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# GRANULOCYTE COLONY-STIMULATING FACTOR: MOLECULAR MECHANISMS OF ACTION DURING STEADY STATE AND 'EMERGENCY' HEMATOPOIESIS

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## Abstract

Neutrophils are phagocytes whose principal function is to maintain anti-bacterial immunity. Neutrophils ingest and kill invading bacteria, releasing cytotoxic, chemotactic and inflammatory mediators at sites of infection. This serves to control the immediate host immune response and attract other cells, such as macrophages and dendritic cells, which are important for establishing long-term adaptive immunity. Neutrophils thus contribute to both the initiation and the maintenance of inflammation at sites of infection. Aberrant neutrophil activity is deleterious; suppressed responses can cause extreme susceptibility to infection while overactivation can lead to excessive inflammation and tissue damage. This review will focus on neutrophil regulation by granulocyte colony-stimulating factor (G-CSF), the principal cytokine controlling neutrophil development and function. The review will emphasize the molecular aspects of G-CSF-driven granulopoiesis in steady state (healthy) conditions and during demand-driven or 'emergency' conditions elicited by infection or clinical administration of G-CSF. Understanding the molecular control of granulopoiesis will aid in the development of new approaches designed to treat disorders of neutrophil production and function.

### Keywords

G-CSF; neutrophils; granulopoiesis; STAT3; infection

## General features of cytokines, neutrophils and their regulation by G-CSF

Infections create physiologic stress in host organisms, threatening both the quality and duration of life. Successful resolution of infection requires a functional immune system to recognize and destroy the invading microbe, without eliciting unnecessary damage to the host. Cytokines are proteins found in vertebrates [1,2] that regulate the development and homeostasis of the immune system. Cytokine production is induced during infection to control the body's immune response. A great deal of attention has focused upon the characterization of cytokines, from

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their three-dimensional atomic structures to whole animal responses. In many cases, however, we lack a detailed understanding of the molecular pathways that mediate a cytokine response under physiologic conditions. The elucidation of these pathways is important for developing future therapies to modulate host immunity during infection and to discover new ways to treat immune system disease.

Granulocyte colony-stimulating factor (G-CSF) is the major cytokine regulator of neutrophilic granulocytes [3]. Neutrophils are an essential cell type in the innate immune system necessary for the clearance of bacterial pathogens. Their critical role is demonstrated by the profound immunodeficiency of individuals with neutropenia, or low circulating neutrophil numbers, a condition that can stem from acquired or congenital origin [4–6]. Recombinant G-CSF has become the main therapeutic agent for the treatment of neutropenia, although recently its use has come under scrutiny for potential connections with leukemia [7–11]. This review will present basic features of G-CSF and its signaling mechanisms in steady state and 'demand-driven' granulopoiesis, and will conclude with a brief summary of the clinical aspects of G-CSF therapy.

## Identification of G-CSF as a neutrophil growth factor

G-CSF was discovered by virtue of its ability to stimulate the growth of neutrophil colonies from human bone marrow progenitor cells *ex vivo*. Human G-CSF (molecular weight ~30,000) was purified from placental-conditioned medium [12] and murine G-CSF (molecular weight ~25,000) was subsequently isolated from medium conditioned by mouse lung samples following in vivo endotoxin treatment [13]. The use of distinct cell types for G-CSF isolation reveals the diversity of tissues that express this cytokine, which include fibroblasts, endothelial cells, monocytes, macrophages and bone marrow stromal cells (reviewed in [14]). The cDNA for human G-CSF was cloned in 1986 using partial amino acid sequence information and oligonucleotide probes to screen the CHU-2 and 5637 tumor cell line libraries [15,16]. The murine G-CSF cDNA was cloned by cross-reactivity to the human cDNA [17], confirming a close relationship between human and mouse proteins.

Isolation of the G-CSF cDNA enabled the production of recombinant protein, which greatly facilitated the study of its biologic properties. Purified and recombinant G-CSF stimulate the proliferation of neutrophil precursors and other myeloid progenitor cells in vitro [12,13,18–21]. G-CSF, however, selectively supports the terminal differentiation of neutrophils relative to other myeloid lineages. G-CSF has also been shown to promote the differentiation of certain myeloid leukemia cell lines [13], but the profound maturation block of most human leukemias precludes its use as an anti-leukemia therapy. Deletion of the murine G-CSF gene revealed physiologic roles for this cytokine in steady state granulopoiesis and in the production of neutrophils under 'emergency' conditions of infection [3,22,23]. A comprehensive review of these topics can be found in references [14,24].

## Expression and action of G-CSF in vivo

Healthy individuals express low G-CSF levels in serum (range, <30–163pg/mL) [25–27]. By comparison, infection induces a substantial elevation in serum G-CSF in humans (range, 30–3,199pg/mL) and mice [25–27]. The high levels of G-CSF produced during infection are coupled with an increase in hematopoietic growth factor activity in serum, as judged by colony-stimulating assays and $^{3}H$ thymidine uptake experiments with bone marrow samples ex vivo [27]. The receptor for G-CSF is expressed in granulocytic precursors and mature neutrophils, with higher receptor levels detected at later stages of maturation [28]. Therefore, the induction of G-CSF serum levels during infection directly stimulates increased proliferation of granulocytic progenitor cells. Committed neutrophil precursors at the myeloblast, promyelocyte and myelocyte stages show elevated rates of proliferation during exposure to G-

CSF in vivo [29,30], indicating that G-CSFR expression in the neutrophil lineage correlates with G-CSF sensitivity. Granulocytic progenitors respond to G-CSF by shortening their passage through the cell cycle, and therefore dividing more frequently [30]. G-CSF is also required for the survival of granulocytic lineage cells [22]. The increased proliferation rate of granulocytic precursors in response to G-CSF results in an elevated proportion of early neutrophil forms (e.g., myeloblast and promyelocyte) in the bone marrow relative to steady state conditions [29,30]. This ability of the bone marrow to respond to the physiologic demand of infection or pharmacological levels of G-CSF during clinical administration is termed 'emergency' or 'demand-driven' granulopoiesis. This response is necessary for a sustained output of circulating neutrophils during infection or G-CSF therapy [3,6,23,31].

