

STAT3 Is a Negative Regulator of Granulopoiesis but Is Not Required for G-CSF-Dependent Differentiation

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Summary

STAT3 has been described as an essential component of G-CSF-driven cell proliferation and granulopoiesis. This notion was tested by conditional gene ablation in transgenic mice. Contrary to expectation, granulocytes developed from STAT3 null bone marrow progenitors, and STAT3 null neutrophils displayed mature effector functions. Rather than a deficit in granulopoiesis, mice lacking STAT3 in their hematopoietic progenitors developed neutrophilia, and bone marrow cells were hyperresponsive to G-CSF stimulation. These studies provide direct evidence for STAT3-independent granulopoiesis and suggest that STAT3 directs a negative feedback loop necessary for controlling neutrophil numbers, possibly through induced expression of the signaling inhibitor, SOCS3.

Introduction

Mammalian hematopoiesis follows an orchestrated program of cell proliferation, differentiation, and apoptosis, resulting in expansion of progenitor stem cells that become progressively restricted along distinct developmental lineages. The signals that control these steps remain poorly understood, but cytokines have been implicated in many aspects of this process, including as mitogens, survival factors, inducers of differentiation, and stimulators of cell migration and effector functions. Cytokines function through specific cell surface receptors that stimulate multiple intracellular signaling pathways, largely through activation of receptor-associated and cytoplasmic protein kinases (Ihle et al., 1995).

At least four signaling pathways contribute to cytokine action. All of these pathways require activation of protein tyrosine kinases of the JAK family and depend at least in part on phosphorylation of specific tyrosine docking

sites on receptor cytoplasmic tails. Activation of the PI3 kinase pathway through recruitment of adaptor proteins such as GAB2, SHP2, or Grb2 contributes to cell survival (Hibi and Hirano, 2000), while the Ras-MAP kinase cascade is activated during cell proliferation (Ihle, 1996). A largely uncharacterized signaling pathway leads to *c-myc* gene induction, also required for cell cycle progression. Finally, activation of STAT transcription factors by specific tyrosine phosphorylation is a common feature of cytokine signaling and adds potential for considerable specificity given the presence of seven family members that are differentially activated by distinct receptors (Ihle, 2001). Each of these signaling pathways requires receptor docking of JAK enzymes; distinct distal receptor cytoplasmic regions mediate downstream signals.

Selective mutagenesis of receptor signaling motifs coupled with ectopic expression of dominant-negative signaling molecules has been used to dissect the individual requirements of distinct pathways in cell responses. However, results of such studies have been contradictory and controversial. A case in point is analysis of the role of STAT3 during response of cells to G-CSF, a growth factor critical for granulopoiesis (Smithgall et al., 2000). Early work using G-CSF-responsive cell lines demonstrated potent activation of STAT3 as well as weaker activation of STAT1 and STAT5 (Tidow and Welte, 1997). However, activation of STAT3 was linked to both proliferation and terminal differentiation and arrest of granulocytes, possibly reflecting cell type-specific differences among the cell lines tested (Coffer et al., 2000; Takeda and Akira, 2000). Analysis in transgenic mice held out the prospect of precise definition of the role of individual STAT proteins in cytokine responses.

Unlike other STAT proteins, none of which is required for viability, ablation of STAT3 produced early embryonic lethality (Takeda et al., 1997). In fact, STAT3 is required for maintenance of embryonic stem cells, at least in vitro (Boeuf et al., 1997; Matsuda et al., 1999; Niwa et al., 1998; Raz et al., 1999), suggesting that it might be generally required for cell proliferation or survival. G-CSF plays a critical role in granulopoiesis in vivo, as demonstrated by severe neutropenia in animals lacking the gene for G-CSF or for G-CSFR (Lieschke et al., 1994; Liu et al., 1996). Moreover, it was recently reported that ablation of the STAT3 recruitment site on the G-CSFR or ectopic expression of DN-STAT3 led to a similar neutropenic phenotype, suggesting STAT3 is a major target for G-CSFR signaling (McLemore et al., 2001). However, selective ablation of STAT3 expression in committed myeloid cells through Cre recombinase-dependent gene deletion directed by the macrophage lysozyme promoter did not prevent normal production of neutrophils (Takeda et al., 1999), limiting any STAT3 requirement to an earlier, progenitor stage.

