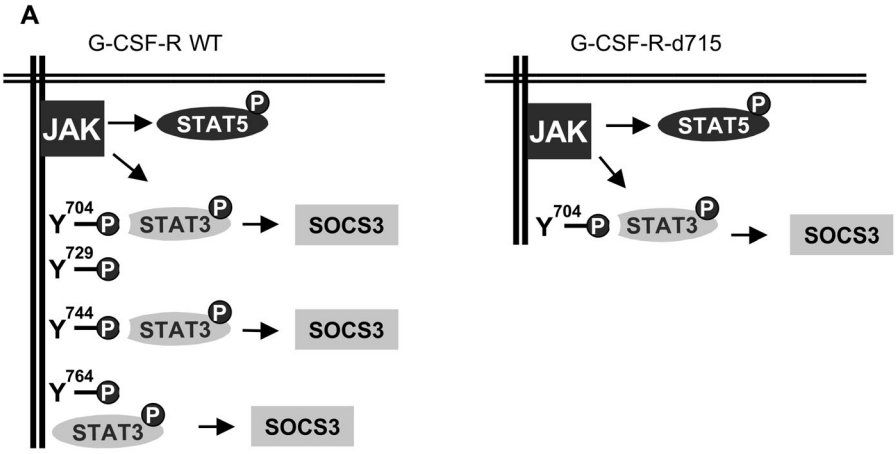


Figure 3 A: Reduced SOCS3 expression in G-CSF-R-d715 cells. Since SOCS3 is a STAT3 target gene (35), the reduced STAT3 activation of G-CSF-R-d715 contributes to the reduced expression of SOCS3 in G-CSF-R-d715 cells (Chapter 4).

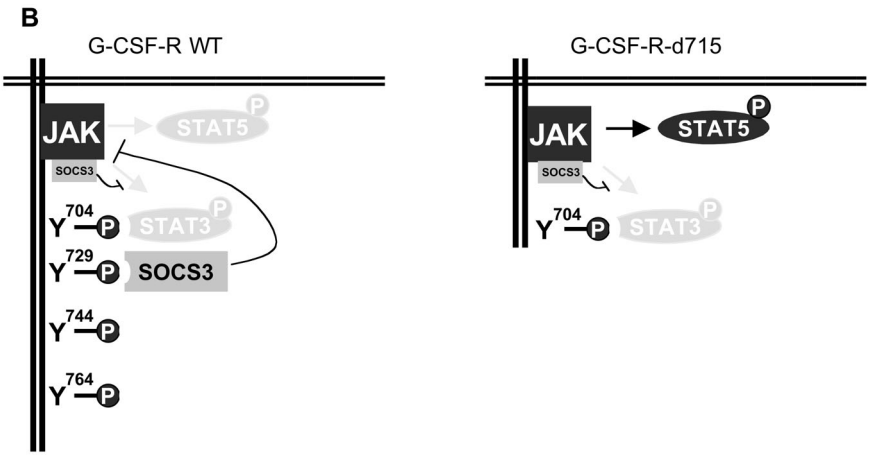


Figure 3 B: SOCS3 inhibits STAT3 but not STAT5 activation by G-CSF-R-d715. SOCS3 inhibits STAT3 and STAT5 activation by the WT G-CSF-R, depicted in light grey in the left panel. STAT3 activation by the G-CSF-R-d715 is still sensitive to inhibition by SOCS3. It is conceivable, based on the SOCS3 titration experiments in luciferase assays, that even at low SOCS3 levels, inhibition of STAT3 activation remains largely intact (shown in light grey). This suggests that SOCS3, in the absence of Y729, is able to interact with JAK and thus inhibits STAT3 activation. In contrast, STAT5 activation by G-CSF-R-d715 is completely resistant for negative feedback by SOCS3.

