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Socs3 maintains the specificity of biological responses to cytokine signals during granulocyte and macrophage differentiation

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Abstract

Granulocyte colony stimulating factor (G-CSF) and interleukin-6 (IL-6) play key roles in regulating emergency granulopoiesis and inflammation, and are both negatively regulated by the inducible intracellular protein suppressor of cytokine signaling-3 (Socs3). Mice with *Socs3* deleted specifically in hematopoietic cells succumb to severe neutrophil and macrophage driven inflammation by one year of age, and responses to G-CSF are grossly exacerbated. In order to determine which elements of cellular responses to cytokines require Socs3, we have examined the differentiative and proliferative capacity of hematopoietic progenitor cells stimulated by G-CSF and IL-6. The differentiation of Socs3-deficient progenitor cells is skewed towards macrophage production in response to G-CSF or IL-6, whereas wild-type progenitor cells produce mainly neutrophils. The proliferative capacity of Socs3-deficient progenitor cells is greatly enhanced in response to G-CSF at all concentrations but only at low concentrations for IL-6. Strikingly, synergistic responses to co-stimulation with stem cell factor and IL-6 (but not G-CSF) are lost at higher concentrations in Socs3-deficient progenitor cells. Cytokine-induced expression of transcriptional regulators including *cebpb*, *Ets2*, *Bcl3*, *c-Myc*, *Jun* and *Fosl2* are differentially regulated in Socs3-deficient cells. The tight regulation by Socs3 of STAT3 phosphorylation and gene transcription after cytokine receptor ligation significantly influences the fate of myeloid progenitor cells.

Keywords

Suppressor of cytokine signaling-3; cytokines; signal transduction; hematopoiesis; G-CSF

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Introduction

The proliferation and differentiation of hematopoietic progenitor cells to macrophages and granulocytes requires cytokine stimulation. Although the cytokines that drive myeloid cell production are well documented, much less is known about how hematopoietic progenitor cells interpret and integrate cytokine signals to form a granulocyte or a macrophage. The magnitude and duration of cytokine receptor activation is likely to influence the decision-making process of the progenitor cell by altering the profile of transcriptional regulators present in the cell during differentiation^{1,2}.

Suppressor of Cytokine Signaling 3 (Socs3) is a key negative regulator of cytokine signaling³. Socs3 is induced by cytokine or Toll-like receptor stimulation and is essential for the negative regulation of LIF, IL-6, leptin and G-CSF signaling^{4–10}. Socs3 is a member of the Socs family of proteins, comprising 8 members (CIS, Socs1–7), and is characterised by a C-terminal Socs box, an N-terminal SH2 domain and a kinase inhibitory region^{3,11,12}. The Socs box is important for interactions with the elongin B/C ubiquitin ligase complex and is essential for the negative regulation of G-CSF responses *in vivo*^{13–15}. The SH2 domain of Socs3 interacts with phosphorylated tyrosine residues on receptors including the G-CSF receptor (G-CSFR) (Tyr729) and the gp130 subunit (Tyr759) of the IL-6R, LIFR and IL-11R^{16–20}. Mutations in critical phosphotyrosine residues of the G-CSFR (Tyr704, Tyr729, Tyr744, Tyr764) resulted in aberrant proliferation and differentiation of a hematopoietic progenitor cell line suggesting a critical role for Socs3 in these processes^{21,22}.

In the absence of Socs3, the magnitude and duration of G-CSF-induced signal transducer and activator of transcription 3 (STAT3) activation in bone marrow cells is greatly increased and *in vivo* administration of G-CSF, rather than being well tolerated, becomes toxic⁵. We and others have previously shown that the absence of Socs3 in hepatocytes and macrophages results in an increase in the magnitude and duration of STAT3 and STAT1 activation in response to IL-6^{4,6,7}. At the transcriptional level, this amounts to a change in signal quality, with significant increases in interferon-inducible genes in response to IL-6^{4,7}. However, the biological consequences of the quantitative changes in signaling, and the qualitative changes in gene expression are not obvious. Preliminary observations suggested a skewing of differentiation of Socs3-deficient hematopoietic progenitor cells toward macrophages in response to G-CSF⁵. In this study, we have further examined the consequences of altered regulation of G-CSF and IL-6 signaling in hematopoietic progenitor cells, a cellular system with a broad degree of proliferative and differentiative plasticity.

Methods

Mice with Socs3-deficient hematopoiesis

vavCre⁺Socs3^{-fl} mice, in which *Socs3* is deleted in all hematopoietic cells (*Socs3^{-/-}*), were generated and maintained on a pure C57BL/6 genetic background, as previously described⁵. In initial experiments, hematopoietic cells (*Socs3^{+fl}*) from *vavCre⁻Socs3^{+fl}* mice were used as controls. After repeated demonstration that their responsiveness was identical to that of wild-type cells, *Socs3^{+/+}* hematopoietic cells from C57BL/6 mice were used in subsequent experiments.

Hematopoietic progenitor cell purification

For hematopoietic progenitor cell purification, bone marrow (BM) cells were labeled with biotinylated antibodies specific to Ter119, Mac1, Gr1, CD3, CD4, CD8, B220 and CD127 (BD Biosciences, CA, USA) and lineage-committed cells depleted using streptavidin-coated magnetic beads (BD Biosciences, CA, USA). The remaining cells were labelled with anti-

CD19-PE, anti-NK1.1-PE, anti-DX5-PE, streptavidin-PE and c-kit-APC. PE-negative APC-positive cells (Lin⁻Kit⁺) were sorted with a MoFlo (Dako, Denmark). The purity of the sorted population was greater than 95% as assessed by microscopic examination following May-Grünwald Giemsa staining.

Immunofluorescence analysis of phosphorylated STAT3 and p42/44

Purified Lin⁻Kit⁺ hematopoietic progenitor cells were incubated for 1 h at 37°C prior to stimulation with 10³ U/mL recombinant human G-CSF, 10³ U/mL recombinant mouse IL-6 or 100 ng/mL recombinant mouse SCF. Cells were fixed after stimulation with 4% paraformaldehyde (PFA) for 15 min at room temperature. Cells were stored in methanol at -20°C after removal of PFA. For confocal studies, cells were stained with rabbit polyclonal anti-phospho-STAT3 (Cell Signaling Technology, MA, USA) diluted 1:50 in PBS/0.1% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. Cells were washed with PBS/0.1% (w/v) BSA and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, CA, USA) and 0.5 µg/mL DAPI (Sigma-Aldrich, MO, USA). Cells were attached to a glass slide by cytocentrifugation and a fluorescent mounting medium applied prior to analyses using a Leica TCS-SP2 confocal microscope. The average cell fluorescence was determined using Image J software. For flow cytometric analysis, cells were incubated for 1 h with 0.3 µg/mL Alexa Fluor 488 anti-phospho-STAT3, 0.3 µg/mL Alexa Fluor 488 anti-phospho-STAT5 or 0.1 µg/mL PE-anti-phospho-ERK1/2 (BD Biosciences, CA, USA) diluted in PBS/0.1% (w/v) BSA. Cells were washed and analysed using a LSR flow cytometer (BD Biosciences, CA, USA).