We have examined the role of STAT3 in hematopoiesis by selective ablation of the STAT3 gene in hematopoietic progenitor cells. In contrast to an expected neutropenic phenotype, these mice produced excess neutrophils in both bone marrow and the periphery. STAT3 null pro-

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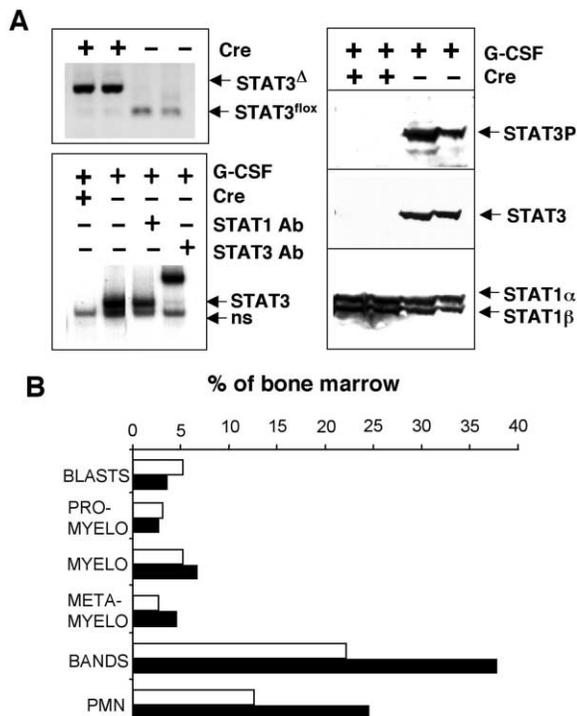


Figure 1. STAT3 Is Deleted in Bone Marrow

(A) Mx-Cre:STAT3^{fllox} (Cre) or STAT3^{fllox} mice were sacrificed 10 days after poly(I:C) treatment. DNA from BM was analyzed for STAT3 by PCR (upper left panel). BM cells were stimulated with G-CSF (10 ng/ml), and protein extracts were analyzed by EMSA (lower left) and by immunoblotting with antibodies against phospho-STAT3, STAT3, and STAT1, as indicated (right panel). ns, nonspecific. (B) Increased late-stage neutrophils in BM lacking STAT3. Neutrophil lineage cells were enumerated from control and STAT3KO marrow following Wright-Giemsa staining.

genitors differentiated normally in response to G-CSF and produced neutrophils that were morphologically and functionally mature. These data demonstrate that STAT3 is not required for G-CSF-dependent proliferation, differentiation, or survival of neutrophils. Instead, STAT3 is necessary for a negative feedback loop that controls neutrophil numbers.

Results

Generation of Mice Lacking STAT3 in Hematopoietic Progenitors

To overcome the STAT3 requirement during embryogenesis, we created conditionally mutant mice by using the *loxP*-Cre recombinase system (Zou et al., 1994). Mice were created carrying a conditional STAT3 allele with exons 16–21 flanked by *loxP* sites (STAT3-fllox), thus targeting critical portions of the STAT3 DNA binding and SH2 domains for Cre-mediated excision to functionally ablate both STAT3 α and STAT3 β (Raz et al., 1999). These mice were mated with a transgenic line bearing Cre recombinase driven by the IFN-inducible Mx promoter (Kuhn et al., 1995). Induction of Cre expression following injection of the IFN-inducer poly(I:C) or of recombinant IFN α led to effective deletion of STAT3 gene segments in bone marrow (Figure 1A). A PCR-based genotyping

assay of total bone marrow cells of poly(I:C)-treated mice showed efficient loss of the STAT3-fllox allele and creation of a novel, ablated STAT3 null allele (STAT3 Δ , lanes 1 and 2). Similar treatment of STAT3^{fllox} mice lacking the Mx-Cre transgene showed no change in the structure of the STAT3 gene (lanes 3 and 4). Only trace amounts of STAT3-fllox were detectable following Mx-Cre induction. Quantitation of the comparative levels of STAT3 null to STAT3-fllox indicated that the efficiency of STAT3 deletion after poly(I:C) treatment was >95% in the marrow (data not shown). Deletion of STAT3 by poly(I:C) was also observed in other organs, with highest efficiency in liver (~100%), moderate efficiency in kidney, spleen, lung, and heart (40%–60%), and lower efficiency in skeletal muscle and testis (10%–20%) (data not shown). For experiments in this paper, comparisons were made between littermate STAT3^{fllox} mice with or without the Mx-Cre transgene, treated equivalently with poly(I:C). STAT3^{fllox} heterozygous mice containing Mx-Cre responded to poly(I:C) in a manner similar to that of Cre-negative mice (data not shown), demonstrating that the phenotypes observed were not due to expression of Cre or presence of the transgene but rather to loss of STAT3. While adverse effects of Cre expression have been noted in experiments with fibroblasts (Silver and Livingston, 2001), hematopoietic cells do not appear to be affected (de Alboran et al., 2001).

