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SOCS3 Is a Critical Physiological Negative Regulator of G-CSF Signaling and Emergency Granulopoiesis

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Summary

To determine the importance of suppressor of cytokine signaling-3 (SOCS3) in the regulation of hematopoietic growth factor signaling generally, and of G-CSF-induced cellular responses specifically, we created mice in which the *Socs3* gene was deleted in all hematopoietic cells. Although normal until young adulthood, these mice then developed neutrophilia and a spectrum of inflammatory pathologies. When stimulated with G-CSF in vitro, SOCS3-deficient cells of the neutrophilic granulocyte lineage exhibited prolonged STAT3 activation and enhanced cellular responses to G-CSF, including an increase in cloning frequency, survival, and proliferative capacity. Consistent with the in vitro findings, mutant mice injected with G-CSF displayed enhanced neutrophilia, progenitor cell mobilization, and splenomegaly, but unexpectedly also developed inflammatory neutrophil infiltration into multiple tissues and consequent hind-leg paresis. We conclude that SOCS3 is a key negative regulator of G-CSF signaling in myeloid cells and that this is of particular significance during G-CSF-driven emergency granulopoiesis.

Introduction

Granulocyte colony-stimulating factor (G-CSF) is the primary extracellular regulator of granulopoiesis, affecting the proliferation, survival, and differentiation of all cells within the granulocytic lineage, from hematopoietic stem cells through to mature neutrophils (Lieschke et al., 1994). Clinically, this activity is exploited to accelerate neutrophil recovery following chemotherapy and to mobilize stem and progenitor cells into the peripheral blood for later use during hematopoietic stem cell transplantation (Duhrsen et al., 1988; Grigg et al., 1995; Sheridan et al., 1992).

Dimerization of the extracellular domains of the G-CSF receptor (G-CSFR) results in activation of Janus kinases (Jaks) and phosphorylation of one or more tyrosine residues in the C-terminal region of the receptor and activation of multiple intracellular signaling proteins, including the signal transducers and activators of transcription (STAT) and mitogen-activated protein (MAP) kinases (Akbarzadeh et al., 2002; Hermans et al., 2002; Lee et al., 2002; McLemore et al., 2001; Suzuki et al., 1999). Jak1, Jak2, and Tyk2 are tyrosine kinases recruited to the G-CSF receptor, and these in turn activate the STAT1, STAT3, and STAT5 transcription factors, of which STAT3 is the most important (Tian et al., 1994).

As is the case with other cytokine signaling pathways, the activation of intermediates which propagate key signals is now well characterized, but understanding of the negative regulation of intracellular signaling is limited. The suppressor of cytokine signaling (SOCS) proteins are attractive candidates as negative regulators of cytokine signaling. They are induced as part of the cellular response to cytokine stimulation and are thought to act within a classical negative feedback loop to inhibit activation of signaling pathways and target signaling components for proteasomal degradation (Endo et al., 1997; Kamura et al., 1998; Naka et al., 1997; Starr et al., 1997; Zhang et al., 1999).

With respect to G-CSF, current data suggest that of the SOCS family, SOCS3 is the most likely candidate as a physiological regulator of cellular responses to this cytokine. SOCS3 expression is induced in primary myeloid cells when stimulated by G-CSF (Hortner et al., 2002; Lee et al., 2002). SOCS3 binds selectively to the human G-CSFR at tyrosine 729 (Y728 for the murine G-CSFR) when this residue is phosphorylated (pY729) (Hortner et al., 2002). In cell-based overexpression systems, binding of SOCS3 to pY729 inhibits STAT-dependent gene expression after stimulation of the cell with G-CSF (Hortner et al., 2002). Evidence from mutational analyses in two distinct systems suggests that Y729-dependent regulation of STAT signaling is important for normal G-CSFR function in primary cells (Akbarzadeh et al., 2002; Hermans et al., 2002). First, primary hematopoietic progenitor cells exclusively expressing a mutant form of G-CSFR that has Y729 as the only functional tyrosine residue responded to G-CSF in an aberrant fashion: there was reduced G-CSF-induced colony formation in semisolid cultures, and the number of cells within each colony was reduced by 50% on average (Hermans et al., 2002). In other experiments where progenitor cells were stimulated through hybrid receptors (EGF extracellular domain and G-CSFR intracellular domain) bearing inactivating mutations of Y729 (Y729F), there was increased tritiated thymidine incorporation by bone marrow cells and an increase in the proportion of macrophage-containing colonies emergent after in vitro culture (Akbarzadeh et al., 2002). Taken together, these data indicate that SOCS3 activity relies on binding to Y729, and that the presence of Y729 diminishes proliferative signals from the G-CSFR. A recent report has postulated that the increased granulocyte production observed in mice with a conditional deletion of *STAT3* was related to the reduced SOCS3 expression observed in

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STAT3-deficient myeloid cells for these mice (Lee et al., 2002). A logical implication of these observations is that SOCS3 is likely to be a negative regulator of proliferative signals from the G-CSFR.

However, SOCS3 is not likely to be the only molecule to bind to phosphorylated Y729. For example, both SOCS3 and the tyrosine phosphatase SHP-2 bind to the same phosphorylated tyrosine residue on the gp130 receptor (Bode et al., 2003; Lehmann et al., 2003; Nicholson et al., 2000; Schmitz et al., 2000). In addition, much of the key data summarized above derive from overexpression systems, which may not accurately reflect primary cell physiology. This latter point is particularly pertinent for SOCS3 because negative effects on signaling from at least twenty other cytokine receptors have been observed when SOCS3 is overexpressed in cell lines (Alexander, 2002). Indeed, several reports have suggested a role for SOCS3 in the negative regulation of interferon-γ receptor signaling (Karlsen et al., 2001; Song and Shuai, 1998; Stoiber et al., 1999; Woldman et al., 2001). However, this role could not be confirmed when tested in the more physiological context of SOCS3 deficiency in hepatocytes and macrophages (Croker et al., 2003; Lang et al., 2003).

Therefore, to directly study the role of SOCS3 in the regulation of G-CSF-induced cellular responses in vivo, and to more broadly determine which hematopoietic growth factors are regulated by SOCS3, we generated mice in which hematopoietic cells lack SOCS3. A conditional gene targeting approach was necessary because mice with germline deletion of *Socs3* die of placental failure in utero (Marine et al., 1999; Roberts et al., 2001; Takahashi et al., 2003), thereby preventing analysis of adult hematopoiesis. To overcome this, we created a *loxP*-flanked *Socs3* allele (*Socs3^{fl}*) (Croker et al., 2003) and transgenic mice in which expression of Cre recombinase was directed to hematopoietic cells by use of the *vav1* promoter.

Results

The *vavCre* Transgene Targets Cre Recombinase Activity to Hematopoietic and Endothelial Cells

To determine the cell type and tissue distribution of Cre activity to be expected in subsequent experiments targeting *Socs3*, *vavCre*$^{+}$ mice were first intercrossed with *R26R* reporter (*loxP-stop-loxP-lacZ*) mice (Mao et al., 1999). β-galactosidase activity in adult tissues was used as an indicator of Cre-mediated deletion. For each of three independent founder *vavCre* lines, high β-galactosidase expression was observed in all hematopoietic cell lineages (Figure 1A), endothelial cells, and testis (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/2/153/DC1), consistent with previous reports for this promoter (Ogilvy et al., 1999) and for other transgenic mice using the same *vav* promoter to drive Cre expression (De Boer et al., 2003; Georgiades et al., 2002). β-galactosidase activity was not observed in other adult cell types and tissues including hepatocytes, myocytes, adipocytes, fibroblasts, epithelial cells, and renal parenchymal cells (Supplemental Figure S1). Importantly, β-galactosidase expression was observed in $>$99% of neutrophils, macrophages, T cells, and in greater than 90% of B cells and nucleated erythroid cells (Figure 1A).