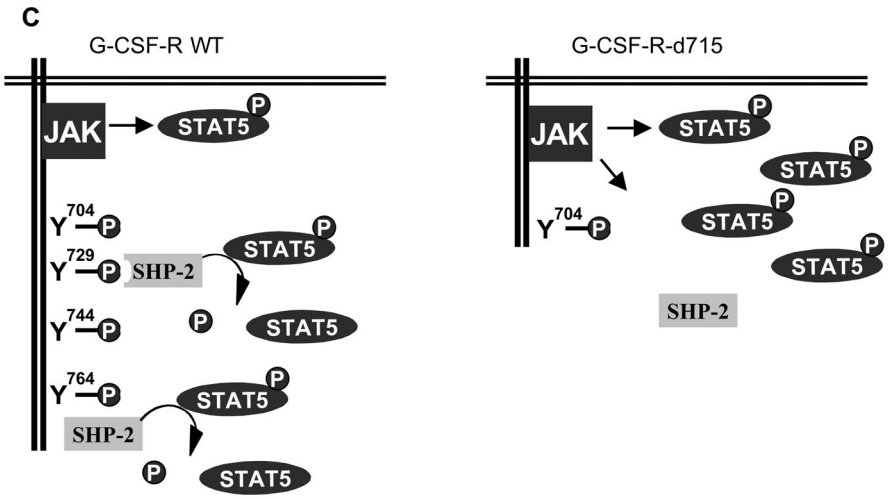


Figure 3 C: Loss of SHP-2 recruitment contributes to increased STAT5 activity by G-CSF-R-d715. Recruitment of SHP-2 is drastically impaired in the absence of Y729 and the G-CSF-R C-terminus. Because SHP-2 has been demonstrated to dephosphorylate STAT5, this will further contribute to loss of inhibition of STAT5 activation.

Via the mechanisms shown in Figure 3, STAT3 activation by G-CSF-R-d715 is inhibited whereas STAT5 activation is not. This results in increased levels of activated STAT5. Since STAT3 is implicated in mediating cell cycle arrest (36) whereas STAT5 acts a positive regulator of G-CSF-induced cell proliferation and survival (37), the increased STAT5/STAT3 activation ratio results in enhanced proliferation and survival and reduced growth arrest, which interferes with proper neutrophil differentiation.

5.7 Concluding remarks

While initially regarded as specific inhibitors for JAK-STAT signaling, it has become clear that SOCS proteins are involved in different signal transduction pathways as well, and that they may also act via cooperation with other signaling intermediates. Since the SOCS proteins are potent inhibitors capable of interfering with known critical players in different diseases, it is interesting to speculate about possible therapeutic applications of these proteins. Given the important roles of SOCS family members in signaling by a large number of cytokines and growth factors, the potential use of SOCS-derived signaling

inhibitors requires development of techniques that allow exact targeting of the affected cell populations in patients. In addition, achieving the right time kinetics of the inhibition is likely to be crucial in mediating appropriate negative feedback as well. Interestingly, Shouda et al., were able to show proof of principle (38). They demonstrated that injection of an adenoviral construct encoding SOCS3 into the joints of mice suffering from antigen-induced arthritis drastically reduced the severity of the arthritis compared to mice injected with control adenovirus. Although these results look promising, successful application of SOCS-derived inhibitors in myeloid progenitors expressing truncated G-CSF receptors will be considerable more complicated. However, the identification of lack of SOCS- and SHP-2-mediated negative feedback in G-CSF-R-d715 signaling is important for future studies aimed at correcting the aberrant granulopoiesis caused by loss of the G-CSF-R C-terminus.

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Summary & Samenvatting
(Summary in Dutch)

Summary

G-CSF is the most important growth factor involved in the production of neutrophilic granulocytes. Signaling routes activated upon ligand binding to the G-CSF receptor (G-CSF-R) control survival, proliferation and differentiation of myeloid progenitor cells towards mature neutrophils. Elucidation of the signaling pathways involved in this process is important for understanding normal neutrophil development and for better insights in diseases with perturbed neutrophil production such as neutropenia and myeloid leukemia.

In Chapter 1 of this thesis an overview of the current knowledge on the G-CSF-R is given and the signaling pathways activated by G-CSF are introduced. Upon G-CSF binding, kinases of the JAK and Src kinase families become active and the four tyrosines of the receptor, located at positions 704, 729, 744 and 764 are phosphorylated. Multiple signaling pathways are subsequently activated via recruitment of intermediates to these phosphorylated tyrosines as well as via tyrosine independent mechanisms. A well known signaling route activated by the G-CSF-R is the JAK-STAT pathway. The most prominent STATs that are activated by G-CSF are STAT3, implicated in mediating a cell cycle arrest, and STAT5, which contributes to proliferation and cell survival.