To further confirm a functional deletion of STAT3 in bone marrow from Mx-Cre animals, nuclear extracts prepared from poly(I:C)-treated, G-CSF-stimulated bone marrow cells were subjected to gel mobility shift analysis in the absence or presence of anti-STAT antibodies. As shown in Figure 1A (lower left panel), a G-CSF-inducible protein-DNA complex, which was absent in cells from Mx-Cre animals treated with poly(I:C), was detected in extracts from control cells. This complex contained STAT3 as indicated by reactivity with anti-STAT3 antibodies but not anti-STAT1 antibodies, consistent with previous reports that STAT3 is the principal STAT activated in response to G-CSF. Significantly, no STAT3-DNA complex was detected in extracts from G-CSF-treated cells isolated from Mx-Cre mice, confirming loss of STAT3. A similar conclusion was supported by Western blotting. STAT3 protein and tyrosine-phosphorylated STAT3, a signature for activation, were readily detected in STAT3^{fllox} cells, but no signal was detected in cells from Mx-Cre:STAT3^{fllox} animals (Figure 1A, right panel). Antibodies that recognized either N-terminal or C-terminal regions of STAT3 that were not deleted in the mutant allele failed to detect STAT3 in mutant cells, showing that no cryptic product was expressed from the targeted allele. In particular, no immunoreactive protein of a size consistent with the targeted deletion (~57 kDa) was detected using antibodies directed against nondeleted regions. STAT1 α and STAT1 β proteins were detected at equal levels in cells of both genotypes, demonstrating the specificity of the disruption of STAT3. These results demonstrated that deletion of STAT3 DNA in Mx-Cre animals effectively ablated STAT3 protein in the marrow.

Granulopoiesis in the Absence of STAT3

To examine the consequence of STAT3 loss in Mx-Cre:STAT3^{fllox} mice, peripheral white blood cells were pre-

Table 1. WBC Differential Count and Hematological Parameters

WBC Differential Count	Control	STAT3KO
Neutrophils (%)	11 ± 4	69 ± 12
Lymphocytes (%)	85 ± 4	28 ± 12
Macrophages (%)	N.D.	2.7 ± 1
Eosinophils (%)	4 ± 0	N.D.
Hematological Parameters		
Hematocrit (%)	54 ± 0	54 ± 2
Hemoglobin (g/dl)	15.2 ± 0.7	15 ± 0.8
Total WBC ($\times 10^{-3}/\mu\text{l}$)	6.5 ± 0.7	7.2 ± 0.7
Platelets ($\times 10^{-6}/\mu\text{l}$)	1.2 ± 0	1.6 ± 0.2

Peripheral blood from STAT3^{fl/fl} (control) and Mx-Cre:STAT3^{fl/fl} mice (STAT3KO) treated with poly(I:C) was analyzed by Wright-Giemsa staining and enumerated for granulocyte cell types and various hematological parameters, by standard methods. N.D., not detected.

pared for Wright-Giemsa staining 3 weeks after induced deletion of STAT3. Morphologically identifiable neutrophils, lymphocytes, and myeloid cells were detected in both mutant and control animals (Table 1). Normal numbers of mature red blood cells were also detected (data not shown). In contrast to animals lacking G-CSF or G-CSFR that displayed a decrease in peripheral neutrophils (Lieschke et al., 1994; Liu et al., 1996), an increased percentage of neutrophils and a corresponding decreased percentage of lymphocytes were detected in STAT3 mutant mice relative to controls. Increased neutrophils were not likely due to an overt inflammatory response because the total white blood cell count was comparable between control and mutant mice and would be expected to increase during infection (Table 1). Moreover, animals were maintained on antibiotic throughout these experiments to suppress infections that could complicate the analysis. Neutrophils were also detected in spleen where a similar phenomenon of a 4-fold increase in neutrophil numbers was detected in mutant mice relative to controls (data not shown).