Hematopoietic Cells from *vavCre*$^{+}$*Socs3*$^{-/fl}$ Mice Are SOCS3 Deficient

We next generated *vavCre*$^{+}$ transgenic mice and *vavCre*$^{-}$ controls bearing various combinations of wild-type (+), null (−), or conditional (*fl*) *Socs3* alleles. *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice, in which *Socs3* is deleted in hematopoietic cells (*Socs3*$^{-/\Delta}$), appeared healthy at birth, developed normally, and proved fertile. In *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice, the *Socs3* conditional allele was not detected in genomic DNA of hematopoietic cells of all lineages in the adult bone marrow (BM), thymus, spleen, lymph nodes, and progenitor cells, having been replaced by the deleted allele (Δ) lacking the entire *Socs3* coding region (Figure 1B). While *Socs3* transcripts are detectable at low levels in steady-state *Socs3* wild-type bone marrow and are markedly induced by stimulation with G-CSF, *Socs3*$^{-/\Delta}$ hematopoietic cells did not express *Socs3* RNA at baseline or when stimulated with G-CSF (Figure 1C). Such high efficiency deletion is consistent with our previous experience of this conditional allele (Croker et al., 2003) and greatly simplifies interpretation of the data to follow.

Perturbed Hematopoiesis and Inflammatory Disease Develop in Aging *vavCre*$^{+}$*Socs3*$^{-/fl}$ Mice

The majority of 8-week *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice displayed normal hematocrit, platelet numbers, peripheral blood counts, bone marrow cellularity, and splenic architecture and cellularity (Table 1). One (of 70) 2-week-old *vavCre*$^{+}$*Socs3*$^{-/fl}$ mouse displayed a neutrophil leukocytosis and splenomegaly. However, from 17 weeks of age, an illness characterized by inflammation in the pleural and peritoneal cavities, neutrophil leukocytosis, and infiltration of liver and lungs by hematopoietic cells from multiple lineages (Figure 2) became increasingly prevalent (11 of 13 mice analyzed to date). As detailed in Table 1, hematopoiesis was perturbed, with neutrophilia (*vavCre*$^{+}$*Socs3*$^{-/fl}$, $6.0 \pm 4.8 \times 10^9$ cells/liter; *vavCre*$^{-}$*Socs3*$^{+/fl}$, $1.7 \pm 1.9 \times 10^9$ cells/liter, $n = 8$–11, $p < 0.05$), increased myelopoiesis in the bone marrow, and splenomegaly with prominent extramedullary hematopoiesis. Erythropoiesis was diminished in the bone marrow but increased along with myelopoiesis in the spleen. Aging *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice maintained a normal hematocrit (*vavCre*$^{+}$*Socs3*$^{-/fl}$, $45 \pm 3\%$; *vavCre*$^{-}$*Socs3*$^{+/fl}$, $46 \pm 3\%$, $n = 7$–9 per group).

Enhanced G-CSF-Induced Colony Formation by SOCS3-Deficient BM Cells

To investigate whether late onset neutrophilia could be a consequence of the aberrant actions of cytokines on hematopoietic progenitors, we tested the responses of hematopoietic progenitor cells in vitro to a range of hematopoietic growth factors. When maximally stimulated by GM-CSF, M-CSF, IL-3, IL-6, SCF, or combinations thereof, the frequency of myeloid progenitor cells arising from the bone marrow of young, healthy mice was normal. However, a selective increase was observed in the number of *vavCre*$^{+}$*Socs3*$^{-/fl}$ clonogenic cells capable of forming colonies in response to G-CSF (Figure 3A). Strikingly, the cellular content of the colonies induced

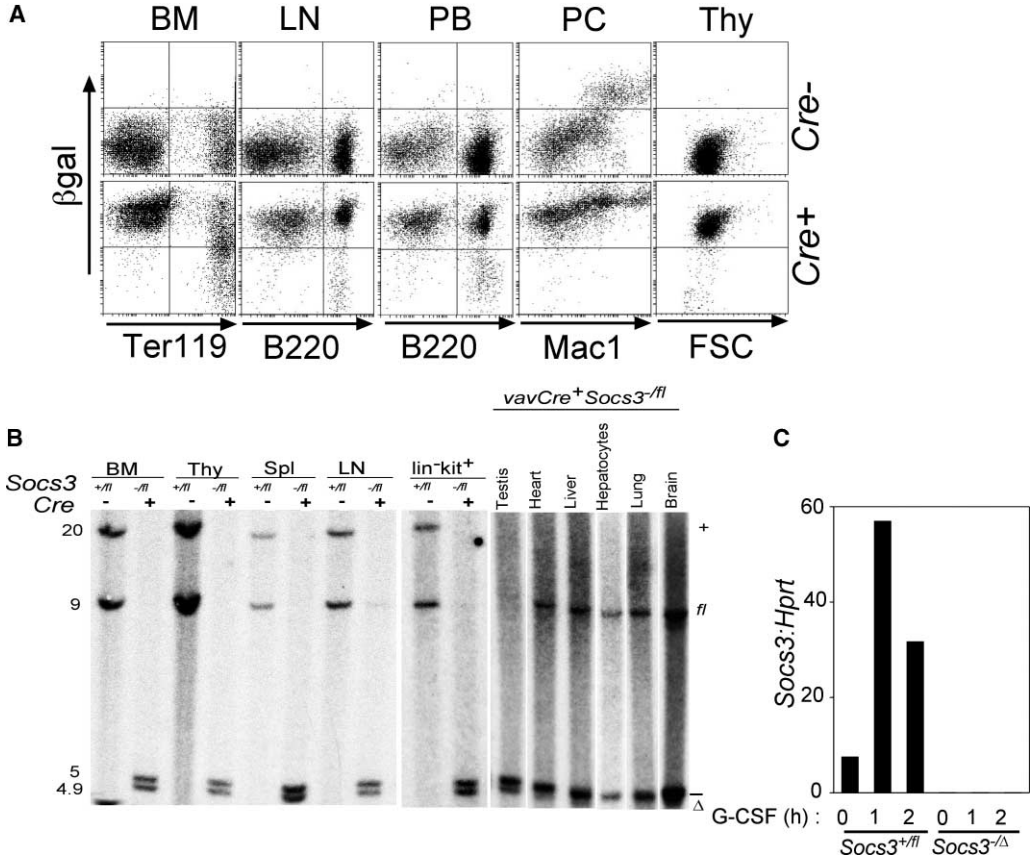

Figure 1. *vavCre* Transgene Directs Expression of Cre Recombinase to Hematopoietic Cells

(A) The *vav* promoter drives Cre expression in hematopoietic cells from the bone marrow (BM), lymph nodes (LN), peripheral blood (PB), peritoneal cavity (PC), and thymus (Thy) deleting a *loxP*-flanked stop sequence in cells from *R26R* reporter mice. Resultant β-galactosidase (β-gal) expression indicates this deletion. Ter119 staining identifies nucleated erythrocyte precursors and enucleate erythrocytes. The latter cannot express β-galactosidase whereas the vast majority of nucleated precursors express β-galactosidase. B220 and Mac1 identify B lymphocytes and macrophages, respectively. FSC represents cell size.

(B) Efficient deletion of *Socs3*^{fl} allele in hematopoietic tissues by Cre. DNA was extracted from BM, thymus, spleen, lymph nodes, and hematopoietic progenitor cells (lin$^-$kit$^+$) of *vavCre*$^+$*Socs3*$^{-/fl}$ or *vavCre*$^-$*Socs3*$^{-/fl}$ mice. DNA was extracted from testis, liver, hepatocytes, heart, lung, and brain of *vavCre*$^+$*Socs3*$^{-/fl}$ mice. A 5′ *Socs3* probe distinguishes the wild-type (+, 20 kbp), conditional (fl, 9 kbp), knockout (−, 5 kbp), and excised (Δ, 4.9 kbp) alleles.