In addition to its proliferation- and survival-promoting activity, G-CSF treatment induces a rapid (within 4–24 h) and sustained elevation in absolute peripheral neutrophil numbers. G-CSF shortens the transit time of developing granulocytes through the bone marrow compartment and accelerates the release of neutrophils that have undergone recent cell division [18,29,30,32]. The properties of G-CSF were quickly recognized for their clinical value [33–40] (for reviews, see [6,24,31]). Recombinant G-CSF was shown to decrease the duration of neutropenia in cancer patients receiving chemotherapy [36,37] (for reviews, see [6,31]), which permits more frequent chemotherapeutic treatments to promote cancer regression. G-CSF also served to accelerate the resolution of foot infections in diabetics, thus shortening antibiotic use and hospital stay [41]. Moreover, G-CSF has been found to stimulate an increase in the absolute number of hematopoietic progenitor cells (HPC) in peripheral blood in a process that is known as "stem cell mobilization" [38,39]. Hence, G-CSF is used to treat myelosuppression and its resulting immunodeficiency, as well as to mobilize HPC to peripheral blood, facilitating their collection for transplantation. In this review, we focus solely on neutrophil regulation by G-CSF; excellent reviews of G-CSF-mediated HPC mobilization and clinical ramifications can be found in references [42–45].

## Pathways regulating G-CSF expression

G-CSF production is induced by several inflammatory stimuli that become rapidly elevated during infection such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNFα) and lipopolysaccaride (LPS) [24]. Therefore, pathogen-mediated activation of host pattern recognition receptors via LPS, and the host cytokine response to infection, serve to induce circulating levels of G-CSF (Fig. 1). This in turn stimulates neutrophil production in the bone marrow and neutrophil mobilization to the peripheral circulation. The synthesis of G-CSF appears to be controlled via post-transcriptional and transcriptional mechanisms [46,47]. The G-CSF promoter contains binding sites for NF-κB p65 and NF-IL6/C/EBPβ [48], which are well-characterized transcription factors that participate in many inflammatory and immune 'stress' response pathways. These promoter elements mediate inducible expression of G-CSF, as well as its constitutive regulation in carcinoma cells [49–51]. A recent profiling of signaling protein activation and cytokine release in RAW 264.7 cells led to the prediction that the p38 MAP kinase pathway also plays an important role in G-CSF production [52], a hypothesis that requires formal examination via gene knockdown or deletion experiments.

Interleukin-17 (IL-17) has emerged as an important regulator of G-CSF and neutrophil production in vivo. IL-17 is synthesized by a specialized class of activated $CD4^+$ T cells (Th17) that are best known for their contribution to pathologic inflammation [53–56]. Th17 cells reside in the intestinal lamina propria under steady state conditions [57], and can be induced during infection with *Candida albicans* [58]. Recently a specialized subset of intestinal dendritic cells was found to elicit Th17 differentiation via TGFβ and IL-10 [59], indicating that the gut may be a key source of IL-17 production in basal conditions.

IL-17 signaling through the IL-17 receptor induces expression of G-CSF (Fig. 1), as well as certain CXC-related chemokines [60]. IL-17 stimulates G-CSF- and stem cell factor- (SCF) dependent granulopoiesis and activates neutrophil recruitment during bacterial infection [60–62] (Fig. 1). Thus, in Th17-mediated pathologic inflammation, the neutrophil response elicited by IL-17-dependent regulation of G-CSF may play a role in the initiation and maintenance of inflammation. It is less clear how IL-17 production by resident intestinal Th17 cells affects G-CSF synthesis and neutrophil production under steady state conditions. This is an area that should be explored to determine the contribution of IL-17-elicited neutrophils to mucosal immunity. Regardless, a major function of the IL-17/G-CSF/neutrophil axis is likely to operate during the adaptive phase of an immune response since it is elicited by activated $CD4^+$ Th17 cells. This cytokine cascade may provide a mechanism to stimulate neutrophils during chronic or unresolved infections.

Neutrophil turnover has been shown recently to play an important role in the homeostatic regulation of IL-17 and its control of granulopoiesis [56]. When neutrophil migration into tissues is blocked by adhesion molecule deficiency, macrophages and dendritic cells secrete excessive levels of IL-23, a key cytokine that drives Th17 development and IL-17 production [53–55], leading to increased G-CSF-dependent granulopoiesis (Fig. 1). Neutrophil phagocytosis by macrophages and dendritic cells suppresses their production of IL-23, thus decreasing IL-17 synthesis and G-CSF-dependent granulopoiesis [56] (Fig. 1). Therefore, IL-17 regulates granulopoiesis by its control of G-CSF expression; circulating neutrophils can act in a negative feedback loop to block excessive production of Th17 cells and IL-17. These studies demonstrate that G-CSF synthesis and neutrophil production are under exquisite control. Moreover, G-CSF-dependent neutrophil production is linked to responses in both the innate and adaptive immune systems (Fig. 1). The molecular details of the pathways leading to G-CSF production remain poorly understood, and particularly how these pathways operate in vivo in steady state and infection settings. For example, the signaling mechanisms that are used by IL-17, and its function in natural infection settings, are unresolved. Similarly, we have little knowledge of the mechanisms controlling the transcriptional activation of the G-CSF promoter or G-CSF mRNA turnover, despite the importance of this cytokine in immunity.

## Characterization of the G-CSF receptor (G-CSFR) and its signal transduction mechanisms

The G-CSFR (molecular weight ~100,000–130,000) shares structural similarity with Type I cytokine receptors, containing a conserved cytokine receptor homologous (CRH) domain, an Ig-like domain and three fibronectin type III-like domains in the extracellular region; a transmembrane region; and an intracellular region lacking intrinsic catalytic activity [63–66]. Atomic resolution of the complex between human G-CSF and the ligand-binding domain of the murine or human G-CSFRs revealed a 2:2 ligand:receptor stoichiometry. The human G-CSFR forms a “cross-over” complex in association with G-CSF, which is tethered at the membrane distal Ig-like domains [67]. Ligand binding is mediated by contacts with residues in the Ig domain of one receptor monomer and the membrane-proximal CRH region from the second receptor monomer [67]. Although the G-CSFR operates as a homo-oligomer, the 3-dimensional structure of the human G-CSF/G-CSFR complex shows similarity with the IL-6/IL-6Rα/gp130 complex rather than other homodimeric type I cytokine receptor structures such as the erythropoietin (Epo)/Epo receptor or growth hormone receptor complexes [67–69]. Moreover, the structural configuration for the human G-CSF/human G-CSFR complex differs from the human G-CSF/murine G-CSFR complex [67,70], although both are capable of eliciting biologic responses. Thus, the high degree of primary structure homology between human and murine ligands renders distinct structures of an activated receptor complex, indicating flexibility in the mechanisms to initiate the G-CSFR signal transduction cascade.

This is useful knowledge for the prospective design and characterization of small molecule G-CSF mimetics.