Clonogenic assays

2.5 × 10⁴ bone marrow cells or 5 × 10⁴ spleen cells or 300–600 Lin⁻Kit⁺ cells were resuspended in 0.3% (w/v) agar and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 20% (v/v) fetal calf serum²³. Cells were incubated for 7 days at 37°C with a final concentration of 10 ng/mL recombinant murine (rm) GM-CSF, rm M-CSF or recombinant human (rh) G-CSF, 25 ng/mL rm IL-3, 100 ng/mL rm IL-6, rh IL-11 or rm SCF, or combinations thereof. At day 7, colonies were enumerated and either fixed in glutaraldehyde and stained for differential counting²³, or picked, pooled and counted to determine the cellular content of colonies. Peripheral blood progenitors were enumerated by culturing 5 – 20 µL of blood in standard colony assays for 7 days, prior to fixation, staining and counting at 40x magnifications²⁴.

For clone mapping experiments, gridded plates were used and cultures were seeded at 300 Lin⁻Kit⁺ cells per mL. Emergent clones (>2 cells) were tracked on Days 1–4. Beyond Day 4, the cultures became too crowded to accurately score.

CFSE proliferation

For CFSE experiments, Lin⁻Kit⁺ hematopoietic progenitor cells were stained with 5 µM CFSE. 10³ cells were added to wells containing DME/20% (v/v) BCS and the indicated stimuli. Propidium iodide-stained cells were analysed by flow cytometry after 1–4 days incubation at 37°C/10% (v/v) CO₂.

Microarray analysis

Purified Lin⁻Kit⁺ hematopoietic progenitor cells were treated with 10 ng/mL G-CSF or 100 ng/mL IL-6 or saline for 4 h. Experiments were designed to enable performance of multiway comparisons between *Socs3*^{-/-} cells stimulated with G-CSF or IL-6 or saline and *Socs3*^{+/+} cells stimulated in parallel. Total RNA was prepared with TRIzol (Invitrogen, CA, USA) and purified using RNeasy columns (Qiagen, CA, USA). Two separate experiments were performed and RNA pooled for analysis from progenitor cells purified from 5–10 mice. cDNA

was prepared from 100 ng total RNA according to Affymetrix methodology and purified using Phase Lock Gels (Eppendorf, Germany). Biotin-labeled RNA was synthesized using BioArray HighYield RNA Transcript Labeling Kit (Enzo, NY, USA), fragmented to obtain a cRNA size of approximately 200 bases, and hybridised to Affymetrix murine genome V2 chips in accordance with the manufacturer's instructions. Biotin-labeled RNA was stained with phycoerythrin-streptavidin (Invitrogen, CA, USA) and fluorescence intensity analysed with a GeneChip scanner (Affymetrix, CA, USA). The raw expression values were analysed using Robust Multichip Average (<http://rmaexpress.bmbolstad.com>)²⁵. Further analysis of microarray data was performed using the Affy package of the R project for Statistical Computing (<http://www.r-project.org>).

Quantitative real-time PCR

To validate the differential expression of genes identified by microarray analysis, two independent Lin⁻Kit⁺ hematopoietic progenitor cell samples were treated with 100 ng/mL IL-6, 10 ng/mL G-CSF or saline for 4 h, and total RNA was prepared using TRIzol and RNeasy columns. cDNA was generated using Superscript II reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an Applied Biosystems GeneCard and a 7900HT scanner (Applied Biosystems, CA, USA).

Results

Altered progenitor cell differentiation induced by IL-6 from Socs3-deficient bone marrow

In our initial study, when Socs3-deficient BM cells were grown in clonal culture with G-CSF (but not GM-CSF or IL-3), three prominent abnormalities were identified: a three-fold increase in the frequency of colony formation, a two-fold increase in the cellular content of the colonies, and a propensity for the colonies to contain macrophages as well as neutrophils⁵. IL-6 signaling is also negatively regulated by Socs3 in hepatocytes and macrophages^{4,6,7}, but when Socs3-deficient BM cells were grown in clonal culture with IL-6 stimulation, no increase in colony number was observed but a change in the colony composition was apparent. To explore further whether lack of Socs3 altered the behaviour of hematopoietic progenitor cells in response to IL-6, we therefore studied the composition of emergent colonies, and their dose-responsiveness to IL-6.

Table 1 summarises the types of colonies present after 7 days stimulation with IL-6, G-CSF, or two potent combinations of cytokines which can induce both neutrophil and macrophage maturation (SCF and IL-3, M-CSF and GM-CSF). Consistent with previous observations, G-CSF induced an increased number of macrophage colonies, while there was no perturbation in distribution of myeloid colony types in cultures stimulated with either SCF + IL-3 or GM-CSF + M-CSF. IL-6 also increased the proportion of colonies containing macrophages, to the apparent expense of numbers of pure neutrophil-containing colonies.

Although the overall number of colonies produced by IL-6 was not different between Socs3-deficient and wild-type cells (Table 1), an increased sensitivity to IL-6 was evident (Figure 1A). The differences between Socs3-deficient and wild-type cells were most obvious when the composition of the colonies was analysed (Figure 1B). At low concentrations of IL-6, macrophage-containing colony formation was highly favoured by cells lacking Socs3.

IL-6 and G-CSF induce aberrant macrophage production from purified progenitor cells

To reduce the possibility that this disturbance in myeloid cell differentiation trivially reflected either enrichment for different progenitor cell populations between the genotypes or the abnormal activity of some other cell type within the Socs3-deficient bone marrow, cultures of

purified Lin⁻Kit⁺ progenitor cells were prepared and analysed (Table 2). Again, a skewing of the composition of colonies toward macrophage development was observed. At low concentrations of IL-6 (Figure 2A), a greater than ten-fold difference in colony number was observed. A five-fold increase in the number of colonies was also observed in response to IL-11 (Figure 2B), a cytokine that, like IL-6, uses gp130 to transduce signals.