(C) *Socs3*$^{-/\Delta}$ cells are *Socs3* deficient. *Socs3* expression in bone marrow from *vavCre*$^+$*Socs3*$^{-/fl}$ or *vavCre*$^-$*Socs3*$^{+/fl}$ mice either unstimulated or after G-CSF stimulation for 1–2 hr was analyzed by real-time PCR. Expression of the control housekeeping gene, *Hprt*, was assessed to control for RNA integrity.

by G-CSF was 2-fold greater from SOCS3-deficient bone marrow than for controls (Figure 3B). Again, selectivity was observed for this phenotype, with no changes in the capacity for mature cell generation occurring when the progenitor cells from the same bone marrow sample were stimulated with GM-CSF, M-CSF, SCF, or combinations of these other cytokines. The increased size of the developing colonies was not unique to G-CSF stimulation, however, and was also observed following activation by IL-6 (Figure 3B).

To reduce the possibility that this 2-fold increase in number of G-CSF-responsive progenitors simply reflected enrichment for committed neutrophil progenitors or the abnormal activity of some other cell type within the SOCS3-deficient bone marrow, cultures of purified lin$^-$kit$^+$ progenitor cells were analyzed and an increased number of G-CSF-responsive cells, both colony-forming cells and total cells, was again observed (Figure 3C). This increased response was observed at both supramaximal and submaximal concentrations of G-CSF.

In addition to the increased proliferation by SOCS3-deficient progenitor cells stimulated by G-CSF, a subtle shift in differentiation was also evident. There was a significant increase in the number and proportion of macrophages within these colonies, as compared with the nearly exclusively neutrophil composition of colonies derived from control progenitors. G-CSF-responsive SOCS3-deficient progenitor cells generated higher proportions of macrophage and granulocyte-macrophage colonies than did control progenitor cells (Table 2).

SOCS3-Deficient Myeloid Cells Are Hyperresponsive to G-CSF

To further exclude the possibility that the above observations reflected aberrant composition of the myeloid progenitor cell pool in young adult *vavCre*$^+$*Socs3*$^{-/fl}$

Table 1. Evolution of Abnormal Granulopoiesis in Mice with SOC3-Deficient Hematopoiesis

Age (wk)	Organ	*Socs3* Genotype	Cellularity* ($\times 10^6$)	Weight (mg)	Differential (%)						
					Blast	Myeloblast/ Myelocyte	Metamyelocyte/ Neutrophil	Lymphocyte	Monocyte	Eosinophil	Nucleated Erythroid
8	Peripheral blood	+/fl	6.9 ± 1.9				9 ± 3	89 ± 5	3 ± 3	0 ± 1	
		−/Δ	4.4 ± 1.3				15 ± 3	83 ± 4	2 ± 2	0 ± 1	
	Bone marrow	+/fl	44 ± 14		2 ± 1	9 ± 3	23 ± 5	34 ± 6	5 ± 2	2 ± 1	24 ± 2
		−/Δ	38 ± 4		2 ± 2	12 ± 2	29 ± 13	25 ± 9	7 ± 1	2 ± 2	23 ± 1
	Spleen	+/fl	110 ± 18	91 ± 13	1 ± 1	1 ± 1	3 ± 2	81 ± 6	2 ± 2		13 ± 3
		−/Δ	137 ± 61	96 ± 34	2 ± 1	3 ± 1	4 ± 1	63 ± 15	4 ± 2		25 ± 13
17–40	Peripheral blood	+/fl	7.7 ± 1.6				22 ± 24	73 ± 23	4 ± 2	3 ± 2	
		−/Δ	13 ± 4**				44 ± 26	47 ± 27**	8 ± 4**	2 ± 3	
	Bone marrow	+/fl	38 ± 18		3 ± 2	7 ± 2	30 ± 6	24 ± 2	9 ± 4	4 ± 2	24 ± 7
		−/Δ	37 ± 10		6 ± 3	9 ± 4	50 ± 10**	9 ± 4**	8 ± 3	5 ± 5	13 ± 8**
	Spleen	+/fl	85 ± 25	76 ± 18	2 ± 2	0 ± 0	3 ± 4	90 ± 8	2 ± 2	0 ± 1	3 ± 5
		−/Δ	200 ± 117**	239 ± 154**	3 ± 2	3 ± 4**	14 ± 8**	54 ± 24**	3 ± 2	1 ± 2	21 ± 19**

Results represent mean ± SD from four to eleven mice of each genotype.
*Peripheral blood, per ml; bone marrow, per two femurs; spleen, total spleen.
**$p < 0.05$, *vavCre*$^+$*Socs3*$^{-/fl}$ versus *vavCre*$^+$*Socs3*$^{-/fl}$.

mice, additional evidence of enhanced in vitro responses to G-CSF were sought. As stated above, the distribution of morphologically identifiable myeloid precursors was normal in young *vavCre*$^+$*Socs3*$^{-/fl}$ mice. Therefore, Gr-1-expressing myeloid cells (promyelocytes, myelocytes, metamyelocytes, and neutrophils) were sorted, and their proliferative responses to G-CSF were assayed by tritiated thymidine incorporation. No differences were noted in the composition of the sorted precursor populations between genotypes (data not shown). As illustrated in Figure 3D, thymidine incorporation by *Socs3*$^{-/\Delta}$ Gr-1$^+$ cells was significantly increased in cells stimulated with several concentrations of G-CSF but not IL-3. Furthermore, the survival of *Socs3*$^{-/\Delta}$ Gr-1^{hi} cells (a population highly enriched for neutrophils and metamyelocytes, but not precursors with mitotic potential) was enhanced after 48 hr in culture in response to G-CSF (Figure 3E).

To examine G-CSFR expression, bone marrow cells from *vavCre*$^+$*Socs3*$^{-/fl}$ and control mice were incubated with radiolabeled G-CSF in the presence and absence of excess unlabeled G-CSF. No differences in the numbers of G-CSFR were observed between genotypes (*vavCre*$^+$*Socs3*$^{-/fl}$, 1699 ± 72 cpm; *vavCre*$^-$*Socs3*$^{+/fl}$, 1884 ± 248 cpm), eliminating this as an explanation for the differences in cellular responses. In contrast, the duration and intensity of STAT3 phosphorylation was increased in lysates of bone marrow cells after a 15 min pulse with G-CSF in vitro (Figure 3F). Activation of the ras-MAPK pathway downstream of the G-CSF receptor was not substantially affected in SOCS3-deficient cells. Similar patterns of induction of phosphorylation of p42/44 MAP kinase were observed for both SOCS3-deficient and control cells. G-CSF-induced phosphorylation of STAT1 or STAT5 was not detectable in lysates from either SOCS3-deficient or control bone marrow cells (data not shown). These in vitro data prove that expression of SOCS3 regulates both STAT3 phosphorylation and the proliferation and survival of myeloid cells in response to G-CSF.

SOCS3 Is Required to Negatively Regulate Emergency Granulopoiesis

These in vitro data predict that SOCS3 might be required to negatively regulate granulopoiesis under stress conditions characterized by high levels of circulating G-CSF, such as bacterial infection or burns. To mimic such an emergency situation, *vavCre*$^+$*Socs3*$^{-/fl}$ mice (with SOCS3-deficient hematopoiesis) and *vavCre*$^-$*Socs3*$^{+/fl}$ control mice were injected with either pharmacological doses of G-CSF or endotoxin-free diluent. Typically in such an experiment, G-CSF is injected for 5 days (Roberts and Metcalf, 1994). However, *vavCre*$^+$*Socs3*$^{-/fl}$ mice developed severe lethargy and hind-leg paresis after 4 days of G-CSF injection. As a result, mice were analyzed after 4 days of G-CSF injection rather than 5 days of G-CSF injection. *Socs3* wild-type mice never display such toxicity from G-CSF, even at substantially higher doses, or after prolonged injection (Roberts et al., 1997; Roberts and Metcalf, 1994).