G-CSFR expression has been detected in both hematopoietic and non-hematopoietic cell types including hematopoietic stem and progenitor cells, the placenta, neurons within the central nervous system, adult neural stem cells, endothelial cells, and cardiomyocytes [63,71–74]. In the hematopoietic system, the G-CSFR shows predominant expression within the myeloid lineage [63–65]. The G-CSFR is detected on granulocytic lineage cells, as well as a subset of monocytes and blast cells, but is not found on erythrocytes, lymphocytes or eosinophils [28]. Activation of G-CSFR signal transduction proceeds by a mechanism that is shared with other cytokine receptors, in which associated Jak protein tyrosine kinases undergo trans-phosphorylation and full kinase activation upon cytokine binding. Jak1 and Jak2 are activated by the G-CSFR [75,76]. While individual deletion of the Jak1 or Jak2 genes does not affect the level of fetal liver granulocytes or granulocytic progenitors (e.g., CFU-G), respectively, [77,78], Jak1, but not Jak2, appears to play a non-redundant role in the G-CSFR signaling pathway [79]. Early structure/function studies of the G-CSFR showed that the cytoplasmic domain contained regions with distinct signaling functions. The membrane-proximal region, which is now known to couple to the Jak kinases, is essential for G-CSF-mediated proliferation. The membrane-distal region of the G-CSFR regulates inducible gene expression, neutrophil marker protein induction and granulocytic differentiation [80–82]. This region of the G-CSFR contains 4 tyrosine residues (in humans, Y704, Y729, Y744, Y764; in mice, Y703, Y728, Y743, Y763; see Fig. 2), which undergo G-CSF-dependent phosphorylation mediated by the receptor-associated Jak kinases. The G-CSFR phosphotyrosines serve to recruit intracellular signaling molecules to the ligand-activated receptor complex via SH2 domain/phosphotyrosine interactions [83–90] (for review, see [91]) (Fig. 2).

The precise role of G-CSFR tyrosine residues in mediating proliferation and differentiation signals from the receptor has been examined by enforced expression of engineered G-CSFRs in myeloid tissue culture cells and primary bone marrow progenitors [84–86,92], as well as experiments with genetically recombined G-CSFR loci expressing mutated receptors [93–96]. Figure 2 summarizes the results of studies that have examined the role of G-CSFR-coupled signaling cascades, the majority of which become activated through their association with receptor phosphotyrosines. The G-CSFR cytoplasmic tyrosines are particularly crucial in delivering the G-CSF signal at sub-saturating ligand concentrations [88]. In saturating amounts of G-CSF, the receptor cytoplasmic tyrosine residues appear to be dispensable ex vivo [88], although it is not clear if this is the case in vivo since knock-in studies with a mutated G-CSFR lacking all 4 cytoplasmic tyrosines in the context of the full-length receptor have not been reported. In the background of a truncated G-CSFR, which lacks sequences encoding the 3 C-terminal tyrosine residues, mutation of the remaining tyrosine residue at position 704 (G-CSFR d715F) caused defects in neutrophil production and mobilization [94]. Minimally, it is possible that the G-CSFR tyrosine residues enable an amplification or enhancement of signaling responses in the context of the full-length receptor; this may be particularly crucial in delivering the G-CSF signal under conditions of demand or cellular stress. By contrast, it would not be surprising if the receptor tyrosines are absolutely necessary for G-CSF-responsive neutrophil regulation in vivo. Future studies to determine the function of G-CSFR tyrosines in the context of the full-length receptor are important for elucidating the structural requirements of a functional G-CSFR.

The G-CSFR stimulates numerous signal transduction proteins, including the STAT 1, 3 and 5 transcription factors, the Ras/Mek/Erk1/2 pathway, the Src-related kinases Lyn and Hck, the serine/threonine kinase Akt, and the Syk tyrosine kinase [76,87,90,97–102] reviewed in [91] (see Fig. 2). These pathways are generally considered to transmit signals that induce and/or enhance granulocytic proliferation and differentiation [83,85,87,102–106]. In addition, the G-

CSFR physically associates with integrin α9β1, which augments receptor signaling and granulopoiesis in vivo [107]. The G-CSFR also couples to pathways that negatively regulate its signaling output and granulopoiesis-inducing function, including those mediated by the SHP-1 tyrosine phosphatase (e.g., hematopoietic cell phosphatase, HCP) and the suppressor of cytokine signaling 3 (SOCS3) protein [108,109] (Fig. 2). Negative regulation of G-CSFR signaling is also accomplished by ligand-mediated internalization and degradation of the receptor [110].

Aside from immediate signaling cascades elicited by G-CSF, investigators have elucidated G-CSF-responsive genes. Several of these have established roles in cellular proliferation and/or myeloid biology. G-CSF-responsive genes include Mac1 (CD18/CD11b), FcγII/III, and neutrophil secondary granule proteins, which are important for neutrophil function [92,104, 111]; immediate early genes including c-fos, Oncostatin M (OSM), IRF-1, egr-1, fibrinogen and SOCS3 [99]; and the myeloid transcription factors PU.1, C/EBPα, C/EBPβ and Gfi-1 [104,112–114]. Surprisingly, however, in most cases we have a poor understanding of the mechanisms that connect the immediate G-CSFR signaling responses to the induction of G-CSF-responsive gene expression, and therefore to the precise cellular functions that are driven by specific G-CSFR signaling cascades. Certain genes have been identified as direct STAT-responsive genes, including SOCS3 and OSM [99,115,116]. A putative STAT binding site has been reported in the human Gfi-1 promoter but its function is unknown [117]. In other cases, such as PU.1 and C/EBPα, STATs are known to play a role but their exact mechanism is less clear [104,114]. Beyond the STATs, the function of Ras/Mek/Erk1/2, Lyn, Hck, Akt, and Syk in G-CSF-dependent gene expression is even less well understood. In addition, many studies have used tissue culture systems to study candidate gene expression, and therefore we lack a comprehensive view of G-CSF-dependent transcriptional responses in primary granulocytes. Furthermore, our knowledge of the specific role of individual signaling pathways in steady state and ‘emergency’ conditions of granulopoiesis remains quite limited due to the lack of appropriate model systems to explore in vivo functions. The following section will discuss the available information.