STAT3-Deficient Neutrophils Mature Normally and Are Functional

Granulopoiesis in the absence of G-CSF or G-CSFR, in the presence of a mutant G-CSFR unable to activate STAT3, or in the presence of dominant-negative STAT3 resulted in a profound neutropenia accompanied by accumulation of immature precursors (Lieschke et al., 1994; Liu et al., 1996; McLemore et al., 2001). In contrast, an excess of morphologically mature neutrophils was present in the periphery of STAT3 null animals. Also in contrast to the results observed in G-CSF or G-CSFR mutant animals, no significant increase in precursors or immature granulocytes was detected in STAT3 null bone marrow. Rather, increased numbers of relatively mature band and segmented stage PMN were observed (Figure 1B).

To further examine functional parameters of STAT3 mutant neutrophils, the ability of these cells to emigrate to the peritoneal cavity was measured in response to thioglycollate (TGA). STAT3 mutant neutrophils trafficked normally to the peritoneum, with 4- to 8-fold greater numbers accumulating within 4 hr compared to control animals. TGA-elicited neutrophils stained normally for Gr-1 and CD11b, late-stage markers of neutro-

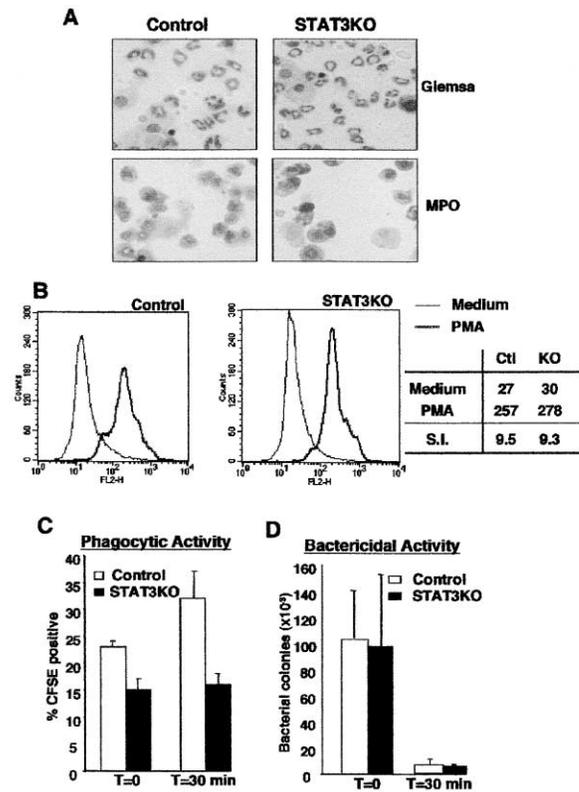


Figure 2. Peripheral Neutrophils Lacking STAT3 Are Functional

(A) In vitro differentiated granulocytes were stained with Giemsa (upper panels) or for myeloperoxidase (lower panels). (B) Peritoneal exudate cells from thioglycollate-treated mice were analyzed for oxidation of dihydrorhodamine by FACS with or without treatment with PMA. Mean fluorescence intensities and stimulation index (S.I.) are indicated. (C) STAT3-deficient neutrophils are phagocytic. CFSE-labeled bacteria were incubated with peritoneal neutrophils at 37°C for 30 min (T = 0), and gentamycin was added for another 30 min (T = 30 min). Cells washed free of extracellular bacteria at T₀ or T₃₀ were analyzed by FACS for engulfed bacteria. (D) STAT3-deficient neutrophils are bactericidal. Bacteria engulfed by neutrophils were enumerated by serial dilution.

phil differentiation, and cell surface expression of these proteins was enhanced by stimulation with PMA (data not shown).