The synergistic effect of SCF and gp130 signaling on progenitor cell proliferation and differentiation

Synergy is observed between SCF and hematopoietic regulators such as IL-3, G-CSF, IL-6 or IL-11, with an increased number of stem and progenitor cells that proliferate and differentiate into mature myeloid cells, compared to stimulation with either cytokine alone²⁶. The colonies arising from combinations of SCF and IL-3, G-CSF, IL-6 or IL-11 were larger than with any single cytokine. In sharp contrast to the patterns observed with SCF and G-CSF, the synergistic effect of SCF and IL-6 was lost in progenitor cells lacking Socs3 (Table 2), with significant reductions in both the number and size of emergent colonies at concentrations of IL-6 which gave maximal synergy in wild-type cells. This striking effect was highly dependent on the concentration of IL-6 in the culture (Figure 2C). In the presence of low concentrations of IL-6 (1 ng/mL) the synergistic effect on colony number was restored and was similar to that observed for wild-type cells at that concentration (Figure 2C). In contrast to progenitors stimulated with SCF and IL-6, a significant increase in colony number was observed when progenitors were stimulated with SCF and IL-11 (Figure 2D), irrespective of IL-11 concentration.

The lack of synergy between SCF and IL-6 (100 ng/mL) was associated with a significant skewing to colonies containing macrophages and a reduction in colonies containing neutrophils (Table 2). This skewing was also observed when progenitors were stimulated with SCF and G-CSF, with a significant reduction in pure neutrophil colonies and an increase in pure macrophage colonies. When Socs3-deficient progenitors were stimulated with SCF and low concentrations of IL-6 (1 ng/mL), the same propensity for colonies to contain significant numbers of macrophages was observed.

Progenitor cell hyper-responsiveness to G-CSF manifests as an additional division during maturation

To further investigate the cellular basis of altered responsiveness to G-CSF and IL-6 when Socs3 is lacking, we monitored the division of progenitor cells in agar and liquid cultures. Clone mapping experiments in agar were performed on Days 1, 2, 3 and 4, using gridded culture dishes. The total number of clones (defined as >2 cells) initiated within days 1–4 was identical between wild-type and Socs3-deficient cultures when stimulated with IL-6, and no differences in the size of these clones could be discerned (data not shown). For G-CSF, a greater number of new clones were observed on Day 3 (18 clones per 300 Lin⁻Kit⁺ cells for *Socs3*^{-/-} vs 9 clones per 300 Lin⁻Kit⁺ cells for *Socs3*^{+/+}, *p* = 0.05), with no difference in average cell number per clone (9 cells per clone for *Socs3*^{-/-} vs 8 cells per clone for *Socs3*^{+/+}, *p* > 0.1). By Day 4, the average size of the emergent clones was also significantly greater for Socs3-deficient cells than wild-type (20 cells per clone for *Socs3*^{-/-} vs 14 cells per clone for *Socs3*^{+/+}, *p* < 0.005).

Consistent with these patterns, liquid cultures revealed no difference between wild-type and Socs3-deficient cells stimulated with IL-6, SCF, GM-CSF, SCF+IL-3, SCF+G-CSF, SCF+IL-11, SCF+IL-6 or saline on Days 1–4, but increased numbers of Socs3-deficient cells was noted after stimulation with G-CSF or IL-11 (Figure 3A). No differences were observed in the rate of cell death between genotypes (Figure 3A). To confirm the rate of cell division as the primary explanation for the increased production of cells in response to G-CSF, we labelled progenitor cells with CFSE to monitor cell division in culture. The average cellular fluorescence of CFSE-labeled G-CSF-stimulated Socs3-deficient cells was significantly lower

than control cells but not when using SCF+IL-3 (Figure 3B). Together these data suggest that the observed proliferation advantage induced by G-CSF accrues predominantly through an extra division per progenitor, rather than increased survival of cells capable of cell division, or of cells generated, in response to cytokine.

Progenitor cell responses to IL-6 and G-CSF are associated with prolonged phosphorylation of STAT3, but not MAPK

To investigate further the signaling pathways perturbed in progenitor cells lacking *Socs3*, confocal microscope (Figure 4A and 4B) and flow cytometric (Figure 4C) analyses of phosphorylation of STAT3, STAT5 and Mapk3/Mapk1 (also known as p42/44 or Erk1/2) were performed after stimulation with G-CSF or IL-6. As demonstrated in Figure 4, stimulation of progenitor cells with either G-CSF or IL-6 for 30 minutes reproducibly results in detection of phosphorylated STAT3, and that this signal is significantly reduced after 2 h in wild-type cells. In *Socs3*-deficient cells however, phosphorylated STAT3 remains at high levels at 2 h, consistent with data from other primary *Socs3*-deficient cells^{4,5}, and returns to baseline after 4 hours (data not shown). In contrast, no differences in phosphorylation of Mapk3/Mapk1 induced by SCF, G-CSF or IL-6 could be discerned between genotypes (Figure 4C). No differences in STAT5 phosphorylation in wild-type or *Socs3*-deficient cells were detected after stimulation with G-CSF, IL-6, GM-CSF or IL-3 (data not shown).

Changes in gene expression in *Socs3*-deficient hematopoietic progenitor cells in response to G-CSF and IL-6

The prolonged duration of activation of the STAT3 transcription factor in hematopoietic progenitor cells stimulated by G-CSF may contribute to their aberrant proliferation and differentiation. To identify genes that may mediate this phenomenon, purified Lin⁻Kit⁺ hematopoietic progenitor cells were stimulated for 4 h with G-CSF and gene expression analysed by microarray. Except for the expression of *Socs3*, no significant differences (greater than 1.7-fold) in gene expression were noted between genotypes treated with saline for 4 h, indicating that the populations of progenitors were very similar in the absence of cytokine stimulation. Figure 5A and Figure 5B show changes in the transcriptional profile of *Socs3*-deficient progenitor cells compared to control progenitor cells after 4 h of G-CSF stimulation. To ensure that we identified differences in gene expression that were specific to G-CSF stimulation rather than broader differences related to more generalized responses in the absence of *Socs3*, we performed multi-way comparisons of replicate biological data. 58 genes were induced more than 1.7-fold in *Socs3*-deficient cells compared to wild-type cells (lower circle Figure 5A), of which 38 were clearly induced by G-CSF in *Socs3*-deficient cells. Of this subset, only 4 were also convincingly induced by G-CSF in wild-type cells. Conversely, only 9 genes were differentially suppressed by G-CSF in *Socs3*-deficient progenitors relative to wild-type cells. In parallel microarray experiments, we also analysed the gene expression of *Socs3*-deficient progenitors after IL-6 induction, and noted that for the majority of the genes differentially induced or suppressed by G-CSF, the pattern of response was similar for IL-6 (Table 3).