The responses to G-CSF expected of wild-type mice were observed, and these were markedly accentuated in *vavCre*$^+$*Socs3*$^{-/fl}$ mice, which exhibited enhanced

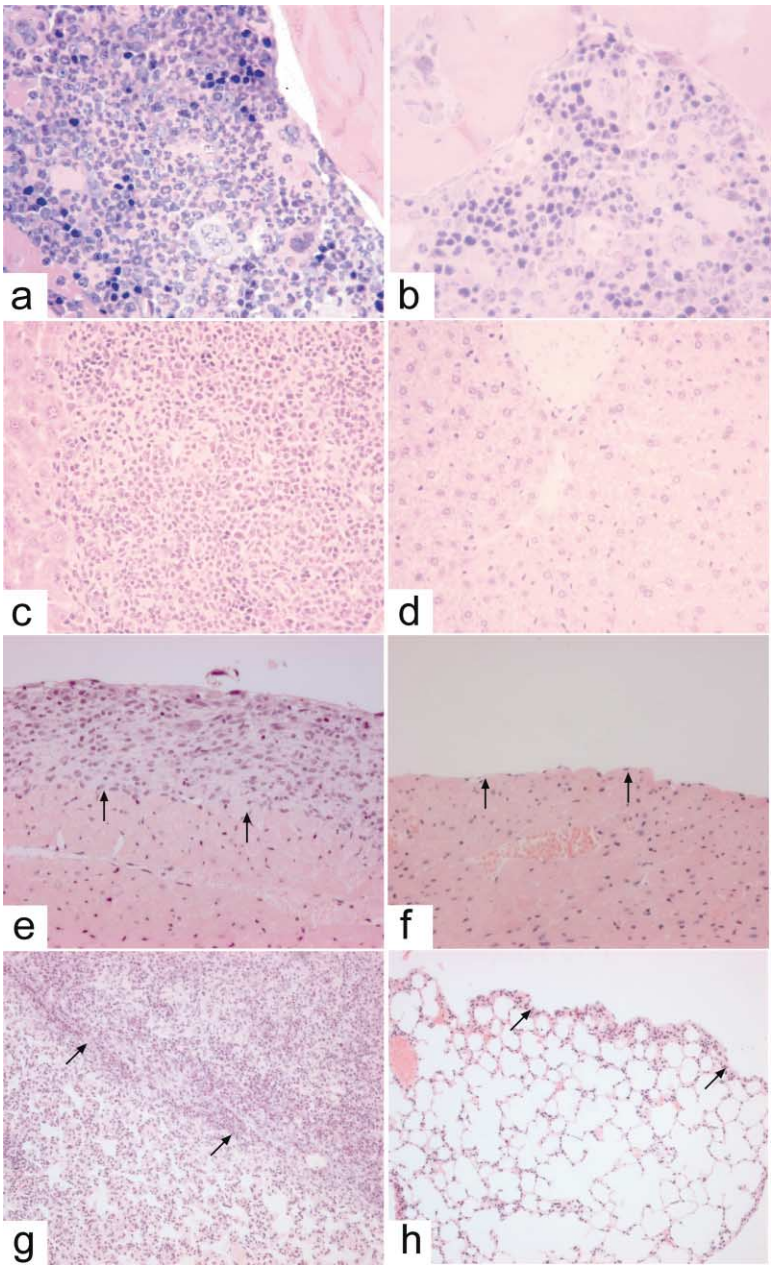

Figure 2. Neutrophil Infiltration and Chronic Inflammation in Aging *vavCre*$^{+}$*Socs3*$^{-/fl}$ Mice

Photomicrographs of hematoxylin and eosin-stained sections of various tissues from 17- to 40-week-old *vavCre*$^{-}$*Socs3*$^{+/fl}$ (b, d, f, and h) and *vavCre*$^{+}$*Socs3*$^{-/fl}$ (a, c, e, and g) mice demonstrating: (a) a dominant granulocytic population within the bone marrow; (c) a large hematopoietic focus in the liver containing predominantly neutrophils but also eosinophils, lymphocytes, macrophages, plasma cells, and megakaryocytes; (e) an extensive pericardial infiltrate of lymphocytes, macrophages, and fibroblasts; and (g) a chronic inflammatory infiltrate on the pleural surface of the lung. Lymphocytes, macrophages, fibroblasts, neutrophils, and eosinophils are all prominent. This pathology was not observed in histology from control mice (b, d, f, and h).

neutrophilia, progenitor cell mobilization, and splenomegaly (Figure 4A). In addition, all *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice displayed greatly increased neutrophilic infiltration of tissues including the liver, spinal cord, and muscle (Figure 4B). This was particularly marked in two of the four mice examined, which displayed prominent focal aggregates of neutrophils within the liver parenchyma. In one mouse, whole areas of the bone marrow were replaced with degenerating neutrophils and debri (Figure 4B). Evidence of pathological neutrophil death was also seen in the liver of this particular mouse. No such changes were observed in control mice injected with G-CSF. In addition, the hematological and histological parameters of vehicle-injected *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice were identical to vehicle-injected control mice (data not shown).

On the basis of our detailed survey of *vavCre*$^{+}$*R26R* lacZ reporter mice, endothelial cells in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice are very likely to be *Socs3* deficient, and therefore it is possible that the pathological responses observed in the above experiment were contributed to by loss of SOCS3 expression in tissues other than the hematopoietic system. A further caveat in interpreting these data is the hemizygosity of SOCS3 in all other tissues and the possibility that this may have contributed to the breadth of pathology observed. To confirm that the enhanced responses were principally intrinsic to the hematopoietic system, radiation chimeras were created in which the hematopoietic compartment was SOCS3 deficient, and all other tissues were wild-type. *Socs3* wild-type C57BL/6.SJL *(Ptprc*^{a} *Pep3*^{b} *[Ly5.1])* recipient mice were reconstituted with C57BL/6 (Ptprc^{b} Pep3^{a} [Ly5.2]) SOCS3-deficient or control fetal liver cells and then