Functional competence of STAT3-deficient neutrophils was confirmed by four different assays: differentiation marker staining, respiratory burst, phagocytosis, and bacterial killing. Bone marrow of control or mutant mice was differentiated in vitro with G-CSF for 1 week followed by staining with Giemsa and myeloperoxidase; control and mutant neutrophils were comparable (Figure 2A). When neutrophils are exposed to appropriate stimuli, NADPH oxidase is activated, resulting in the consumption of oxygen and its conversion to superoxide in a respiratory burst. Production of reactive oxygen intermediates was measured in peritoneal and peripheral blood neutrophils by oxidation of dihydrorhodamine to fluorescent rhodamine following stimulation with PMA. Neutrophils from both control and STAT3 null mice displayed an equivalent oxidative burst (Figure 2B). A similar equivalence of respiratory burst potential was

observed using wild-type and mutant peripheral blood (data not shown).

Neutrophils are phagocytic and bactericidal. To examine these parameters, carboxyfluorescein succinimidyl ester (CFSE)-labeled bacteria were incubated with TGA-elicited peritoneal granulocytes, and bacterial uptake was monitored by FACS (Figure 2C). Bacterial uptake was monitored before ($T = 0$) and after a further incubation with antibiotic to kill noninternalized bacteria ($T = 30$). Both wild-type and STAT3 null cells were capable of engulfing bacteria, although the STAT3-deficient cells displayed a 30%–50% reduced phagocytic activity. Bacterial killing was measured by determining the viability of engulfed bacteria following lysis of bacteria-loaded neutrophils (Figure 2D). Despite the reduction in phagocytic activity observed in STAT3 null neutrophils, bacteria that were engulfed were effectively killed during a 30 min incubation. Taken together, the results show that STAT3 null neutrophils are functional.

Normal Granulocyte Precursor Frequency in the Absence of STAT3

Myeloid cells are derived from bone marrow progenitors, and granulocytic differentiation is potentiated by G-CSF. Granulocyte precursor frequencies were assessed in the absence of STAT3 by colony formation assays. Bone marrow from poly(I:C) treated control or Mx-Cre mice was seeded in semisolid medium containing a cocktail of IL-3, SCF, IL-6, and EPO (complete medium), supporting growth of a panoply of myeloid progenitors. Alternatively, cells were cultured with G-CSF as the only supportive cytokine to enumerate granulocyte precursors. A comparable number of colonies formed from bone marrow cells of both control and STAT3 mutant mice in complete medium (Figure 3A, left panel) and produced a mixture of mature cell types (right panel), including morphologically mature neutrophils (arrows). Similarly, medium containing only G-CSF supported increased colony numbers in a dose-dependent manner from bone marrow of both genotypes (Figure 3B, left panel), demonstrating equal numbers of general precursors and of granulocyte precursors despite loss of STAT3. Similar to the result with cells matured in response to complete medium, cells with typical mature neutrophil morphology developed in response to G-CSF, regardless of STAT3 status (right panel).

Since poly(I:C)-induced deletion may not be 100%, we tested whether cytokine-dependent colony formation favored selective outgrowth of residual wild-type cells from mutant marrow. PCR assays performed on isolated individual colonies verified the presence of cells with the expected genotypes (Figure 3C). Colonies developed from control marrow carried the STAT3-flox allele while Mx-Cre:STAT3-flox marrow produced only STAT3 null colonies. These data confirm that G-CSF-dependent granulopoiesis is not impaired in the absence of STAT3, but the neutrophilia observed in STAT3 null animals cannot be explained by increased progenitor frequency.

STAT3 Is Not Required for Response to G-CSF In Vivo, but STAT3 Null Granulocytes Are Hyperproliferative

The similar numbers of granulocytic progenitors detected in STAT3 mutant marrow suggested that the in-