## G-CSF-responsive signaling pathways that control steady state and G-CSF-driven ‘emergency’ granulopoiesis

G-CSF and its receptor are essential for basal and stress-induced granulopoiesis [3,23,118, 119]. Mice deficient in G-CSF or G-CSFR have severe neutropenia and reduced levels (~50%) of late-stage neutrophil precursors in bone marrow under resting conditions [3,118]. G-CSF$^{-/-}$ mice are unable to respond to infection with *Listeria monocytogenes* by stimulating granulopoiesis and induction of circulating neutrophils, thereby resulting in a markedly reduced ability to control the infection [3]. G-CSFR$^{-/-}$ mice fail to mount an increase in bone marrow or circulating neutrophils in response to G-CSF administration [93,120]. These data highlight the necessity of the G-CSF/G-CSFR pathway in the anti-bacterial immune response, and furthermore demonstrate that the G-CSFR is solely responsible for transmitting G-CSF signals in vivo. A residual level of neutrophil production does continue in G-CSF$^{-/-}$ or G-CSFR$^{-/-}$ mice [3,118], indicating separate mechanisms to induce neutrophil production. Interleukin-6 was identified as an independent regulator of granulopoiesis, as loss of IL-6 function worsened the neutropenia observed in G-CSFR$^{-/-}$ animals [121]. Conversely, G-CSF was found to provide a compensatory signal for the myeloid progenitor deficiency in mice lacking thrombopoietin [122]. It is presently unclear whether and how additional cytokine-dependent or –independent pathways contribute to the residual granulopoiesis found in G-CSF$^{-/-}$ or G-CSFR$^{-/-}$ mice. Regardless, the G-CSFR signaling pathway is absolutely crucial for regulating terminal neutrophil differentiation since the residual neutrophils found in G-CSFR$^{-/-}$ animals exhibit defective functional responses including impaired adhesion and chemoattractant-induced migration [123]. G-CSFR function is required throughout

development of the neutrophil lineage, beginning with myeloid-committed progenitors in vivo [124]. The G-CSFR elicits specific signals to regulate granulopoiesis; for example, these can not be replaced by signaling through the cytoplasmic region of the related cytokine receptor, EpoR [93]. The identity of G-CSFR-mediated signaling cascades important for granulopoiesis have been relatively elusive, although a first glimpse came through experiments with G-CSFR d715F mice. These experiments revealed a role for the G-CSF-responsive transcription factor STAT3 in neutrophil production and function [94].

Initial studies investigating STAT3 function in vivo demonstrated an essential role in embryonic development and viability [125], therefore it was necessary to generate conditional knockout mice to examine STAT3 function in granulopoiesis. Several groups accomplished this using tissue-specific or inducible Cre recombinase mouse strains and mice carrying floxed STAT3 alleles. Surprisingly deletion of STAT3 in the hematopoietic compartment led to neutrophilia [103,126–128] rather than neutrophil deficiency. Hematopoietic-specific STAT3 deletion results in a specific increase in late stage neutrophils (e.g., band/segmented) in bone marrow and elevated uptake of $^{3}$H-thymidine in G-CSF-stimulated granulocytes ex vivo [126,127]. By contrast, normal levels of G-CSF-responsive colony-forming progenitor cells and immature granulocytic precursors, including promyelocytes, myelocytes and metamyelocytes, were found in STAT3-deficient bone marrow [126,127]. These results point to a specific role for STAT3 in negatively regulating the terminal stages of bone marrow granulopoiesis and circulating neutrophil levels.

This negative regulatory role for STAT3 was surprising in light of studies that had pointed to its important function during neutrophil development [94,104,105], yet it meshed well with the finding that STAT3 controlled the expression of SOCS3 [115,126], an inducible negative regulator of cytokine signaling responses [129,130]. STAT3 deletion abrogates the ability of G-CSF to activate SOCS3 and causes enhanced G-CSFR signaling via STAT1 and Erk1/2 [126,127]. Conditional deletion of SOCS3 in bone marrow progenitors leads to a phenotype in older mice (aged >17 weeks) that resembles aspects of the phenotype of hematopoietic conditional STAT3 knockout mice, albeit one that is observed much earlier in development (ages 3–8 weeks) [109,128]. Aged bone marrow SOCS3-deficient mice contain elevated levels of late stage bone marrow granulocytes (e.g., metamyelocytes and neutrophils) and exhibit peripheral neutrophilia under steady state conditions. This is similar to the increase in late stage bone marrow granulocytes and circulating neutrophils found in bone marrow STAT3-deficient mice [103,126–128]. Furthermore, SOCS3-deficient bone marrow cells show prolonged G-CSF-dependent STAT3 activation and enhanced responses to G-CSF ex vivo (e.g., proliferation, cloning frequency) [109], resembling STAT3-deficient granulocytes [126]. Following G-CSF injection, mice carrying hematopoietic SOCS3 deletion have elevated neutrophil production and mobilization in vivo, and they develop an inflammatory neutrophil infiltration into tissues, indicative of enhanced neutrophil activity [109]. Bone marrow STAT3-deficient mice also have increased levels of mature neutrophils in the peripheral blood after prolonged exposure (5 d) to G-CSF in vivo [103,127]. These data collectively indicate that SOCS3 is a critical G-CSF/STAT3 target gene that controls the magnitude and duration of signaling from the G-CSFR, thereby regulating the production of neutrophils from the bone marrow. The conserved SOCS box domain of SOCS3, which mediates binding to the elongin B/C ubiquitin ligase complex, is responsible for controlling STAT3 activation via the G-CSFR and for maintaining regulated output of neutrophils during exposure to pharmacologic levels of G-CSF in vivo [131]. The SOCS box is dispensable for regulating steady state circulating neutrophil levels, although increased levels of G-CSF-responsive progenitors were found in bone marrow samples of mice expressing the SOCS box-deficient SOCS3 allele (SOCS3$^{\Delta SB}$) suggesting that the SOCS box does control homeostatic progenitor growth/survival mechanisms [131]. SOCS3 attenuates G-CSFR-mediated signals by interacting with

a membrane-proximal lysine residue in the receptor cytoplasmic region, to regulate receptor ubiquitination and routing to lysosomes [132].

Elucidation of the G-CSFR/STAT3/SOCS3 axis did not explain the previously identified roles for STAT3 in granulocytic differentiation, myeloid transcription factor expression, and neutrophil function [94,104,112,114,133]. Moreover, the role of STAT3 in G-CSF-driven 'emergency' granulopoiesis and neutrophil function had not been examined thoroughly. To address these questions, we utilized mice with bone marrow conditional STAT3 deletion (Tie2cre/STAT3$^{\Delta/flox}$ mice, referred to herein as STAT3-deficient) [103]. We found that STAT3 was critical for neutrophil chemotaxis toward ligands for the CXCR2 receptor (e.g., MIP-2 and KC) and for regulating chemoattractant-stimulated actin polymerization, demonstrating essential roles for STAT3 in cellular migration pathways that are vital to the mature neutrophil. Furthermore, STAT3-deficient mice fail to induce acute neutrophil mobilization from the bone marrow in response to G-CSF or MIP-2 (reference [103] and ADP and SSW, unpublished results). Following prolonged exposure to G-CSF (e.g., 5 d), STAT3-deficient mice are unable to upregulate the frequency or absolute numbers of immature neutrophils in the bone marrow or peripheral blood, unlike wild type animals. Rather, STAT3-deficient mice demonstrate a skewed long-term mobilization response which is characterized by excessive mature neutrophil numbers and reduced immature neutrophils in the peripheral circulation [103]. We found that these functions of STAT3 were independent of SOCS3, as mice with myeloid-specific deletion of SOCS3 exhibit a normal frequency of immature neutrophils in response to G-CSF, normal G-CSF-driven neutrophil mobilization and intact neutrophil chemotactic function [103]. In addition, estimation of total neutrophil numbers in hematopoietic tissues indicated that STAT3 regulates the quality of the neutrophil response (e.g., excessive mature neutrophils and impaired immature neutrophils relative to wild type), but does not control the total neutrophil output following exposure to G-CSF [103]. These data indicate that G-CSF and STAT3 control additional genes beyond SOCS3 that regulate crucial steps in G-CSF-driven granulopoiesis and neutrophil function. The identity of these genes is important for understanding the molecular mechanisms of G-CSF action, which may lead to novel approaches for diseases associated with neutrophil deregulation.