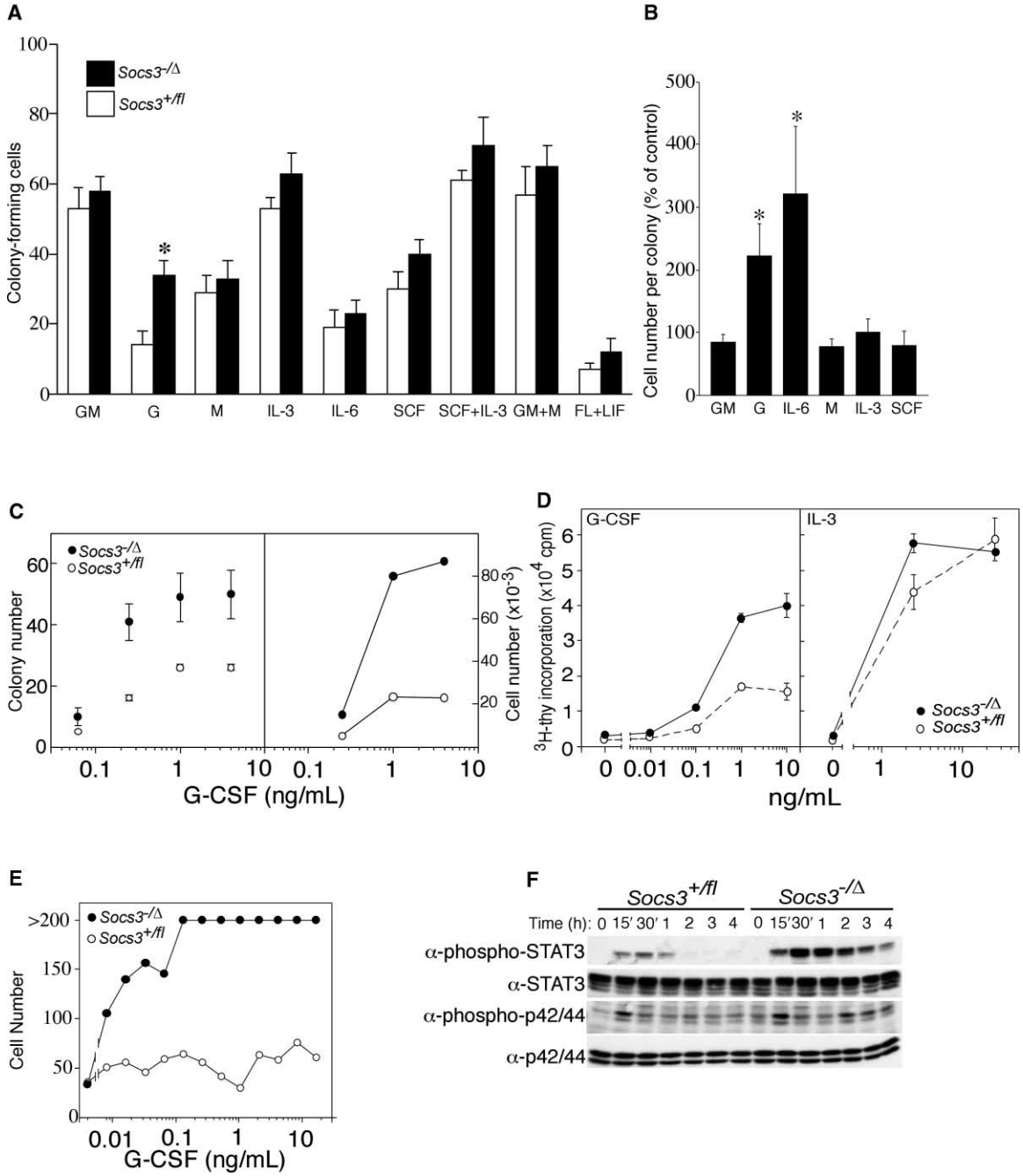

Figure 3. SOCS3-Deficient Granulocytes Are Hyperresponsive to G-CSF

(A) Frequency of colony-forming cells (CFC) in response to a range of cytokines. 2.5 × 10^4 SOCS3-deficient or control BM cells were cultured with specific stimuli in supramaximal concentrations for 7 days.

(B) Cellular content of SOCS3-deficient colonies relative to controls. Ten to eighty consecutive colonies from parallel cultures of BM cells of each genotype were picked, pooled, and counted to calculate an average cellular content. Mean ± SD of results from four to six mice per genotype. *$p < 0.01$.

(C) Number of G-CSF-responsive CFC (left) and total cells (right) generated after 7 days from 600 lin^-kit^+ progenitor cells in response to supramaximal and submaximal concentrations of G-CSF.

(D) Proliferation of SOCS3-deficient Gr-1^+ myeloid cells in response to G-CSF and IL-3. 10^5 SOCS3-deficient or control Gr-1^+ myeloid BM cells were cultured with G-CSF or IL-3 in various concentrations for 48 hr before cells were pulsed with ^{3}[H]thymidine for 16 hr. Mean ± SD of triplicate cultures from one of three representative experiments.

(E) Survival of SOCS3-deficient and control Gr-1^{hi} BM cells in response to G-CSF. Two hundred cells were cultured with G-CSF in various concentrations, and the number of viable cells was counted on day 2.

(F) Activation of STAT3 and p42/44 MAP kinase in response to G-CSF. 2.5 × 10^6 bone marrow cells were pulsed for 15 min with 10 ng/ml G-CSF. Lysates were analyzed by immunoblot using antibodies specific for phosphorylated and total STAT3 or p42/44 MAP kinase. Representative data are from three independent experiments.

Table 2. Aberrant G-CSF-Induced Production and Differentiation of Progenitor Cells Lacking SOCS3

			Colony Composition					
Stimulus	*Socs3* Genotype	Total	Blast	Neutrophil	Granulocyte/ Macrophage	Macrophage	Eosinophil	Megakaryocyte
GM-CSF	+/fl	58 ± 12		17 ± 6	9 ± 3	29 ± 6	2 ± 1	
	−/Δ	76 ± 12	1 ± 1	27 ± 4	14 ± 4	33 ± 8	2 ± 2	
G-CSF	+/fl	11 ± 4		10 ± 3	1 ± 1	1 ± 1		
	−/Δ	32 ± 7*	2 ± 1	22 ± 3*	3 ± 2	6 ± 4*		
M-CSF	+/fl	55 ± 12		4 ± 3	3 ± 1	48 ± 13		
	−/Δ	75 ± 23		6 ± 2	4 ± 2	65 ± 24		
IL-3	+/fl	61 ± 12	6 ± 2	13 ± 4	11 ± 2	21 ± 7	2 ± 1	8 ± 1
	−/Δ	76 ± 5	6 ± 1	20 ± 5	16 ± 5	23 ± 10	2 ± 3	8 ± 2

Unfractionated bone marrow cells (2.5×10^4) were cultured in the presence of a single cytokine in 0.3% agar/DME/FCS. After 7 days of incubation, cultures were fixed, stained, and enumerated at 200× magnification. Results represent mean ± SD from four mice of each genotype. * $p < 0.05$, *Socs3*$^{+/fl}$ versus *Socs3*$^{-/\Delta}$.

treated with the same dose of G-CSF for 4 days and analyzed on day 5. Recipients of either SOCS3-deficient or control *vavCre*$^{-}$*Socs3*$^{+/fl}$ cells demonstrated efficient reconstitution by donor cells (>90% of cells in all hematopoietic lineages as judged by expression of Ly5.2).

Mice reconstituted with SOCS3-deficient hematopoietic cells were clearly hyperresponsive to G-CSF, with augmented progenitor cell mobilization and greater splenomegaly than recipients of control cells (Figure 5A), but some differences in hematological parameters and in the degree of tissue pathology were evident between these mice and *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice (Figure 5B). After G-CSF injection for 4 days, there was a near absence of mature neutrophils in the bone marrow of mice reconstituted with SOCS3-deficient cells (data not shown). Surprisingly, these mature neutrophils were not detected in increased numbers in the blood (Figure 5A) as was observed for *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice injected with G-CSF, but rather were found in large numbers in the tissues, particularly the liver and lungs (Figure 5B). Mice injected with G-CSF for 8 days did display a neutrophilia greater than observed for controls, ultimately recapitulating the pattern of differences observed in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice. The degree of enhancement of G-CSF response observed in transplant recipients of SOCS3-deficient cells was not as marked as observed for *vavCre*$^{+}$*Socs3*$^{-/fl}$ over control mice. Further, neutrophil infiltration of the spinal cord following G-CSF injection was not observed in mice reconstituted with SOCS3-deficient cells. These data indicate that, while the hematopoietic cell hyperresponsiveness to G-CSF is primarily responsible for the aberrant tissue infiltration and pathology, loss of SOCS3 expression by other cells, for example, endothelial cells, must also contribute to the severity of the phenotype.

Recruitment and Activation of SOCS3-Deficient Neutrophils

The pathological tissue infiltration and damage by neutrophils observed in both G-CSF-injected mice with SOCS3-deficient hematopoiesis and aging *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice suggests that abnormalities in neutrophil recruitment and activity may exist. In order to analyze the survival and function of mature neutrophils in response to an inflammatory stimulus, mice were injected intraperitoneally with casein (Metcalf et al., 1996). Recruitment of neutrophils to the peritoneal cavity 3 hr following the installation of casein was normal (*vavCre*$^{+}$*Socs3*$^{-/fl}$, $5 \pm 4 \times 10^6$ neutrophils; *vavCre*$^{-}$*Socs3*$^{+/fl}$, $5 \pm 4 \times 10^6$ neutrophils, n = 5–10 per group). The percentages of dying cells within the freshly harvested peritoneal lavages were similar between genotypes (data not shown). To further investigate survival of SOCS3-deficient neutrophils after exposure to inflammatory milieux, we then cultured the peritoneal exudate cells at 37°C for 8 hr in media alone or media supplemented with either G-CSF or GM-CSF. The survival of the inflammatory cells in media was unaffected by the absence of SOCS3 (*vavCre*$^{+}$*Socs3*$^{-/fl}$, 40 ± 11% PI^{+}; *vavCre*$^{-}$*Socs3*$^{+/fl}$, 39 ± 10% PI^{+}, n = 4–7 per group), as was survival in the presence of G-CSF or GM-CSF (data not shown). Finally, as a measure of the functional activity of tissue neutrophils from sites of inflammation, superoxide production was measured. Superoxide production by SOCS3-deficient neutrophils was normal in response to fMLP, with or without G-CSF priming, as well as with a maximal stimulation by PMA (data not shown). No superoxide production was detected with G-CSF priming alone (data not shown).