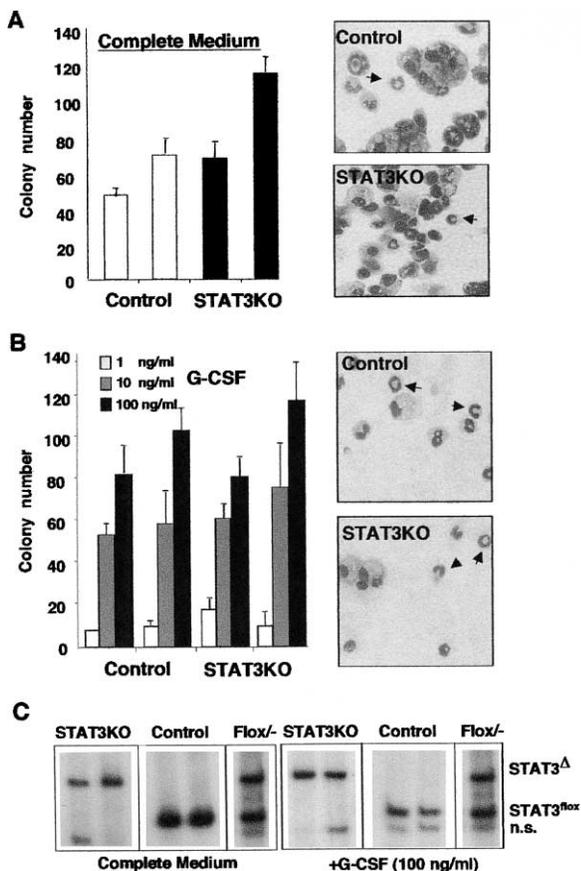


Figure 3. Normal Numbers of Granulocyte Precursors in the Absence of STAT3

Control and STAT3KO BM were analyzed for myeloid precursors by colony formation in methylcellulose.

(A) Colonies developed in complete medium (MethoCult M3434) containing SCF, IL-3, IL-6, and EPO. Example colonies of CFU-GEMM are shown, and mature neutrophils are indicated by arrows. (B) Colonies developed in the presence of G-CSF as the only supporting cytokine.

(C) Individual colonies developed in complete medium (left panel) or in response to G-CSF (right panel) were genotyped for STAT3. A heterozygous flox^{-/-} sample was included for comparison. ns, nonspecific.

creased abundance of neutrophils in the periphery could be due to increased proliferation. G-CSF is known to increase production and release of neutrophils from bone marrow, and it is widely used to treat neutropenia in clinical settings. To directly investigate possible roles for STAT3 in G-CSF action in vivo, an experimental auto-reconstitution assay was used to monitor newly formed bone marrow granulocytes and release of peripheral neutrophils. Mice were deleted for STAT3 by two doses of poly(I:C) treatment, and their marrow was ablated by treatment with 5-fluorouracil. Subsequent hematopoietic repopulation was followed in mice with or without treatment with G-CSF. Peripheral blood Gr-1⁺ leukocytes from autoreconstituted mice were assayed for intracellular STAT3 and quantified by FACS. Figure 4A shows results from a representative experiment in which 9% of peripheral blood cells were Gr-1 positive in a STAT3^{f/f} control animal, and that percentage increased