## Additional transcription factors and cell cycle regulators controlling G-CSF-driven granulopoiesis

Steady state granulopoiesis is dependent upon an array of transcription factors that function at distinct developmental stages (e.g., RUNX1, SCL, PU.1, IRF8, LEF-1, Gfi-1, C/EBPα and C/EBPε) [134–137]. The developmental expression of these transcription factors [138], and their role in basal myelopoiesis has been discussed elsewhere [134–136] and therefore will not be reviewed at length herein.

In comparison, the pathway(s) that control G-CSF-driven 'emergency' granulopoiesis at the transcriptional level have been relatively understudied. This relates, in part, to the early developmental defects in the hematopoietic system or the neutrophil lineage in the absence of key myeloid transcriptional regulators such as PU.1 or C/EBPα [135]. Analysis of the role of these factors in 'emergency' granulopoiesis requires developmentally staged gene deletion/knockdown to bypass the early developmental block and enable the maturation of G-CSF-responsive precursor cells [139]. Certain key myeloid transcriptional regulators such as Gfi-1 and C/EBPε have late-stage developmental defects, which allows investigation into their role in G-CSF-driven 'emergency' granulopoiesis. For example, under steady state conditions, Gfi-1$^{-/-}$ mice lack morphologically mature neutrophils and instead show accumulation of 'atypical' Gr-1$^{+}$/CD11b$^{+}$ myeloid cells. These atypical myeloid cells lack granules and neutrophil granule protein transcripts, yet possess limited neutrophil functions such as oxidative burst and phagocytosis [140]. Gfi-1$^{-/-}$ mice respond to G-CSF treatment by inducing

the level of 'atypical' myeloid cells, yet they fail to upregulate circulating mature neutrophils. Thus, Gfi-1 appears dispensable for a G-CSF-dependent expansion/proliferative phase in myelopoiesis. By contrast, Gfi-1 is critical for regulating the terminal stages of neutrophil maturation during basal and G-CSF-driven granulopoiesis.

The transcription factor C/EBPα is essential for the transition between the common myeloid progenitor (CMP) stage and the granulocyte-macrophage progenitor stage, yet appears dispensable for later developmental stages of granulopoiesis under steady state conditions [139,141]. In addition, neutrophil development could be driven in C/EBPα$^{-/-}$ hematopoietic progenitors or C/EBPα$^{-/-}$ myeloid cell lines in response to other myeloid cytokines such as interleukin-3 and/or granulocyte-macrophage colony-stimulating factor [142–144], indicating the presence of alternative pathways to induce granulopoiesis. To examine the underlying cause, expression of other C/EBP family members was examined in primary myeloid progenitors during cytokine exposure ex vivo [113]. These studies revealed that C/EBPβ mRNA levels were selectively upregulated in granulocytic-macrophage progenitors (GMPs) in response to G-CSF. By contrast, C/EBPα was not induced by G-CSF in hematopoietic stem cells (HSCs), CMPs or GMPs [113]. G-CSF-dependent induction of C/EBPβ suggested it may control important aspects of emergency 'granulopoiesis', a prediction that was confirmed using mice deficient in C/EBPβ [113]. C/EBPβ is required for G-CSF-driven expansion of the granulocytic compartment, G-CSF-dependent induction of circulating neutrophil levels and efficient bacterial clearance in vivo [113], yet is strikingly dispensable for granulopoiesis under basal conditions [145–147]. These results highlight the importance of G-CSF-dependent transcriptional response pathways under conditions of 'demand-driven' granulopoiesis, and furthermore indicate that new studies should not remain limited to previously identified myeloid transcription factors. Moreover, it is well established that the expression level of lineage-specification transcription factors, and the ratio between key regulators within an individual lineage, is essential for directing correct hematopoietic lineage maturation and suppressing malignant transformation [134,135]. While G-CSF has been shown to influence the expression of several important myeloid transcription factors [104,112–114], it is not known if this contributes to neutrophil maturation in basal or 'demand-driven' conditions. This question can be addressed by deletion of the G-CSF-responsive signaling pathways that are necessary for transcription factor regulation, and/or by the genetic manipulation of transcription factor levels in developing granulocytes.

An important consequence of the G-CSF-dependent transcriptional response is how it impinges upon the cell cycle machinery to control the proliferation of myeloid progenitor cells and granulocytic precursors. Cyclin D3$^{-/-}$ mice have a profound defect in neutrophil production, exhibiting neutropenia in steady state conditions and an inability to mount an increase in circulating neutrophil levels in response to G-CSF administration, rendering extreme susceptibility to infection with *Listeria monocytogenes* [148]. Cyclin D3 expression is induced in bone marrow cells upon G-CSF administration in vivo [148], suggesting it is transcriptionally regulated by a G-CSF-dependent signaling cascade. Elucidation of the key molecular elements of G-CSF-mediated cyclin D3 regulation will provide an important step toward understanding the mechanisms that regulate G-CSF-driven granulopoiesis. Unraveling these is crucial for determining the host pathways that mount a functional neutrophil response during infection.

## G-CSF function in infection

Perhaps not surprisingly, G-CSF has distinct functions depending on the type of infection and its route of delivery. Considering the vast array of microbes that elicit disease, and the number of host-derived molecules that are involved in the immune response, it is reasonable to expect that individual cytokines will demonstrate selective and unique functions. Studies with G-CSF

have utilized well-characterized models that mimic natural and harmful infections. G-CSF has a critical role in the 'emergency' response to infection with *Listeria monocytogenes*, a bacterial pathogen that propagates within macrophages. In an intravenous infection model using G-CSF-deficient mice, G-CSF was required for the induction of circulating neutrophil and monocyte levels elicited by *L. monocytogenes*. G-CSF also plays a critical role in suppressing the bacterial load in spleen and liver [3]. Following intraperitoneal infection, G-CSF was required for maintaining total bone marrow cell numbers, a response that is likely to be related to its ability to preserve bone marrow colony-forming (e.g., progenitor) cells in steady state and during *L. monocytogenes* exposure [23]. In addition, G-CSF is necessary for macrophage phagocytosis function and the induction of absolute peritoneal macrophage numbers elicited by *L. monocytogenes* [23,149]. Certain aspects of the response to *L. monocytogenes* are G-CSF-independent, for example neutrophil recruitment into the infected peritoneum is unaffected by G-CSF deficiency [23]. Thus, with *L. monocytogenes*, G-CSF plays a major role in controlling hematopoietic progenitor and macrophage responses, as well as circulating neutrophil levels, which are collectively required for bacterial clearance.