A subset of differentially regulated genes identified by microarray were selected for confirmation using real-time PCR. Selection was based on plausible biological function, availability of primers and need to control for quality of sample and changes in cytokine receptor expression. For the majority of G-CSF differentially induced genes, the real-time PCR data confirmed the microarray data and for most, the differential expression pattern was similar for IL-6 (Fig 5C). Several transcription factors were differentially regulated in *Socs3*-deficient progenitors including *cebpb*, *Jun*, *Fosl2*, *Myc*, *Ets2* and *Bcl3*. Other differentially regulated genes whose protein products are known to affect the proliferation, differentiation and mobilization of hematopoietic progenitor cells included the *Csf2rb1*, *Csf2rb2*, *Ly6a*, *Spp1*,

gp130, *Selp* and *Gzmb*. Several genes that can be induced by TGF-beta including *Tgfb1*, *Thbs1* and *Spp1* were differentially regulated in Socs3-deficient progenitors. Rather than identifying a single or limited number of candidate target genes to predict the change in proliferation and differentiation noted several days following exposure to G-CSF or IL-6, the data reflect the breadth of effect of Socs3 upon the responses of hematopoietic progenitor cells to cytokine stimulation.

Discussion

The generation of macrophages and neutrophils from hematopoietic progenitor cells requires the integration of cytokine signals to coordinate the proliferation, differentiation and survival of progenitor cells to form mature myeloid cells. During infection or after wounding, neutrophils are some of the earliest responding cells and this emergency granulopoiesis is mediated by G-CSF. In patients undergoing chemotherapy, G-CSF is used to mobilize hematopoietic stem and progenitor cells into the blood for later use in transplantation. Exacerbated signaling through gp130-containing receptors including the IL-6R is likely to influence hematopoiesis and immune responses²⁷⁻³⁰. We have previously shown that Socs3-deficient mice display severe morbidity during G-CSF-driven emergency granulopoiesis⁵. This was associated with extensive infiltration and destruction of numerous tissues by neutrophils as well as abnormal mobilization of stem and progenitor cells. In this study, we sought to investigate the defects in Socs3-deficient hematopoietic progenitor cells following cytokine stimulation.

The data reveal three striking abnormalities in cellular behaviour by Socs3-deficient hematopoietic progenitor cells: (i) increased proliferation of myeloid progenitor cells in response to G-CSF; (ii) skewing of mature cell production induced by either IL-6 or G-CSF toward macrophages, rather than purely neutrophils; and (iii) absence of synergy and loss of additive effect by IL-6 when progenitor cells are also stimulated with SCF.

The increased cellular output in both clonal and liquid cultures from Socs3-deficient progenitor cells stimulated with G-CSF is best explained by the occurrence of an additional division during the normally tightly balanced proliferation and maturation pathway that myeloid progenitor cells take to generate large numbers of post-mitotic progeny^{31,32}. The CFSE data that provide the strongest evidence for this extra division do not allow identification of at what stage in the myeloid maturation sequence this occurs. Alternative explanations such as enhanced survival of Socs3-deficient progenitor cells are refuted by the measurement of cell death in the CFSE experiments and by the absence of additional emergent clones in the first few days of the clonal cultures.

Why this increased proliferation occurs with G-CSF, but not with higher concentrations of IL-6 when both cytokines induce similarly perturbed prolongations in STAT3 phosphorylation and similarly normal MAPK phosphorylation cannot be fully explained. However, one obvious difference in gene transcription observed between Socs3-deficient progenitor cells stimulated with G-CSF or IL-6 was the fact that G-CSF markedly increased *c-myc* expression above wild-type whereas no differences in *c-myc* induction were observed between wild-type and Socs3-deficient cells after IL-6 stimulation. In embryonic stem cells and hematopoietic stem cells, *c-myc* is critical for promoting self-renewal and pluripotency while preventing differentiation^{33,34}. Increased *c-myc* expression in G-CSF (but not IL-6)-treated Socs3-deficient hematopoietic progenitor cells may promote self-renewal of hematopoietic progenitors and delay their differentiation to neutrophils. The effect of this change in the balance between self-renewal and differentiation, an increase in the number of neutrophil colonies forming in response to G-CSF and an increase in the size of these colonies, was precisely what was observed in the current experiments.

Another clue may be that at doses of IL-6 below that required for maximal proliferation of wild-type cells, Socs3-deficient cells showed hyper-responsiveness and preferential maturation to macrophages. Induction of *c-myc* expression by either 1 ng/mL or 100ng/mL of IL-6 for 2 or 4 hours did not significantly differ between wild-type and Socs3-deficient progenitors (data not shown), suggesting that a concentration effect is insufficient to explain the differences between G-CSF and IL-6 in Socs3-deficient cells. IL-6-responsive cells may be a different subset of Lin⁻Kit⁺ progenitor cells to those fully responsive to G-CSF as a single cytokine.

Some insight to the altered maturation of progenitor cells resulting in increased macrophage production has been gained through the microarray experiments performed early after stimulation by G-CSF and IL-6. The relative abundance of transcription factors and regulatory proteins is known to influence the outcome of cytokine stimulation^{1,2}. In hematopoietic progenitor cells and also in neutrophils, G-CSF and IL-6 are potent activators of the STAT3 transcription factor^{35,36}. In this study, the duration of G-CSF- and IL-6-induced STAT3 activation was increased in purified hematopoietic progenitor cells lacking Socs3. At the most elementary level, this can be considered to have two effects: increasing the abundance of active STAT3 that can interact with latent transcription factors, and also increasing interactions with transcription factors that are rapidly induced by G-CSF or IL-6 stimulation.

C/EBP β and to a lesser extent C/EBP δ were more highly induced by both IL-6 and G-CSF in Socs3-deficient progenitor cells than wild-type cells. The C/EBP family of transcription factors are induced by STAT3 and can interact with STAT3 to augment G-CSF-induced transcriptional activity³⁷. C/EBP transcription factors are differentially expressed during myeloid cell development and gene-targeting studies have demonstrated central roles for these transcription factors in balancing macrophage and neutrophil development^{38–48}. A loss of temporal control of expression of the C/EBP transcription factors during cytokine-stimulated culture is likely to contribute to the aberrant proliferation and differentiation of Socs3-deficient progenitor cells⁴⁴.

The expression of *Ets2*, *Bcl3*, *Jun* and *Fosl2* (*Fra-2*) transcription factors are also aberrantly increased in Socs3-deficient hematopoietic progenitor cells stimulated with IL-6 or G-CSF, and may also contribute to the altered cell behaviour. Constitutive expression of *Ets2* in a myeloblast cell line induces their differentiation to macrophages suggesting a role for this transcription factor in the skewing of progenitor cell differentiation to macrophages⁴⁹. The *Bcl3* transcriptional regulator is IL-6-inducible and expression is dependent on STAT3⁵⁰. The activator protein 1 (AP-1) transcription factor complex is composed of dimers of Jun family protein homodimers (c-Jun, JunB, JunD) or Jun heterodimers with Fos (c-Fos, FosB, Fra-1, Fra-2)^{51–56}. STAT3 can interact directly with c-Fos and c-Jun transcription factors to enhance IL-6 response element (IRE) transactivation⁵⁷. Cooperation of STAT3 and the MAP kinase pathway can also upregulate expression of *Jun* and *Fos*^{57–59}. Increased STAT3 activation in Socs3-deficient hematopoietic progenitor cells may promote interaction with latent Jun and Fos members to further increase expression of these transcription factors. Enhanced c-Jun and Fra-2 expression may also lead to increased levels of a heterodimeric Fra-2/c-Jun or homodimeric c-Jun AP-1 transcription factor complexes.