Discussion

Taken together, these in vitro and in vivo data provide direct proof that SOCS3 is a key negative regulator of G-CSF-induced cellular responses. Signals emanating from the G-CSFR induce proliferation, differentiation, survival, and mobilization of granulocyte progenitors and neutrophils (Duhrsen et al., 1988; Liu et al., 1996; Roberts and Metcalf, 1994; Semerad et al., 2002). Recent studies using defined receptor mutations and conditional deletion of signaling intermediates, such as STAT3, aimed at identifying the precise composition of signaling cascades have revealed a complex regulatory pathway. Cellular responses to G-CSF clearly depend on signaling events downstream of four tyrosine residues on the G-CSFR. Mutation of Tyr729 on the human G-CSFR, the putative binding site for SOCS3, increases STAT-mediated luciferase reporter activity, cell survival, colony number, and colony size (Hermans et al., 2002). Previous studies of hematopoietic progenitor cells genetically manipulated to receive signals from mutant Tyr729Phe G-CSF receptors (Akbarzadeh et al., 2002)

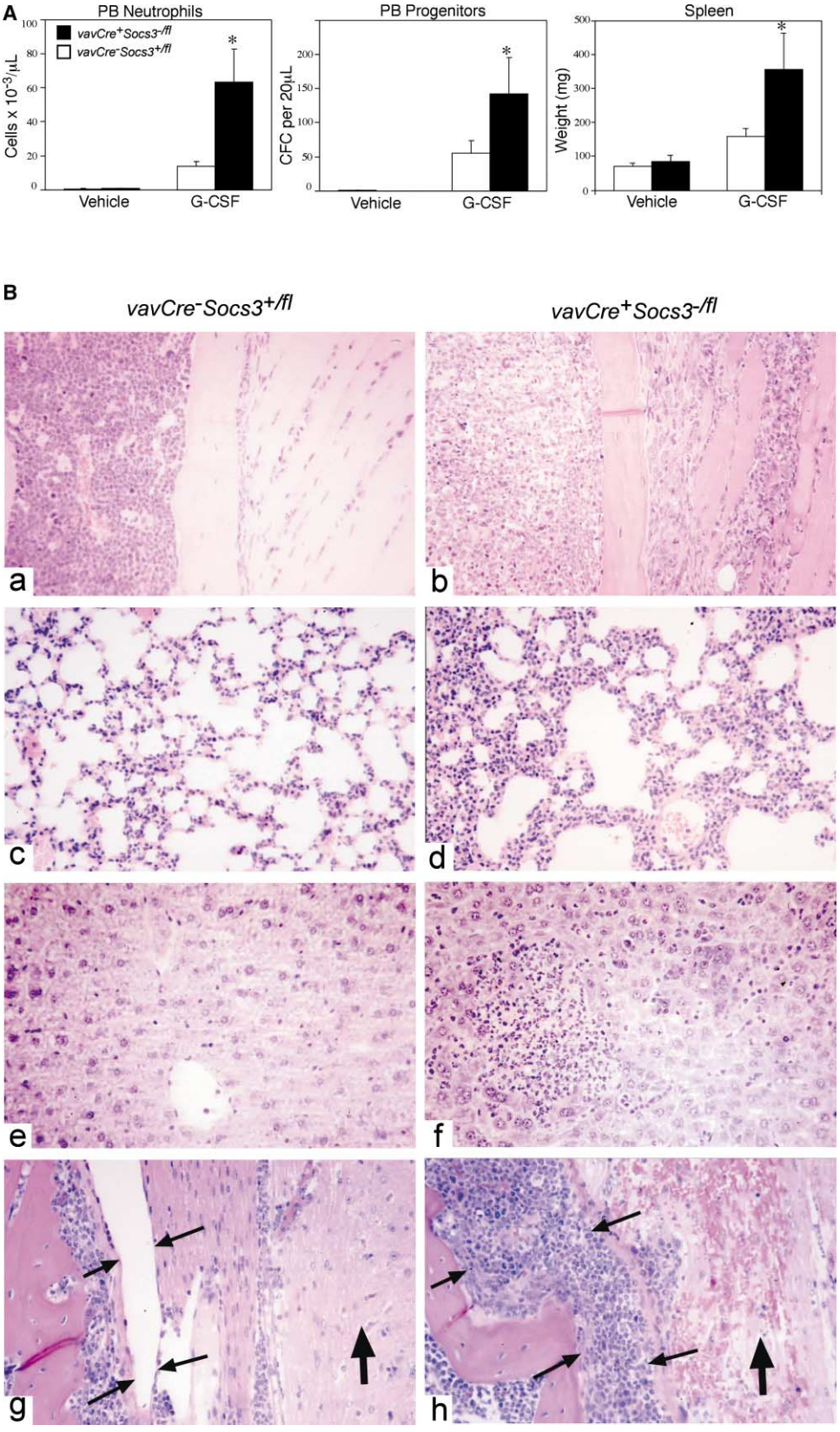

Figure 4. G-CSF-Induced Neutrophil Infiltration into Tissues and Neutrophil Death Are Enhanced in the Absence of SOCS3

(A) Peripheral blood neutrophil and progenitor counts, and spleen weights from mice injected with vehicle or G-CSF (5 μg/day i.p.) for 4 days. Mean ± SD of data from *vavCre*$^{+}$*Socs3*$^{-/fl}$ or *vavCre*$^{-}$*Socs3*$^{+/fl}$ mice. n = 4 mice per group, *p < 0.05.

demonstrated a partial shift in differentiation within emergent colonies from neutrophil to macrophage. Consistent with those experiments, SOCS3-deficient progenitors displayed a similarly aberrant differentiation response. G-CSF-responsive SOCS3-deficient progenitor cells generated macrophage and granulocyte-macrophage colonies in greater proportions than SOCS3-sufficient control progenitor cells. These data provide evidence that qualitative, as well as quantitative, changes in cellular responses to extracellular signals can be modulated by the expression of regulatory proteins such as SOCS3.

In the current study, we have shown that SOCS3 is a critical regulator of G-CSF-induced myeloid progenitor cell proliferation and mature cell survival as well as for establishment of G-CSF-responsive progenitor cell numbers in the bone marrow. In vivo, these defects translate into significant abnormalities in G-CSF-induced emergency granulocyte production. Unexpectedly, this was associated with G-CSF-induced toxicity, a previously unreported observation for G-CSF-treated animals, even at substantially higher doses of G-CSF, or in G-CSF-transgenic animals or animals injected for a longer duration.

Anomalous responses to G-CSF were largely, but not completely, intrinsic to the hematopoietic system. Animals transplanted with SOCS3-deficient fetal liver cells displayed pathological infiltration into multiple tissues when injected with G-CSF for 4 days and signs of distress after injection for 8 days. However, even with an extended injection schedule, these mice did not display the spinal cord infiltration by neutrophils observed in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice. This suggests a role for SOCS3 in the regulation of G-CSF or other cytokine responses in nonhematopoietic tissues. Given that endothelium is a critical mediator of neutrophil migration to the tissues and that the *vavCre* transgene is expressed in endothelium, it is possible that the differences in G-CSF-induced pathology observed between the two models can be attributed to deletion of *Socs3* in endothelium. This will be addressed in more detail by G-CSF treatment of control and SOCS3-deficient mice following reciprocal transplantation of SOCS3-deficient and control bone marrow.

Which hematopoietic cells are responsible for the tissue damage observed in response to G-CSF administration? Neutrophils are certainly involved as they dominate the pathology and are intrinsically hyperresponsive to G-CSF in vitro. It is possible that the tissue infiltration and destruction by neutrophils in G-CSF-treated SOCS3-deficient mice may also be compounded by aberrant recruitment or activation of neutrophils, although our initial data addressing these issues do not indicate enhanced recruitment or superoxide production. Our experiments do not exclude a role for SOCS3-deficient macrophages or lymphocytes and studies where these cell types are made selectively SOCS3 deficient are required to define their contribution. Further studies will also be needed to determine whether the defects in granulopoiesis in SOCS3-deficient progenitors are due to defects or changes in differentiation, cell cycle, or survival.