Studies with G-CSFR-deficient mice have also emphasized the importance of the G-CSF/receptor axis in infection. Gregory et al. [119] recently investigated the role of G-CSFR in response to infection with *Pseudomonas aeruginosa*, a Gram-negative bacteria that poses a major risk to hospital and cystic fibrosis patients. Using an intratracheal delivery model to mimic the pulmonary infection in cystic fibrosis patients, G-CSFR was found to be a necessary component of the host response to *P. aeruginosa*. G-CSFR is required for the systemic induction of circulating neutrophil levels, bacterial clearance from the lung, and the prevention of extensive pulmonary damage during infection. Furthermore, neutrophil survival in the infected lung tissue is impaired in the absence of G-CSFR, suggesting that G-CSF signals are important for controlling neutrophil survival at the site of infection. The neutrophil response to *P. aeruginosa* is thus dependent upon G-CSFR signal transduction, and this response is necessary for the resolution of infection.

G-CSF has more modest function during infection with *Candida albicans*, a fungal pathogen that frequently causes severe infection in immunocompromised individuals. Following intravenous *C. albicans* infection, circulating neutrophil levels were induced in both wild type and G-CSF-deficient animals and the latter showed elevated levels of myeloid progenitors in bone marrow [150]. As suggested by the authors of this report [150], these data strongly imply the presence of additional growth factors or alternative mechanisms that generate neutrophil responses in certain stress situations. This idea is underscored by the fact that G-CSF-deficiency does not cause an absolute blockade in steady state neutrophil production [3]. The identity of G-CSF-independent neutrophil regulatory pathways are unknown, with the exception of IL-6 [121], and are a subject of much interest as they could provide novel approaches for the treatment of neutropenia. In summary, therefore, the function of G-CSF is dependent upon the type and route of infection. Moreover, it is important to consider that any functions revealed are those that have been assayed directly; G-CSF is likely to have broad and varied activity for a number of bacterial pathogens, which future research should reveal.

## Clinical use of G-CSF and future avenues for research and development

G-CSF is used to treat neutropenia resulting from immunosuppressive chemotherapy or severe congenital disorders [6,36,37,40,151–154], which cause extreme sensitivity to life-threatening bacterial infection. G-CSF reduces the degree and duration of neutropenia in individuals who receive chemotherapy, and protects patients with congenital neutropenia from acute infection and early lethality [6,36,37,40,151–154] (Fig. 3). Concerns have been raised, however, about a potential association between the clinical use of G-CSF and development of acute myeloid leukemia (AML) [7–11]. This was first questioned in individuals with severe congenital

neutropenia (SCN) (Fig. 3), as a subset have acquired a mutant G-CSFR allele that encodes a truncated protein lacking sequences necessary for negative regulation and induction of differentiation signaling; the presence of the mutant G-CSFR correlated with the development of AML in these patients [110,155–162]. The mutant G-CSFR was originally suggested as a potential cause of the neutrophil maturation arrest at the promyelocyte/myelocyte stage of development and the profound absolute neutropenia observed in SCN patients, as well as a potential contributing factor to leukemic transformation [155,157,163,164] (Fig. 3). It is now recognized that distinct molecular mechanisms underlie the neutrophil maturation arrest in SCN, including mutations in the genes for neutrophil elastase and Hax-1, as well as reduced expression of the transcription factor LEF-1 [4,137,164–169]. Thus, the acquired G-CSFR mutation may provide a growth advantage to myeloid progenitor cells that already have a maturation defect, increasing the risk of additional genetic mutations and leukemic transformation [170]. A single nucleotide polymorphism in the G-CSFR gene that encodes Lys785, in place of Glu785, is associated with high-risk myelodysplastic syndrome [171], underscoring the idea that perturbations in G-CSFR signaling function contribute to hematological disease. Importantly, in SCN patients, G-CSF therapy significantly reduces the risk of mortality from sepsis, from 6–7% per year in the pre-G-CSF era to 0.9% per year with G-CSF treatment [9]. SCN patients do have an increased risk of developing MDS/AML over time on G-CSF therapy, with 2.9% per year incidence rate after 6 years of treatment compared with 8.0% per year after 12 years [9]. The risk of sepsis mortality remains constant over time [9]. Moreover, a subset of SCN patients that demonstrated less responsiveness to G-CSF, and have thus been treated with higher levels of the cytokine, have an elevated risk of MDS/AML as well as mortality from sepsis [9]. SCN is a pre-leukemic condition; the extension of lifespan with G-CSF therapy may therefore contribute to the increased risk of MDS/AML transformation. Moreover, inherent differences may exist between SCN patients that show robust or poor responses to G-CSF. More severe forms of the disease (i.e., reduced ability to overcome the neutrophil maturation defect upon G-CSF therapy) may have even greater predisposition to leukemic transformation. G-CSF remains the therapy of choice for SCN since it dramatically prevents the likelihood of mortality from sepsis. Stem cell transplantation may provide an alternative treatment for individuals that show poor responsiveness to G-CSF, as these patients are at the highest risk of developing MDS/AML [9].

## Summary and future prospectives

While this review focused solely upon neutrophil regulation by G-CSF, it is important to highlight recent discoveries that show G-CSF operates in other immune cell types and non-hematopoietic tissues. These include a role for G-CSF in mediating T cell tolerance by influencing the development and/or function of antigen-presenting cells, an activity that could prove useful in multiple clinical applications such as stem cell transplantation to enhance graft-versus-leukemia and concomitantly suppress graft-versus-host responses [45,172–176]. G-CSF has also been shown to improve cardiomyocyte survival and cardiac repair following myocardial infarction in animal models, via a process that appears to require the transcriptional activity of STAT3 [74]. Neuronal survival and recovery from cortical ischemia is similarly protected by G-CSF, which is capable of crossing the blood-brain barrier and of stimulating neuronal progenitor responses in vivo [73,177]. These findings have suggested a potential for broadening the clinical uses of G-CSF; careful in vivo experimentation with animal models and appropriately designed preclinical/clinical studies are required to thoroughly evaluate these possibilities [177–181]. Lastly, within the neutrophil lineage, G-CSF can exacerbate both acute and chronic arthritis, raising the possibility that G-CSF antagonists may be useful for the clinical treatment of joint inflammation [182,183].