A conclusion of the experiments examining changes in transcription consequent upon lack of Socs3 is that a large number of effectors of cellular response are normally tightly regulated during cytokine stimulation. Socs3 appears to have a much broader role in regulating cellular responses than obviously predicted based on previous and current data suggesting that STAT3 is the predominant signaling pathway regulated by this protein. The abnormalities in proliferation and differentiation during stimulation of hematopoietic progenitor cells by G-CSF or IL-6 are likely the sum total of a large number of relatively subtle effects in combination,

rather than due to a single linear pathway. For this reason, it is currently impractical to validate this model by experimentally modulating multiple transcription factors simultaneously in a population of primary hematopoietic progenitor cells. Approaches targeting individual transcription factors by overexpression or RNAi-mediated gene knockdown for this purpose are flawed for several reasons. Firstly, the cytokine stimulation that is required during the *in vitro* targeting process introduces biases that do not exist when freshly isolated cells are tested as in this work. Secondly, achieving gross changes to individual transcription factors as occurs with targeting cannot be expected to resemble the moderate changes evident in the expression data. Thirdly, targeting single transcription factors does not test the situation revealed by the expression data-differential changes in multiple transcription factors. It seems probable that it is similarly the sum of many aberrations in hematopoietic cell function that explain the *in vivo* predilection for inflammation in mice lacking Socs3 in blood cells.

Finally, these studies open new questions about gp130 signaling and progenitor cell physiology. Despite both using gp130 as the signaling moiety of their transmembrane receptor, IL-6 and IL-11 stimulated different aberrations in Socs3-deficient progenitors. IL-11 stimulated heightened proliferation at maximal concentrations, whereas IL-6 stimulated heightened proliferation only at low concentrations. Further, the vast majority of mature cells generated by IL-11 were neutrophils, rather than macrophages, and in sharp contrast to IL-6, IL-11 maintained its synergy with SCF in Socs3-deficient progenitors. We speculate that as well as regulation of gp130 signaling by Socs3, the alpha chain abundance can influence cellular behaviour. How Socs3 regulates synergy between IL-6 and SCF is unknown, and future studies will explore this in detail as an avenue to understanding the molecular basis of synergy in hematopoietic cells.

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References

1. Iwasaki H, Mizuno S, Arinobu Y, et al. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes Dev* 2006;20:3010–3021. [PubMed: 17079688]
2. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol* 2007;7:105–117. [PubMed: 17259967]
3. Starr R, Willson TA, Viney EM, et al. A family of cytokine-inducible inhibitors of signaling. *Nature* 1997;387:917–921. [PubMed: 9202125]
4. Croker BA, Krebs DL, Zhang JG, et al. SOCS3 negatively regulates IL-6 signaling *in vivo*. *Nat Immunol* 2003;4:540–545. [PubMed: 12754505]
5. Croker BA, Metcalf D, Robb L, et al. SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. *Immunity* 2004;20:153–165. [PubMed: 14975238]
6. Yasukawa H, Ohishi M, Mori H, et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol* 2003;4:551–556. [PubMed: 12754507]
7. Lang R, Pauleau AL, Parganas E, et al. SOCS3 regulates the plasticity of gp130 signaling. *Nat Immunol* 2003;4:546–550. [PubMed: 12754506]

8. Robb L, Boyle K, Rakar S, et al. Genetic reduction of embryonic leukemia-inhibitory factor production rescues placentation in SOCS3-null embryos but does not prevent inflammatory disease. *Proc Natl Acad Sci U S A* 2005;102:16333–16338. [PubMed: 16258063]
9. Ridley AJ. Membrane ruffling and signal transduction. *Bioessays* 1994;16:321–327. [PubMed: 8024541]
10. Mori H, Hanada R, Hanada T, et al. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat Med* 2004;10:739–743. [PubMed: 15208705]
11. Hilton DJ, Richardson RT, Alexander WS, et al. Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95:114–119. [PubMed: 9419338]
12. Yoshimura A, Ohkubo T, Kiguchi T, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *Embo J* 1995;14:2816–2826. [PubMed: 7796808]
13. Zhang JG, Farley A, Nicholson SE, et al. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:2071–2076. [PubMed: 10051596]
14. Boyle K, Egan P, Rakar S, et al. The SOCS box of suppressor of cytokine signaling-3 contributes to the control of G-CSF responsiveness in vivo. *Blood* 2007;110:1466–1474. [PubMed: 17510322]
15. Kamura T, Sato S, Haque D, et al. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev* 1998;12:3872–3881. [PubMed: 9869640]
16. Babon JJ, McManus EJ, Yao S, et al. The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability. *Mol Cell* 2006;22:205–216. [PubMed: 16630890]
17. Nicholson SE, De Souza D, Fabri LJ, et al. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:6493–6498. [PubMed: 10829066]
18. Hortner M, Nielsch U, Mayr LM, Johnston JA, Heinrich PC, Haan S. Suppressor of cytokine signaling-3 is recruited to the activated granulocyte-colony stimulating factor receptor and modulates its signal transduction. *J Immunol* 2002;169:1219–1227. [PubMed: 12133942]
19. Nicholson SE, Willson TA, Farley A, et al. Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO Journal* 1999;18:375–385. [PubMed: 9889194]
20. Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. *Journal of Biological Chemistry* 2000;275:12848–12856. [PubMed: 10777583]
21. Akbarzadeh S, Ward AC, McPhee DO, Alexander WS, Lieschke GJ, Layton JE. Tyrosine residues of the granulocyte colony-stimulating factor receptor transmit proliferation and differentiation signals in murine bone marrow cells. *Blood* 2002;99:879–887. [PubMed: 11806990]
22. Ward AC, Smith L, de Koning JP, van Aesch Y, Touw IP. Multiple signals mediate proliferation, differentiation, and survival from the granulocyte colony-stimulating factor receptor in myeloid 32D cells. *J Biol Chem* 1999;274:14956–14962. [PubMed: 10329697]
23. Metcalf D. *Clonal Culture of Hemopoietic Cells : Techniques and Applications*. Amsterdam: Elsevier; 1984.
24. Roberts AW, Metcalf D. Granulocyte colony-stimulating factor induces selective elevations of progenitor cells in the peripheral blood of mice. *Exp Hematol* 1994;22:1156–1163. [PubMed: 7523168]
25. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185–193. [PubMed: 12538238]
26. Metcalf D, Nicola NA. Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors. *Proc Natl Acad Sci U S A* 1991;88:6239–6243. [PubMed: 1712485]