Chapter 2 focuses on the role of the tyrosines residues of the G-CSF-R in primary cells. Bone marrow cells of G-CSF-R knockout mice were transduced with G-CSF-R-constructs: WT, mutant Null (that lacks all four tyrosines), or single tyrosine add-back mutants (that each retain one tyrosine residue). Colony assays of transduced cells showed that G-CSF-R tyrosines are dispensable for proliferation of myeloid progenitor cells but are required for maintenance of clonogenic capacity following repetitive replatings. In addition, the presence of only Y764 strongly increased colony formation, proliferation and self-renewal capacity of the cells. Inhibition of the ERK-MAPkinase pathway in these cells blocked proliferation suggesting that ERK activity mediated via Y764 is important for G-CSF-induced proliferation of primary cells. In contrast, the presence of only tyrosine 704, 729 or 744 reduced colony growth. Tyrosine 729 was identified as the single G-CSF-R tyrosine residue involved in recruitment of SOCS3, a member of the negative regulatory SOCS protein family. SOCS3 is capable of downregulating G-CSF-induced STAT5 activity. Taken together these observations indicate that G-CSF-R tyrosines, although dispensable for G-CSF-

induced colony formation, activate additional signaling pathways that regulate maintenance and outgrowth of myeloid progenitor cells.

In Chapter 3 the role of SOCS-mediated negative feedback on G-CSF-signaling is examined in further detail. SOCS1 and SOCS3, but not CIS and SOCS2 were capable of inhibiting G-CSF-induced STAT3 and STAT5 activation. During G-CSF-induced differentiation of myeloid 32D cells, SOCS3 mRNA levels are upregulated whereas SOCS1 expression remains comparable to levels in proliferating cells. This indicates that SOCS3 is the most important SOCS protein responsible for negative feedback during G-CSF-induced neutrophil development. SOCS proteins contain multiple conserved domains that enable them to inhibit signaling via different mechanisms such as inhibition of JAK kinase activity or targeting of proteins for proteasomal degradation. We demonstrated that presence of the SOCS box of SOCS1 and SOCS3 is required for SOCS protein stabilization and for efficient inhibition of STAT signaling and colony formation in response to G-CSF. These experiments suggest that proteasomal degradation, mediated via the SOCS box is more important for downregulation of STAT5 signaling than for downregulation of STAT3 signaling.

Approximately 20% of severe congenital neutropenia (SCN) patients express truncated G-CSF-receptors, such as G-CSF-R-d715, that lack a significant part of the C-terminus. These receptors show multiple aberrations in G-CSF-induced signaling including lack of receptor internalization and an increased STAT5/STAT3 activation ratio. Mice expressing the truncated G-CSF-R-d715 are neutropenic and hyperreactive to G-CSF-treatment, which results in neutrophilia. In Chapter 4 we addressed whether lack of negative feedback by SOCS proteins contributes to the phenotype of G-CSF-R-d715 mice. The expression of SOCS3 mRNA is significantly reduced in cells expressing G-CSF-R-d715. This is probably, at least in part, due to the reduced activation of STAT3 by G-CSF-R-d715. STAT5 activation by G-CSF-R-d715 is insensitive for inhibition by SOCS3, which is likely due to the loss of the SOCS3 recruitment site Y729 in the receptor C-terminus. In contrast, inhibition of STAT3 activation by SOCS3 remains almost completely intact upon loss of the G-CSF-R C-terminus. In addition, we demonstrated that G-CSF-R Y729 is not only a SOCS3 recruitment site but is also important for recruitment of the phosphatase SHP-2 to the G-CSF-R and for the formation of a complex between SHP-2 and STAT5. Truncation of the G-CSF-R at aa 715 therefore disrupts recruitment of both

SOCS3 and SHP-2. Since SHP-2 can act as a STAT5 phosphatase, it is conceivable that loss of SHP-2 recruitment contributes to increased activation of STAT5 of G-CSF-R-d715 as well. These results give an explanation for the increased STAT5/STAT3 activation ratio of G-CSF-R-d715, which is implicated in increased survival and proliferation and decreased differentiation of this SCN-derived G-CSF-R mutant.

In chapter 5 the findings of this thesis are discussed in a broader perspective. Additional data are provided that suggest a possible role for SOCS-mediated feedback in directing lineage choice between neutrophilic and macrophage differentiation. In addition, a model is introduced that explains how loss of SHP-2 and SOCS3-mediated negative feedback contributes to the phenotype of the G-CSF-R-d715 mouse.

Samenvatting

G-CSF is de belangrijkste groeifactor betrokken bij de aanmaak van neutrofiele granulocyten. Signaalroutes die geactiveerd worden na binding van G-CSF aan de G-CSF receptor (G-CSF-R) controleren overleving, celdeling en uitrijping van myeloide voorloper cellen tot rijpe neutrofielen. Het in kaart brengen van de signaal-transductieroutes die betrokken zijn bij deze processen is belangrijk voor een goed begrip van de normale ontwikkeling van neutrofielen. Dit is ook van belang voor het verkrijgen van een beter inzicht in ziektes met afwijkingen in de aanmaak van neutrofielen zoals neutropenie en myeloide leukemie.

In hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van de huidige kennis over de G-CSF-R en worden de signaalroutes die geactiveerd worden door G-CSF geïntroduceerd. Na binding van G-CSF aan de receptor worden JAK- en Src kinases geactiveerd en de 4 tyrosines, op posities 704, 729, 744 en 764 gefosforyleerd. Diverse signaal-transductieroutes worden geactiveerd, zowel via recrutering aan de gefosforyleerde tyrosines, als via tyrosine-onafhankelijke mechanismen. Een van de best bestudeerde signaalroutes van de G-CSF-R is JAK-STAT activatie waarbij STAT3 en STAT5 het meest prominent geactiveerd worden. STAT3 activatie draagt bij aan het tot stand komen van een celcyclus-arrest dat vooraf gaat aan terminale neutrofiele uitrijping van myeloide cellen. Activatie van STAT5 is daarentegen betrokken bij overleving en proliferatie van myeloide cellen.

In studies beschreven in hoofdstuk 2 werd de rol van de tyrosines van de G-CSF-R bestudeerd in primaire cellen. Beenmergcellen van G-CSF-R knockout muizen werden getransduceerd met G-CSF-R constructen: WT, mutant Null (alle 4 de tyrosines ontbreken) of mutanten die ieder nog slechts 1 tyrosine hebben. Koloniekweken van getransduceerde cellen lieten zien dat de tyrosines niet nodig zijn voor de proliferatie van myeloide voorlopercellen, maar dat tyrosines wel vereist zijn voor het behouden van de capaciteit tot kolonievorming bij herhaald her-uitplaten van de cellen. Aanwezigheid van alleen Y764 gaf een sterke toename van kolonieaantallen, -groei en het vermogen tot zelf-vermeerdering van de cellen. Het remmen van de ERK MAP kinase route in deze cellen blokkeerde de proliferatie. Dit duidt erop dat ERK-activatie via Y764 een belangrijke rol speelt bij de groei van primaire cellen. Aanwezigheid van alleen tyrosine 704, 729 of 744 daarentegen verminderde het aantal G-CSF-

geïnduceerde kolonies. Tyrosine 729 blijkt de enige bindingsplaats te zijn voor SOCS3, een eiwit uit de suppressor of cytokine signaling (SOCS) familie, die betrokken is bij negatieve terugkoppeling van signaal-transductie van cytokine receptoren. SOCS3 remt de activatie van STAT5 door G-CSF.

In hoofdstuk 3 wordt de remmende werking van SOCS eiwitten op de G-CSF-R verder beschreven. SOCS1 en SOCS3 remden de activatie van STAT3 en STAT5 door G-CSF; CIS en SOCS2 hadden geen remmende werking. Tijdens G-CSF-geïnduceerde differentiatie van myeloide 32D cellen ging de expressie van SOCS3 mRNA omhoog terwijl de mRNA niveaus van SOCS1 vergelijkbaar waren met cellen die bleven prolifereren. Dit wijst erop dat SOCS3 het belangrijkste SOCS eiwit is dat verantwoordelijk is voor negatieve feedback tijdens G-CSF-geïnduceerde differentiatie van neutrofielen. SOCS eiwitten hebben meerdere geconserveerde domeinen die ze gebruiken om op verschillende manieren signaling te remmen. Dit kan onder andere door het induceren van proteasomale afbraak van eiwitten wat gemedieerd wordt door de C-terminale, geconserveerde, SOCS box. In dit hoofdstuk is aangetoond dat de SOCS boxen van SOCS1 en SOCS3 nodig zijn voor de stabilisatie van deze SOCS eiwitten en voor een efficiënte remming van G-CSF-geinduceerde STAT-activatie en kolonievorming. Deze experimenten impliceren verder dat regulatie van proteasomale afbraak via de SOCS box een grotere rol speelt bij remming van activatie van STAT5, dan van STAT3.

Ongeveer 20% van patienten die lijden aan ernstige aangeboren neutropenie brengen getrunceerde G-CSF-receptoren, zoals G-CSF-R-d715, tot expressie. Deze receptoren missen een deel van de C-terminus en vertonen afwijkingen in hun signaal-transductie. Muizen die G-CSF-R-d715 tot expressie brengen lijden aan neutropenie en zijn overgevoelig voor G-CSF stimulatie, wat resulteert in neutrofilie. In hoofdstuk 4 hebben we onderzocht of het ontbreken van negatieve feedback door SOCS eiwitten bijdraagt aan het fenotype van de G-CSF-R-d715 muis. De expressie van SOCS3 mRNA was significant lager in G-CSF-R-d715 cellen. Dit komt mogelijk, in ieder geval deels, door de verminderde STAT3-activatie van G-CSF-R-d715. Activatie van STAT5 door G-CSF-R-d715 werd niet geremd door SOCS3, wat zeer waarschijnlijk komt door het ontbreken van Y729 in de G-CSF-R-d715. Remming van STAT3-activatie daarentegen bleef vrijwel intact in afwezigheid van de G-CSF-R C-terminus. In dit hoofdstuk wordt aangetoond dat Y729 niet alleen een bindingsplaats was voor SOCS3, maar ook