The advent of precise genetic tools to investigate G-CSF signaling molecule function and new methods to visualize immune cells in vivo, such as multi-photon microscopy, should yield a

thorough understanding of the molecular mechanisms utilized by G-CSF during an active immune response. In the near future, it will be important to study the function of G-CSF in the host response to pathogens that are prevalent in both healthy and immunocompromised populations, and not to limit investigation to a small number of pathogens that are readily handled in the laboratory. Of course, this will require collaborative arrangements between immunologists, signal transduction experts and microbiologists; however, the efforts should yield information that will facilitate the development of new agents to treat neutropenic individuals and those who are refractory to G-CSF, and may provide novel approaches to common and deadly pathogens. Moreover, a thorough understanding of G-CSF function is necessary to reduce any potential risks that arise from its long-term clinical use and to develop methods to regulate neutrophil-mediated inflammatory disease.

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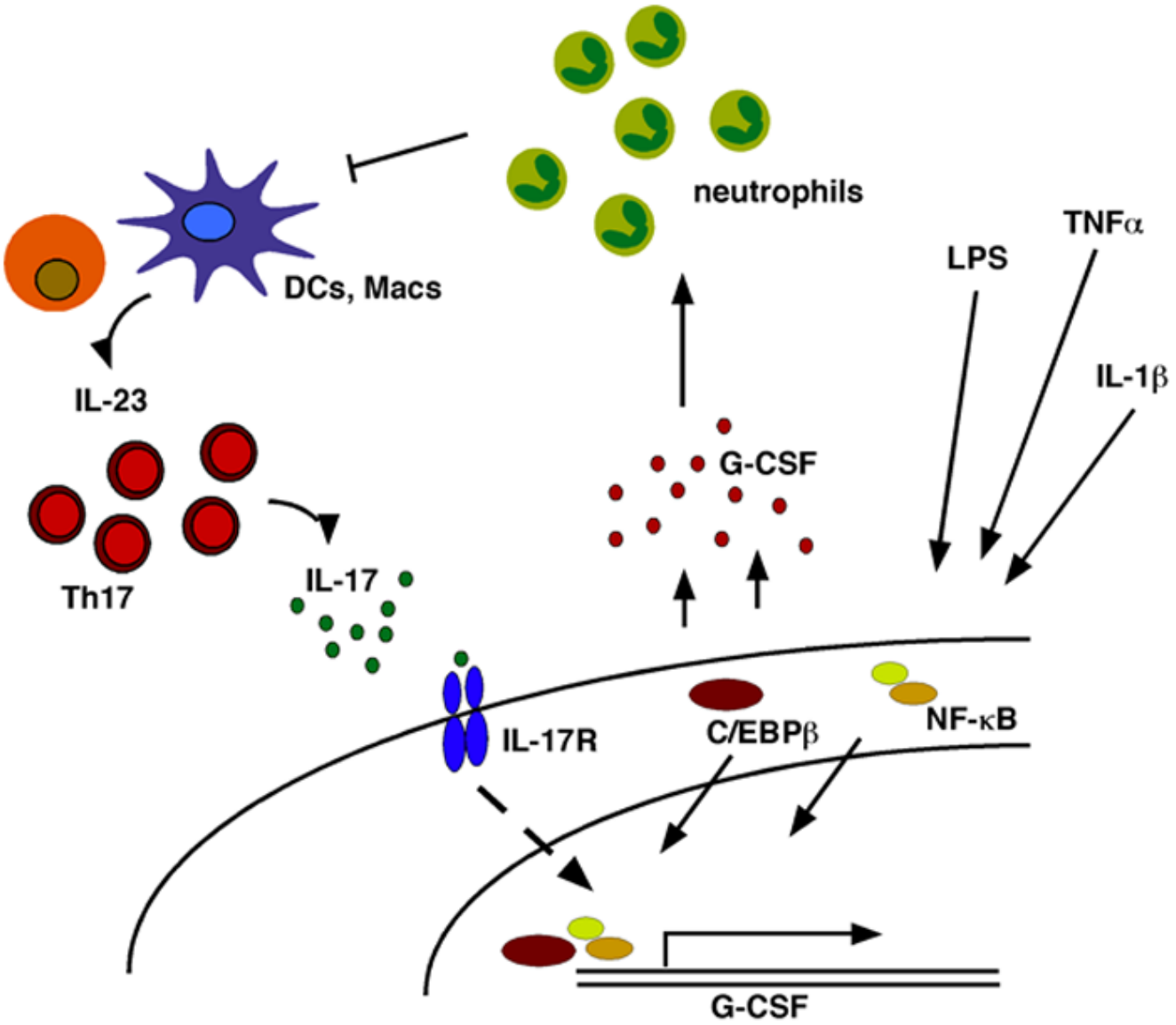


**Figure 1.**

Schematic diagram of pathways regulating G-CSF and neutrophil production. (Right) Inflammatory stimuli in the extracellular microenvironment, such as LPS, TNFα and IL-1β act on target cells (not to scale) to induce G-CSF expression via intracellular signaling molecules such as NF-κB and C/EBPβ. (Left) IL-17 production by Th17 cells activates IL-17R signal transduction, which promotes G-CSF expression. G-CSF can be regulated by transcriptional and post-transcriptional mechanisms. (Center) The increase in G-CSF synthesis stimulates neutrophil production in the bone marrow (anatomical details are not shown). Circulating neutrophils negatively regulate the production of IL-23 and Th17 cells, providing a feedback system to control G-CSF synthesis.