27. Fattori E, Cappelletti M, Costa P, et al. Defective inflammatory response in interleukin 6-deficient mice. *J Exp Med* 1994;180:1243–1250. [PubMed: 7931061]
28. Hilbert DM, Kopf M, Mock BA, Kohler G, Rudikoff S. Interleukin 6 is essential for in vivo development of B lineage neoplasms. *J Exp Med* 1995;182:243–248. [PubMed: 7790819]
29. Schirmacher P, Peters M, Ciliberto G, et al. Hepatocellular hyperplasia, plasmacytoma formation, and extramedullary hematopoiesis in interleukin (IL)-6/soluble IL-6 receptor double- transgenic mice. *Am J Pathol* 1998;153:639–648. [PubMed: 9708822]
30. Yoshida K, Taga T, Saito M, et al. Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proc Natl Acad Sci U S A* 1996;93:407–411. [PubMed: 8552649]
31. Lord BI, Bronchud MH, Owens S, et al. The kinetics of human granulopoiesis following treatment with granulocyte colony-stimulating factor in vivo. *Proc Natl Acad Sci U S A* 1989;86:9499–9503. [PubMed: 2480603]
32. Lord BI, Molineux G, Pojda Z, Souza LM, Mermod JJ, Dexter TM. Myeloid cell kinetics in mice treated with recombinant interleukin-3, granulocyte colony-stimulating factor (CSF), or granulocyte-macrophage CSF in vivo. *Blood* 1991;77:2154–2159. [PubMed: 1709372]
33. Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 2005;132:885–896. [PubMed: 15673569]
34. Wilson A, Murphy MJ, Oskarsson T, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev* 2004;18:2747–2763. [PubMed: 15545632]
35. Tian SS, Lamb P, Seidel HM, Stein RB, Rosen J. Rapid activation of the STAT3 transcription factor by granulocyte colony-stimulating factor. *Blood* 1994;84:1760–1764. [PubMed: 7521688]
36. Zhong Z, Wen Z, Darnell JE Jr. STAT3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 1994;264:95–98. [PubMed: 8140422]
37. Numata A, Shimoda K, Kamezaki K, et al. Signal transducers and activators of transcription 3 augments the transcriptional activity of CCAAT/enhancer-binding protein alpha in granulocyte colony-stimulating factor signaling pathway. *J Biol Chem* 2005;280:12621–12629. [PubMed: 15664994]
38. Dahl R, Walsh JC, Lancki D, et al. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat Immunol* 2003;4:1029–1036. [PubMed: 12958595]
39. Wang D, D'Costa J, Civin CI, Friedman AD. C/EBPalpha directs monocytic commitment of primary myeloid progenitors. *Blood* 2006;108:1223–1229. [PubMed: 16645168]
40. Zhang DE, Hetherington CJ, Meyers S, et al. CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol Cell Biol* 1996;16:1231–1240. [PubMed: 8622667]
41. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 1996;88:1234–1247. [PubMed: 8695841]
42. Heath V, Suh HC, Holman M, et al. C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development in vitro and in vivo. *Blood* 2004;104:1639–1647. [PubMed: 15073037]
43. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A* 1997;94:569–574. [PubMed: 9012825]
44. Jones LC, Lin ML, Chen SS, et al. Expression of C/EBPbeta from the C/ebpalpha gene locus is sufficient for normal hematopoiesis in vivo. *Blood* 2002;99:2032–2036. [PubMed: 11877276]
45. Natsuka S, Akira S, Nishio Y, et al. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* 1992;79:460–466. [PubMed: 1730090]
46. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998;18:4301–4314. [PubMed: 9632814]

47. Scott LM, Civin CI, Rorth P, Friedman AD. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood* 1992;80:1725–1735. [PubMed: 1391942]
48. Hohaus S, Petrovick MS, Voso MT, Sun Z, Zhang DE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol Cell Biol* 1995;15:5830–5845. [PubMed: 7565736]
49. Aperlo C, Pognonec P, Stanley ER, Boulukos KE. Constitutive c-ets2 expression in M1D+ myeloblast leukemic cells induces their differentiation to macrophages. *Mol Cell Biol* 1996;16:6851–6858. [PubMed: 8943340]
50. Brocke-Heidrich K, Ge B, Cvijic H, et al. BCL3 is induced by IL-6 via STAT3 binding to intronic enhancer HS4 and represses its own transcription. *Oncogene* 2006;25:7297–7304. [PubMed: 16732314]
51. Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 1987;238:1386–1392. [PubMed: 2825349]
52. Rauscher FJ 3rd, Sambucetti LC, Curran T, Distel RJ, Spiegelman BM. Common DNA binding site for Fos protein complexes and transcription factor AP-1. *Cell* 1988;52:471–480. [PubMed: 3125983]
53. Franza BR Jr, Rauscher FJ 3rd, Josephs SF, Curran T. The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. *Science* 1988;239:1150–1153. [PubMed: 2964084]
54. Rauscher FJ 3rd, Cohen DR, Curran T, et al. Fos-associated protein p39 is the product of the jun proto-oncogene. *Science* 1988;240:1010–1016. [PubMed: 3130660]
55. Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* 1988;54:541–552. [PubMed: 3135940]
56. Sassone-Corsi P, Lamph WW, Kamps M, Verma IM. fos-associated cellular p39 is related to nuclear transcription factor AP-1. *Cell* 1988;54:553–560. [PubMed: 3135941]
57. Schuringa JJ, Timmer H, Luttikhuisen D, Vellenga E, Kruijer W. c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 response element (IRE). *Cytokine* 2001;14:78–87. [PubMed: 11356008]
58. Higashi N, Kunitomo H, Kaneko S, et al. Cytoplasmic c-Fos induced by the YXXQ-derived STAT3 signal requires the co-operative MEK/ERK signal for its nuclear translocation. *Genes Cells* 2004;9:233–242. [PubMed: 15005710]
59. Clarkson RW, Boland MP, Kritikou EA, et al. The genes induced by signal transducer and activators of transcription (STAT)3 and STAT5 in mammary epithelial cells define the roles of these STATs in mammary development. *Mol Endocrinol* 2006;20:675–685. [PubMed: 16293640]

Figure 1.