Our data complement and clarify recent data describing the role of STAT3 in G-CSF signaling and granulopoiesis. STAT3-deficient mice have a neutrophilia, and their myeloid cells are hyperresponsive to G-CSF and fail to induce expression of SOCS3 in response to G-CSF, suggesting that one important function of STAT3 is to induce SOCS3 (Lee et al., 2002). Our results support this model and indicate that SOCS3 negatively regulates G-CSF signaling by inhibiting STAT3 activation. Further, our data are consistent with the hypothesis that the hyperresponsiveness to G-CSF observed in STAT3-deficient mice is due to the lack of induction of SOCS3. No significant effect on the induction of p42/44 MAP kinase phosphorylation following G-CSF stimulation was observed. Additional studies will be required to determine whether activation of other signaling pathways is substantially affected in the absence of SOCS3 and whether these perturbations contribute to the phenotype we have observed.

These data predict that inhibition of SOCS3 may enhance clinically important G-CSF-dependent processes, including neutrophil recovery after myelosuppressive chemotherapy, or chemoradiotherapy as well as stem and progenitor cell mobilization, and recovery from acute bacterial and/or fungal infection during neutropenia. These data also predict that agonists of SOCS3 may reduce unwanted clinical sequelae of inflammatory processes characterized by neutrophil accumulation and activation, such as engraftment syndrome following allogeneic or autologous stem cell transplantation, pulmonary inflammation observed after recovery from severe neutropenia, acute arthritis, and inflammatory bowel disease (Shouda et al., 2001).

Finally, it is unlikely that hyperresponsiveness to G-CSF fully explains the pathology observed in aging mice lacking expression of SOCS3 in the hematopoietic compartment. As well as cumulative neutrophil infiltration into varied tissues, tissue damage due to chronic inflammation was routinely observed, implying that aberrant responses by SOCS3-deficient T cells, B cells, eosinophils, or macrophages to a variety of other cytokines are likely to be contributing to the final pathology. Given the suggestive data linking SOCS3 in the regulation of numerous cytokines derived from in vitro overexpression systems and the recent data confirming a physiological role for SOCS3 in the regulation of IL-6 signaling

(B) Photomicrographs of hematoxylin and eosin-stained sections of various tissues from G-CSF-injected *vavCre*$^{-}$*Socs3*$^{+/fl}$ control mice (a, c, e, and g) and *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice (b, d, f, and h) demonstrating: (b) neutrophil debris in the bone marrow and pronounced neutrophil infiltration into the adjacent muscle tissue; (d) increased neutrophil numbers in the alveolar wall of the lung; (f) a neutrophil focus in the liver; and (h) increased myelopoiesis, edema, and associated destruction of the spinal cord of *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice. The large arrows (g and h) indicate spinal cord, and the small arrows demarcate the epidural space, which in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice is replaced by myeloid infiltrate (h). None of the pathological features observed in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice injected with G-CSF were observed in *Socs3* wild-type mice injected with G-CSF (a, c, e, and g) or in *vavCre*$^{+}$*Socs3*$^{-/fl}$ mice injected with vehicle (data not shown). Data are representative of four mice.

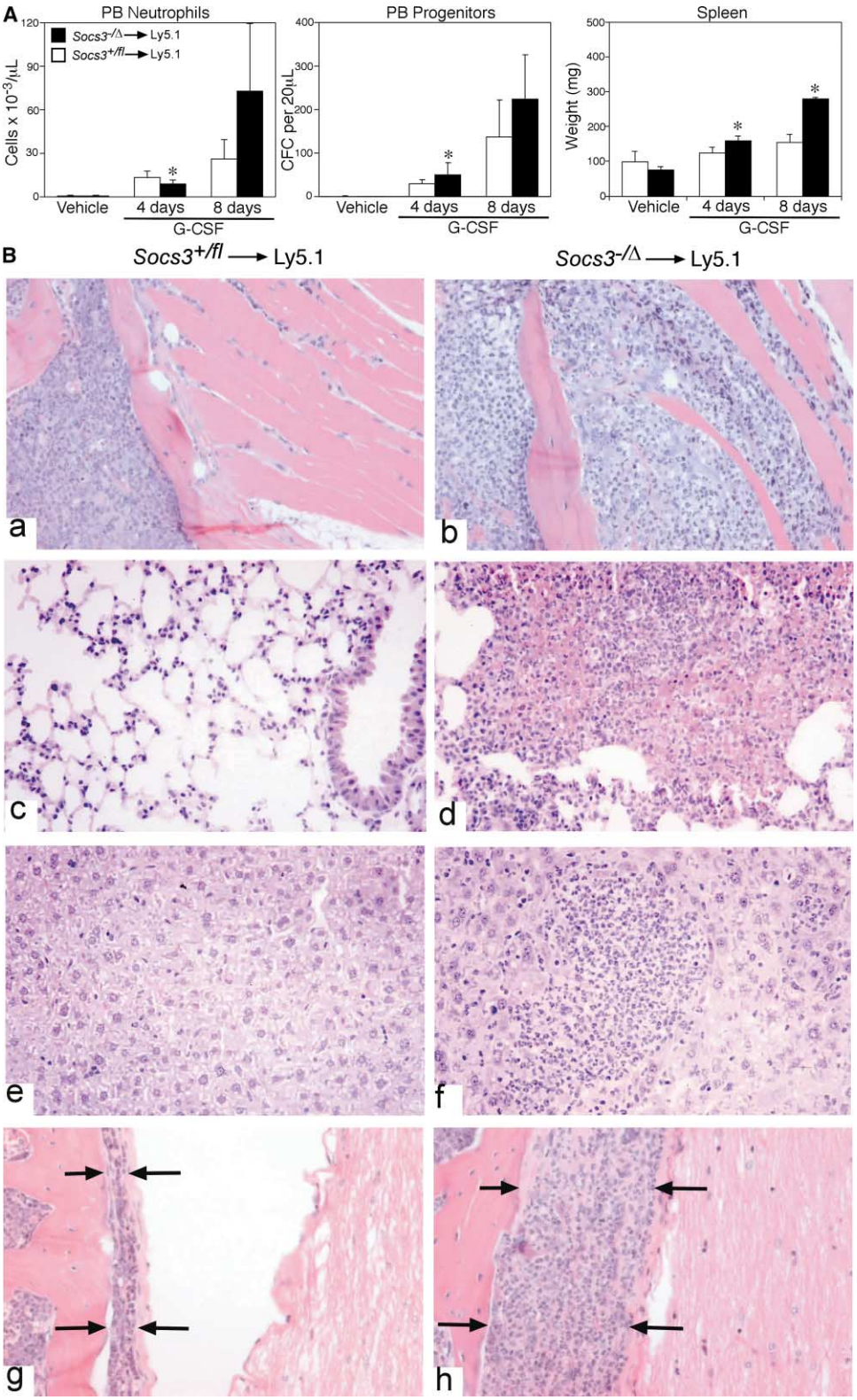

Figure 5. Enhanced In Vivo Responses Induced by G-CSF in Mice with SOCS3-Deficient Hematopoiesis

(A) Peripheral blood neutrophil and progenitor counts, and spleen weights from Ly5.1 mice transplanted with SOCS3-deficient or wild-type Ly5.2 fetal liver cells and injected at least 3 months posttransplantation with vehicle or G-CSF (5 μg/day i.p.) for 4 or 8 days. n = 6–8 mice (vehicle and 4 days) or 2–3 mice (8 days) per group, *$p < 0.05$.

(B) Photomicrographs of hematoxylin and eosin-stained sections of tissues from mice, transplanted with SOCS3-deficient (b, d, f, and h) or wild-type (a, c, e, and g) fetal liver cells, and then injected with G-CSF for 8 days. (b) Neutrophil debris in the bone marrow and pronounced

(Alexander, 2002; Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003), further experiments addressing the role of SOCS3 in specific circumstances are required. Nevertheless, the spontaneous and G-CSF-induced in vivo pathology reported here, in conjunction with the unequivocal in vitro data, clearly indicate that an important physiological function of SOCS3 is the negative regulation of G-CSF signaling.