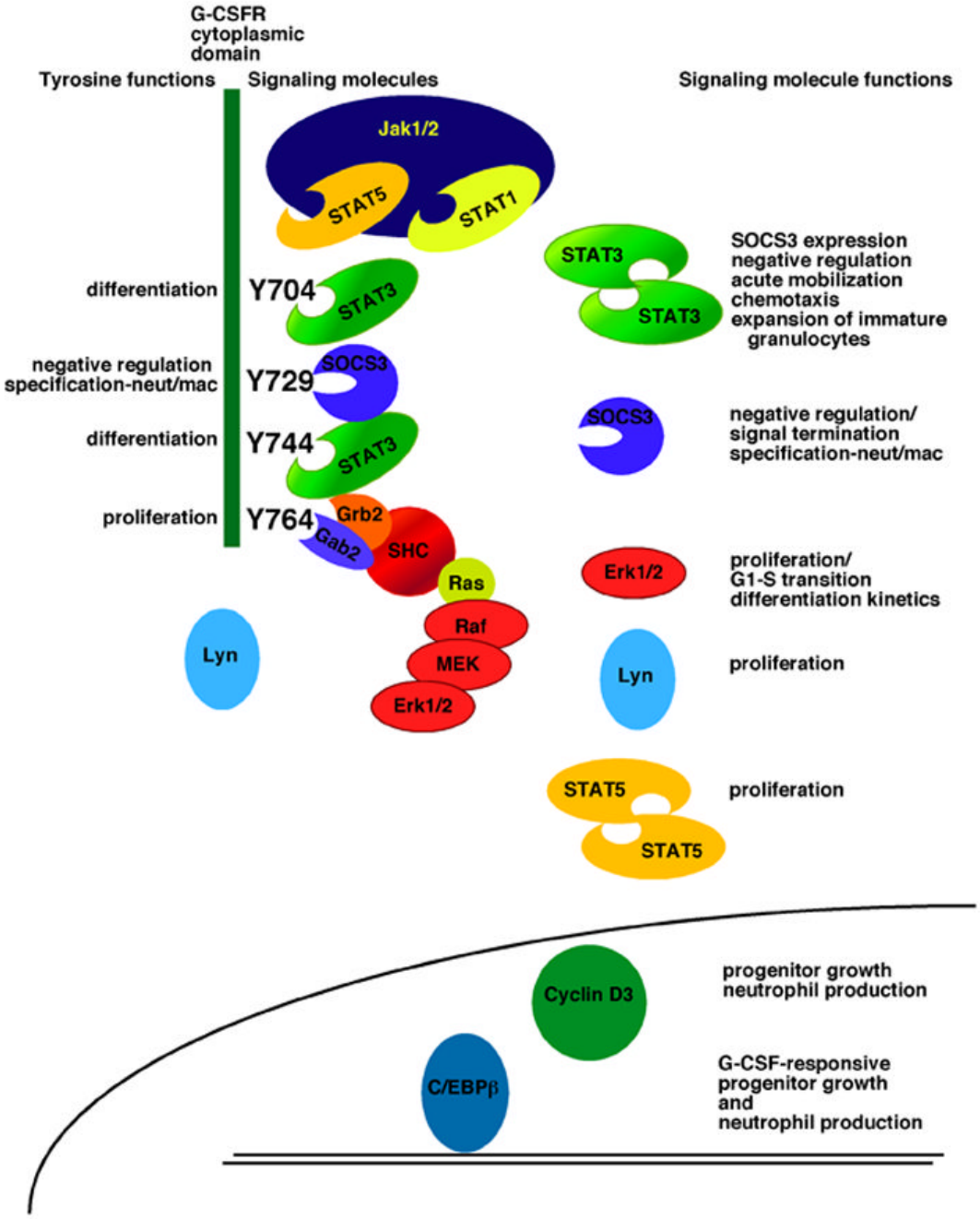


**Figure 2.**

Functions of G-CSFR tyrosine residues and receptor-activated signaling pathways. The intracellular region of the G-CSFR is illustrated (narrow green box, left; not drawn to scale), with the four tyrosine residues highlighted in black. On the left of each tyrosine residue are functions that have been assigned by in vitro and in vivo studies, including proliferation, differentiation and macrophage/granulocyte lineage-specification. On the right of each tyrosine, representations of signaling proteins that couple to specific residues are shown. The function of these molecules is listed on the right side of the figure. At the lower portion of the figure, a schematic diagram of the nucleus of a granulocytic progenitor is shown, along with

additional signaling molecules that are required for ‘emergency’ granulopoiesis. References for tyrosine and signal protein function can be found in the text.

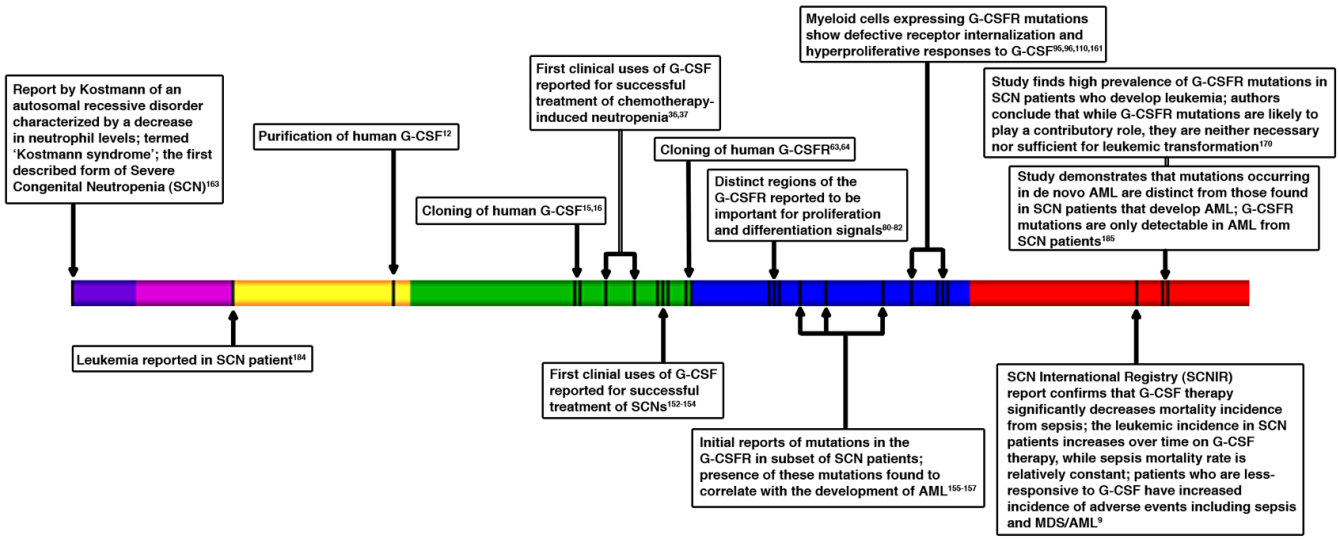


**Figure 3.**

A timeline of major events in the development and use of G-CSF in the clinic. Left to right, the timeline highlights principal findings that are relevant to the therapeutic use of G-CSF including the initial description of congenital neutropenia [163] and leukemia in SCN [184], purification and cloning of human G-CSF [12,15,16], first clinical use of G-CSF for neutropenia resulting from chemotherapy or congenital origin [36,37,152–154], cloning of G-CSFR and identification of receptor functional domains [63,64,80–82], initial reports of G-CSFR mutations in SCN [155–157], functional studies of G-CSFR mutants found in SCN [95,96,110,161], and associations between the therapeutic use of G-CSF and the development of MDS/AML [9,170,185]. Due to space limitations, all significant references could not be included. (Timeline: Purple, 1956-1959; Pink, 1960-1969; Yellow, 1970-1979; Green, 1980-1989; Blue, 1990-1999; Red, 2000-present)