Altered IL-6 dose-response of neutrophil, granulocyte-macrophage and macrophage progenitors in the absence of *Socs3*. (A) 2.5×10^4 unfractionated bone marrow cells were cultured with variable concentrations of IL-6 for 7 days. Colonies containing greater than 50 cells were enumerated and results represented as a percentage of colony number at 100 ng/mL IL-6, the maximal IL-6 concentration in these assays. wild-type, filled circle; *Socs3*-deficient; open circle. Mean and standard deviation of 3–4 independent experiments shown. (B) Colony composition was determined by examination of stained cultures. Mean and standard deviation of 3–4 independent experiments shown. *Socs3*-deficient neutrophil colonies, open circle;

Socs3-deficient macrophage colonies, grey triangle. Wild-type neutrophil colonies, filled circle. Note that no wild-type macrophage colonies develop.

Figure 2.

Altered responsiveness to both IL-6 and IL-11 by purified Socs3-deficient progenitor cells.

(A) Increases in IL-6-responsive Socs3-deficient progenitor cells occur at sub-maximal concentrations of IL-6. (B) Increased numbers of IL-11-responsive Socs3-deficient progenitor cells.

(C and D) Synergistic responses to gp130 cytokines and SCF in the absence of Socs3.

(C) The lack of synergy to SCF and IL-6 in Socs3-deficient cells is dependent on the concentration of IL-6. (D) Synergy is observed in Socs3-deficient progenitor cells in response to SCF + IL-11 but this is not dependent on the concentration of IL-11. wild-type, black bar; Socs3-deficient, white bar. Mean and SEM of 3 independent experiments shown. * p<0.05

Figure 3.

Increased cellular output and altered differentiation of Socs3-deficient hematopoietic progenitor cells in response to G-CSF stimulation. The total cellular output (A) is altered in Socs3-deficient CFSE-stained Lin⁻Kit⁺ hematopoietic progenitor cells in a cytokine-specific fashion. Increased numbers of cells were observed in Socs3-deficient cultures in response to 10 ng/mL G-CSF and 100 ng/mL IL-11 but not other combinations of cytokines. * $p > 0.05$, Mean and standard deviation of 3 independent experiments, wild-type v Socs3-deficient. The number of propidium iodide-positive (PI⁺) Socs3-deficient cells was not different to wild-type controls (live cells, white; PI⁺ cells, grey). (B) Purified hematopoietic progenitor cells were stained with CFSE and then cultured in the presence of 10 ng/mL GM-CSF, M-CSF or G-CSF, 25 ng/mL IL-3, 100 ng/mL IL-6, IL-11 or SCF, or combinations thereof for 4 days. The average CFSE intensity of G-CSF-treated Socs3-deficient cells was lower indicating that at least one

additional division had occurred in these cultures. Wild-type, black line; Socs3-deficient, red line.

Figure 4.

STAT3 activation is enhanced in *Socs3*-deficient progenitor cells in response to 10 ng/mL G-CSF or 100 ng/mL IL-6 but *Mapk3/Mapk1* activation is normal. Purified Lin⁻Kit⁺ hematopoietic progenitor cells were treated with 100 ng/mL SCF, 100 ng/mL IL-6, 10 ng/mL G-CSF or saline. Cells were stained with FITC-conjugated antibodies specific to active phospho-STAT3 transcription factor, *Mapk3/Mapk1* (p-ERK1/2) or isotype controls. (A) Cells were stained with antibody specific to phospho-STAT3 (green) and then analysed using confocal microscopy. DAPI (blue) was used to stain nucleic acid. The fluorescence was quantified using Image J software (B) and flow cytometry was used to analyse both STAT3 and *Mapk* phosphorylation (C).

Figure 5. Aberrant gene expression induced by G-CSF in *Socs3*-deficient progenitor cells. Purified lin⁺kit⁺ hematopoietic progenitor cells were stimulated with 10 ng/mL G-CSF or saline and RNA harvested at 4 h post-stimulation for microarray analysis. (A and B) The Venn diagrams show the number of genes differentially regulated in *Socs3*-deficient cells and wild-type cells (relative to saline-treated samples) and the genes that are differentially regulated by G-CSF in both *Socs3*-deficient and wild-type samples. The greyed section highlights those genes induced by G-CSF in *Socs3*^{-/-} cells that were most highly upregulated (A) or downregulated (B) compared to *Socs3*^{+/+} cells. (C) Quantitative analysis of gene expression from G-CSF- and IL-6-treated wild-type or *Socs3*-deficient hematopoietic progenitor cells by real-time PCR.

Transcriptional responses are plotted as $\log_2$ of the response to G-CSF or IL-6 of Socs3-deficient hematopoietic progenitors, relative to the response to G-CSF or IL-6 by wild-type hematopoietic progenitors. Error bars correspond to the standard error of the mean of two separate experiments.

Table 1
Aberrant IL-6 or G-CSF-induced proliferation and differentiation of progenitor cells lacking *Socs3*.

Stimulus	Socs3 Genotype	Total	Colony Composition					
			Blast	Neutrophil	Granulocyte/Macrophage	Macrophage	Eosinophil	Megakaryocyte
IL-6	+/fl	20 ± 6	1 ± 1	17 ± 7	3 ± 2			
	-/Δ	26 ± 2	2 ± 1	9 ± 2 [*]	13 ± 3 [*]	3 ± 1 [*]		
G-CSF	+/fl	11 ± 4		10 ± 3	1 ± 1	1 ± 1		
	-/Δ	31 ± 9 [*]	2 ± 1	21 ± 3 [*]	3 ± 2	6 ± 5 [*]		
SCF + IL-3	+/fl	75 ± 13	8 ± 6	22 ± 8	15 ± 3	19 ± 3	2 ± 0	9 ± 5
	-/Δ	75 ± 6	7 ± 4	24 ± 5	16 ± 2	17 ± 6	1 ± 1	10 ± 1
M-CSF + GM-CSF	+/fl	60 ± 19	1 ± 2	15 ± 5	12 ± 5	30 ± 8	2 ± 2	
	-/Δ	61 ± 15	4 ± 4	15 ± 5	13 ± 2	28 ± 10	1 ± 1	

2.5 × 10⁴ unfractionated bone marrow cells were cultured in the presence of 10 ng/mL GM-CSF, M-CSF or G-CSF, 25 ng/mL IL-3, 100 ng/mL IL-6 or SCF, or combinations thereof in 0.3% agar/DME/FCS. After 7 days of incubation, cultures were fixed, stained and enumerated at x200 magnification. Results represent mean ± SD from 4 mice of each genotype.

* p<0.05, *Socs3*^{+/*fl*} v *Socs3*^{-/*Δ*}.

Table II
Aberrant IL-6 and G-CSF-induced proliferation and differentiation of purified hematopoietic progenitor cells.