Experimental Procedures

Generation of Mice with a *Socs3*-Deficient Hematopoietic System

Generation of mice bearing null (*Socs3*$^{-}$) and loxP-flanked conditional (*Socs3*^{fl}) alleles of *Socs3* on a C57BL/6 inbred background have been previously described (Croker et al., 2003; Roberts et al., 2001). In the *Socs3*^{fl} allele, the entire *Socs3* coding region is flanked by *loxP* sites, and expression of Cre renders the allele functionally null. In order to generate a hematopoietic-specific deletion of *Socs3*, we created transgenic mice in which Cre recombinase expression was restricted to cells of the hematopoietic and endothelial lineages (*vavCre*$^{+}$ mice). A 11.2 kbp plasmid, containing elements of the *vav1* promoter and a human CD4 reporter (Ogilvy et al., 1999), was digested with SfiI and NotI to replace the human CD4 reporter with a *nls-Cre* (nuclear localization signal-Cre) recombinase cassette. The pIC19H (prokaryotic) sequences were removed by restriction digestion with Hind III and the remaining 8.2 kbp fragment was purified from low-melt agarose using agarase (New England Biolabs). The purified DNA was dialysed for 12 hr in microinjection buffer (10 mM Tris/HCl [pH 7.4], 0.1 mM EDTA) and adjusted to 2 μg/ml for pronuclear injection into C57BL/6 eggs. Digestion of genomic DNA from founders, coupled with a Cre probe, was used to identify transgenic offspring by Southern blot. On the basis of the expression of the *vavCre* transgene in *R26R lacZ* reporter mice (Soriano, 1999), three independent *vavCre* transgenic lines (15, 48, and 71) were selected to use for intercrossing with mice bearing mutant *Socs3* alleles. Mice with a deletion of *Socs3* in the hematopoietic system were generated by crossing mice with two copies of the *loxP*-flanked *Socs3* conditional allele (*Socs3*$^{fl/fl}$) with mice expressing the *vavCre* transgene and bearing one *Socs3* null allele (*Socs3*$^{-}$) and one *Socs3* wild-type allele (*Socs3*$^{+}$). The resultant progeny were analyzed at 8 weeks of age or between 17 and 40 weeks of age. Hematopoietic cells were analyzed by flow cytometry as previously described (Croker et al., 2002) and by cytology following May Grünwald (eosin methylene blue, Merck, Germany) and Giemsa (Merck, Kilsyth, Victoria, Australia) staining. For reconstitution experiments, congenic C57BL/6.SJL *(Ptprc*^{a} *Pep3*^{b} *[Ly5.1])* mice were reconstituted with 3×10^6 C57BL/6 (Ptprc^{b} Pep3^{a} [Ly5.2]) fetal liver cells from E14 embryos of either *vavCre*$^{+}$*Socs3*$^{-/fl}$ or *vavCre*$^{-}$ *Socs3*$^{+/fl}$ genotype after two 5.5 Gy doses of irradiation. Analyses of data were performed using a Student's t test. Animal experiments were done with the approval of the Melbourne Health Research Directorate Animal Ethics Committee.

Real-Time PCR

cDNA was prepared from 2 μg total RNA derived from 3×10^6 *Socs3*$^{+/fl}$ or *Socs3*$^{-/\Delta}$ bone marrow cells stimulated with 10 ng/ml G-CSF. *Socs3* and *Hprt* levels were quantified by real-time PCR analysis using the Rotor Gene 2000 (Corbett Research, Mortlake, Australia). PCR was performed using the following primers and cycling conditions: *Hprt*, 5′-CACAGGACTAGAACACCTGC-3′ (forward) and 5′-GCTGGTGAAAAGGACCTCT-3′ (reverse); *Socs3*, 5′-GGCCAC CCTCCAGCATCTTTGTCG-3′ (forward) and 5′-GTGGCAGCTCCCC CTCCCCTCAG-3′ (reverse), 40 cycles at 94°C for 15 s, 53°C (*Hprt*) or 70°C (*Socs3*) for 30 s, and 72°C for 30 s. Expression of *Socs3* was quantified by comparison with the expression of the single-copy housekeeping gene, *Hprt*.

Clonogenic Assays

2.5×10^4 bone marrow cells or 5×10^4 spleen cells were resuspended in 0.3% agar and modified Dulbecco's medium (DME), supplemented with 20% fetal calf serum (Metcalf, 1984). Cells were incubated for 7 days at 37°C with 10 ng/ml recombinant murine (rm) GM-CSF, rmM-CSF or recombinant human G-CSF, 100 ng/ml IL-6, 25 ng/ml rmIL-3 or 100 ng/ml rmSCF, or combinations thereof. At day 7, colonies were enumerated and either fixed in glutaraldehyde and stained for differential counting (Metcalf, 1984), 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 40× magnifications (Roberts and Metcalf, 1994). In specific experiments, cultures were initiated with 600lin^{-}kit^{+} progenitor cells.

LacZ Assays

β-galactosidase assays were performed as described previously (Elefanty et al., 1999).

Proliferation Assays

Gr1-positive bone marrow cells were sorted, and purity was analyzed by cytology. Cells (10^5) were incubated in 0.01–10 ng/ml G-CSF or 2.5–25 ng/ml IL-3 for 48 hr at 37°C. Cells were pulsed with 1 μCi [^{3}H]thymidine for 16 hr, transferred to Inotech glass fiber filters, and counted in a TopCount NXT Microplate Scintillation Counter (Packard). All samples were analyzed in duplicate or triplicate.

Survival Assays

Survival assays were performed as described (Begley et al., 1986). Bone marrow Gr1^{hi} cells (neutrophils and metamyelocytes but not granulocytes with mitotic potential) were cultured in DME/2%FCS and various concentrations of G-CSF. Viable cell numbers were counted on days 2, 3, and 4. Survival of peritoneal cavity exudate cells from casein-injected mice was assessed using flow cytometry and propidium iodide (PI). Cells were incubated at 37°C/5%CO_2 for 8 hr in Hank's buffered saline solution (HBSS) supplemented with 1% glucose, 0.1% BSA, and either saline, 10 ng/ml G-CSF, or 10 ng/ml GM-CSF.

Superoxide (O_2^-) Production

Superoxide production in neutrophils (3×10^6 cells/ml) was assessed by the superoxide dismutase-inhibitable reduction of cytochrome c (75 μM). Neutrophils were primed with 10 ng/ml G-CSF for 15 min and activated with 1 μM fMLP or 200 pg/ml PMA (Sigma). Changes in absorbance at 550 nm were monitored at 37°C on a Cary 50 UV-Vis spectrophotometer.

Immunoblotting

Bone marrow cells (2.5×10^6) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% bovine serum albumin were pulsed with 10 ng/ml G-CSF for 15 min. SDS-PAGE of total cell lysates and immunoblots to phospho-STAT3, STAT3, phospho-p42/44, and p42/44 (Cell Signaling, Beverly, MA) were performed as described (Brysha et al., 2001).

In Vivo Responses to G-CSF

Seven- to eleven-week-old mice were injected intraperitoneally twice daily with 2.5 μg rhG-CSF (lenograstim, AMGEN). These experiments were performed as previously described (Roberts et al., 1997; Roberts and Metcalf, 1994), except that mice were only injected for 4 days and analyzed on the fifth day. Transplanted mice were injected with G-CSF at least 3 months following reconstitution.

neutrophil infiltration into the adjacent muscle tissue; (d) an extensive neutrophilic infiltrate destroying the lung alveoli; (f) a foci of neutrophils within the liver parenchyma; and (h) myeloid cells in the epidural space adjacent to the spinal cord of Ly5.1 mice with SOCS3-deficient hematopoiesis. None of the pathological changes seen in (b), (d), (f), and (h) were seen in control mice injected with G-CSF (a, c, e, and g) or mutant mice injected with vehicle.

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