voor het fosfatase SHP-2. Truncatie van de G-CSF-R bij aminozuur 715 verstoorde zowel de recrutering van SOCS3 als van SHP-2. Omdat SHP-2 STAT5 kan defosforyleren, kan het verlies van SHP-2-binding ook bijdragen aan de toegenomen STAT5-activiteit van G-CSF-R-d715. Deze resultaten kunnen de toegenomen ration van STAT5/STAT3-activatie van de G-CSF-R-d715 verklaren, die betrokken is bij verhoogde overleving en proliferatie van cellen die G-CSF-R-d715 tot expressie brengen.

In hoofdstuk 5 worden de gegevens uit de experimenten in een breder kader bediscussieerd. Verder worden aanvullende experimentele gegevens gepresenteerd die wijzen naar een mogelijke rol voor SOCS-gemedieerde negatieve feedback in het reguleren van de neutrofiele differentiatie versus macrofaag-differentiatie. Tot slot wordt er een model gepresenteerd dat verklaart hoe het ontbreken van negatieve feedback door SHP-2 en SOCS3 bijdraagt aan het fenotype van het G-CSF-R-d715 muismodel.

Abbreviations

7-AAD	7-amino actinomycin D
aa	amino acid
AML	acute myeloid leukemia
BC	C-terminal barrel
BN	N-terminal barrel
CFU-G	colony-forming unit-granulocyte
CRH	cytokine receptor homology
EGFR	epidermal growth factor receptor
Epo	erythropoietin
ESS	extended SH2 subdomain
FCS	fetal calf serum
FLT3	fms like tyrosine kinase 3
FKHR	forkhead in rhabdomyosarcoma
FNIII	fibronectin type III
G-CSF	granulocyte colony-stimulating factor
G-CSF-R	granulocyte colony-stimulating factor receptor
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRB2	growth factor receptor-binding protein 2
HCK	hematopoietic cell kinase
Ig	immunoglobulin
IL	interleukin
IP	immuno precipitation
JAK	Janus kinase or just another kinase
KIR	kinase inhibitory region
LIF	leukemia inhibitory factor
MAP kinase	mitogen-activated protein kinase
MDS	myelodysplastic syndrome
OSM	oncostatin M
PI-3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
SCN	severe congenital neutropenia
SH2	src homology region 2
SHC	src homologous and collagen
SHIP	SH2-containing inositol phosphatase
SHP	SH2-domain containing tyrosine phosphatase
SOCS	suppressor of cytokine signaling
STAT	signal tranducer and activator of transcription
tg	transgenic
Tpo	thrombopoietin
WB	Western blotting
WT	wild type

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Curriculum Vitae

Gert-Jan van de Geijn werd geboren op 20 juli 1975 in Nijmegen. Na het behalen van zijn VWO diploma aan het Pax Christi College te Druten in 1993, begon hij in datzelfde jaar met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen, afstudeerrichting toxicologie. Tijdens zijn studie heeft hij stage gelopen op de afdeling biochemie onder begeleiding van Prof. Dr. J.J.H.H.M. de Pont, waar hij cholecystokinine-geïnduceerde calcium signalen in pancreascellen heeft onderzocht. Op de afdeling dermatologie van het Universitair Medisch Centrum St. Radboud te Nijmegen heeft hij onder begeleiding van Dr. J. Schalkwijk onderzoek gedaan naar de protease remmer Trappin-2. Hij heeft zijn studie afgesloten met een stage bij Dr. C. J. Thiele, (Pediatric Branch, National Cancer Institute, National Institutes of Health, Bethesda, USA) waar hij het effect van neurotrofines op de gevoeligheid voor chemotherapie van neuroblastoma cellijnen heeft bestudeerd. In november 1998 is hij begonnen als assistent in opleiding (a.i.o.) bij Prof. Dr. I. P. Touw op de afdeling hematologie van het Erasmus Medisch Centrum te Rotterdam waar hij het onderzoek heeft uitgevoerd dat geresulteerd heeft in dit proefschrift.