Stimulus	Socs3 Genotype	Total colonies / 300 cells	Cells per colony	Colony Composition (%)					
				Blast	Neutrophil	Granulocyte/Macrophage	Macrophage	Eosinophil	Megakaryocyte
IL-6	+/ Δ	10 $\pm$ 4	194 $\pm$ 50		64 $\pm$ 3	33 $\pm$ 6	2 $\pm$ 1		1 $\pm$ 1
	-/ Δ	19 $\pm$ 4*	206 $\pm$ 47	6 $\pm$ 6	36 $\pm$ 5*	43 $\pm$ 8*	13 $\pm$ 3*		3 $\pm$ 2
G-CSF	+/ Δ	12 $\pm$ 2	305 $\pm$ 108	4 $\pm$ 0	91 $\pm$ 3	5 $\pm$ 3			
	-/ Δ	20 $\pm$ 9*	693 $\pm$ 147*	9 $\pm$ 6	61 $\pm$ 19*	22 $\pm$ 11*	8 $\pm$ 2*		
SCF	+/ Δ	19 $\pm$ 2	1054 $\pm$ 192	38 $\pm$ 3	62 $\pm$ 3	1 $\pm$ 1			
	-/ Δ	23 $\pm$ 3	849 $\pm$ 77	35 $\pm$ 5	64 $\pm$ 4	23 $\pm$ 1			
SCSF + IL-6	+/ Δ	41 $\pm$ 15	3835 $\pm$ 1828	22 $\pm$ 3	40 $\pm$ 3	37 $\pm$ 7*	5 $\pm$ 1	1 $\pm$ 1	9 $\pm$ 1
	-/ Δ	24 $\pm$ 7*	646 $\pm$ 186*	16 $\pm$ 5	26 $\pm$ 2*	37 $\pm$ 7*	15 $\pm$ 4*		7 $\pm$ 7
SCSF + G-CSF	+/ Δ	32 $\pm$ 14	5144 $\pm$ 2314	38 $\pm$ 6	44 $\pm$ 3	14 $\pm$ 6	4 $\pm$ 0		4 $\pm$ 0
	-/ Δ	39 $\pm$ 10	4854 $\pm$ 656	17 $\pm$ 7*	24 $\pm$ 4*	37 $\pm$ 1*	22 $\pm$ 12*		

300 purified lin⁻kit⁺ hematopoietic progenitor cells were cultured in the presence of 10 ng/mL GM-CSF, M-CSF or G-CSF, 25 ng/mL IL-3, 100 ng/mL IL-6 or SCF, or combinations thereof in 0.3% agar/DME/FCS. After 7 days of incubation, cultures were fixed, stained and enumerated at x200 magnification. Results represent mean $\pm$ SD from 3–4 mice of each genotype.

* p<0.05, Sox3^{+/Δ} v Sox3⁻/Δ.

Table 3**G-CSF-responsive genes that are differentially regulated in *Socs3*^{-Δ} hematopoietic progenitors relative to *Socs3*^{+fl} hematopoietic progenitors**

Genes were identified by microarray analysis of *Socs3*^{-Δ} and *Socs3*^{+fl} hematopoietic progenitors stimulated with either saline or G-CSF (see Figure 5). Responsiveness of genes to IL-6 was determined by microarray analysis of *Socs3*^{-Δ} hematopoietic progenitors stimulated with IL-6 or saline.

Genes differentially induced by G-CSF

Symbol	Title	RefSeq ID	IL-6-responsive
---	---	BB769628	Yes
---	RIKEN cDNA 2010002N04	NM_134133	
Adam8	a disintegrin and metalloprotease domain 8	NM_007403	
Adam9	a disintegrin and metalloproteinase domain 9 (meltrin gamma)	NM_007404	Yes
Aqp9	aquaporin 9	NM_022026	Yes
Basp1	brain abundant, membrane attached signal protein 1	NM_027395	
Bcl3	B-cell leukemia/lymphoma 3	NM_033601	Yes
Ccr5	chemokine (C-C motif) receptor 5	NM_009917	Yes
Cebpβ	CCAAT/enhancer binding protein (C/EBP), beta	NM_009883	Yes
Clec4d	C-type lectin domain family 4, member d	NM_010819	Yes
Clec4e	C-type lectin domain family 4, member e	NM_019948	Yes
Clec5a	C-type lectin domain family 5, member a	NM_021364	Yes
Csf2rb1	colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macrophage)	NM_007780	Yes
Csf2rb2	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	NM_007781	Yes
Cspg2	chondroitin sulfate proteoglycan 2	XM_488510	Yes
Ets2	E26 avian leukemia oncogene 2, 3' domain	NM_011809	
Fosl2	fos-like antigen 2	NM_008037	Yes
Gm1960	gene model 1960, (NCBI)	NM_203320	Yes
Gp49a	glycoprotein 49 A	NM_008147	Yes
Gzmb	granzyme B	NM_013542	
Ier3	immediate early response 3	NM_133662	Yes
Ier5	immediate early response 5	NM_010500	Yes
Ifitm1	interferon induced transmembrane protein 1	NM_026820	Yes
Il6	interleukin 6	NM_031168	Yes
Kcnq1	Tumor-suppressing subchromosomal transferable fragment 8	NM_008434	
Lpp	LIM domain containing preferred translocation partner in lipoma	NM_178665	
Ly6a	lymphocyte antigen 6 complex, locus A	NM_010738	Yes
---	similar to Normal mucosa of esophagus specific gene 1 protein	NM_001004 174	Yes
Nupr1	nuclear protein 1	NM_019738	Yes
Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)	NM_008869	Yes
Ptgs2	prostaglandin-endoperoxide synthase 2	NM_011198	Yes
Selp	selectin, platelet	NM_011347	Yes
Serpib2	serine (or cysteine) proteinase inhibitor, clade B, member 2	NM_011111	
Spp1	secreted phosphoprotein 1	NM_009263	Yes
Srm	spermidine synthase	NM_009272	Yes
Tgm2	transglutaminase 2, C polypeptide	NM_009373	Yes
Thbs1	thrombospondin 1	NM_011580	
Trib3	tribbles homolog 3 (Drosophila)	NM_144554	Yes

Genes differentially suppressed by
G-CSF

Symbol	Title	RefSeq ID	IL-6-responsive
Rasgrp2	RAS, guanyl releasing protein 2	NM_011242	
Pten	phosphatase and tensin homolog	NM_008960	Yes
Zfx1b	zinc finger homeobox 1b	NM_015753	Yes
---	RIKEN cDNA 2310067E08	BF168366	Yes
---	RIKEN cDNA 3300001H21	NM_027334	Yes
Icsbp1	interferon consensus sequence binding protein 1	NM_008320	Yes
Cxcr4	chemokine (C-X-C motif) receptor 4	NM_009911	Yes
---	RIKEN cDNA B430201A12	XM_283903	Yes
Wdr6	WD repeat domain 6	NM_031392	Yes