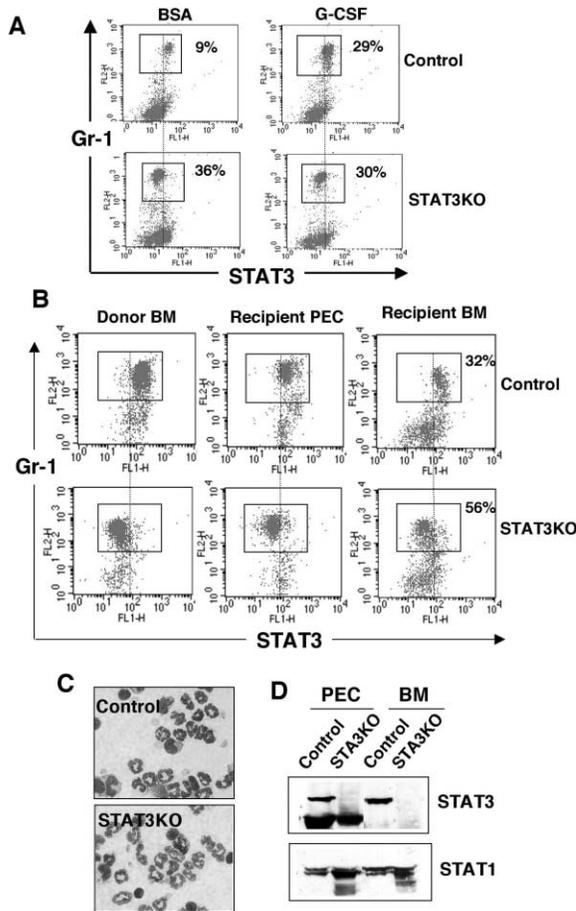


Figure 4. Enhanced Granulopoiesis In Vivo in the Absence of STAT3
(A) Hematopoiesis in the absence of STAT3. 5-Fluorouracil-treated control and STAT3KO mice were treated with G-CSF or BSA (10 μ g/kg) daily for 7 days. Peripheral blood from control (upper panel) or STAT3KO mice (lower panel) was analyzed by FACS for Gr-1 and STAT3.
(B) BM transplantation. Donor BM from control or STAT3KO mice was analyzed by FACS for Gr-1 and STAT3 prior to transplantation (left panels). Five weeks after transplantation, peritoneal exudate cells (PEC, middle panels) and BM cells (BM, right panels) were analyzed by FACS.
(C) PEC from recipient mice were stained with Wright-Giemsa.
(D) Cell extracts from PEC and BM of recipient mice were immunoblotted for STAT3 and STAT1.

approximately 3-fold following G-CSF treatment. In contrast, the percentage of neutrophils in the periphery of STAT3-deficient mice was high even in the absence of cytokine treatment and did not increase further in response to exogenous G-CSF. The cells repopulating mutant mice were not due to inefficient deletion of STAT3 in response to poly(I:C), as indicated by loss of intracellular STAT3 in mutant cells. The response to G-CSF averaged 3-fold in control mice while no increase in peripheral or bone marrow neutrophils was observed in G-CSF-treated STAT3 null animals, although these animals displayed a 3- to 4-fold higher granulocyte abundance despite whether or not they were treated with G-CSF.

Enhanced granulopoiesis from STAT3 null bone mar-

row could be due to the cell-autonomous loss of STAT3 protein in progenitor cells or to alterations in the bone marrow microenvironment, such as from altered cytokine production. The cell-autonomous contribution of STAT3 to granulopoiesis was investigated by bone marrow transplantation. Control and STAT3 null marrow were injected intravenously into lethally irradiated wild-type recipients. Five weeks after transplantation, bone marrow, peritoneal exudate, and peripheral organs were analyzed for granulocytes by FACS (Figure 4B). Gr-1⁺ cells developed from both wild-type and STAT3 null precursors and were detected in both the marrow and the periphery of recipients. The great majority of neutrophils developing from STAT3 null donors lacked detectable STAT3 protein, with the small number of STAT3-positive cells being derived either from residual recipient-derived cells due to incomplete irradiation or from the small number of donor cells that did not undergo recombination. Consistent with the neutrophilia observed in Mx-Cre mice, an increased percentage of granulocytes accumulated in the marrow of recipients reconstituted with STAT3 null cells, and peripheral cells were morphologically mature (Figure 4C). The absence of STAT3 in the reconstituted cells derived from mutant donors was confirmed by immunoblotting (Figure 4D). Increased neutrophils were also observed in spleen and peripheral blood of mice transplanted with STAT3 null cells (data not shown). These data conclusively demonstrate that STAT3 is not required for the development of mature neutrophils and that loss of STAT3 in hematopoietic progenitors rather than in stromal tissue or other organs contributes to increased granulopoiesis.

Enhanced Proliferation, Prolonged Signaling, and Reduced Induction of SOCS3 by G-CSF in the Absence of STAT3

Proliferation of control and STAT3 null bone marrow cells cultured in vitro was measured in response to increasing amounts of G-CSF (Figure 5A, left panel) or of IL-3 (right panel). Proliferation of control bone marrow cells plateaued at approximately 1 ng/ml G-CSF while proliferation of STAT3 null cells continued to increase with increasing G-CSF. In contrast, control and STAT3 null cells responded similarly to IL-3. The increased proliferation of mutant cells could not be accounted for by decreased apoptosis, since no significant difference in TUNEL-positive or Annexin V-positive cell numbers was observed between control and STAT3 null cultures (data not shown). Importantly, no decrease in cell proliferation was observed in the absence of STAT3 as would be expected if it contributed an essential component to G-CSF-driven cell growth.

It must be noted that bone marrow cells from wild-type and STAT3-ablated mice are not identical populations due to enhanced mutant granulopoiesis in vivo. To determine whether enhanced response to G-CSF was cell autonomous or reflected different starting cell populations, we isolated immature granulocytes from mice shortly after gene ablation in response to poly(I:C). Following enrichment by density gradient for immature cells, equivalent numbers of Gr-1/CD11b⁺ cells (Figure 5B) were analyzed for proliferation. Similar to unfractionated bone marrow, STAT3 null cells showed enhanced

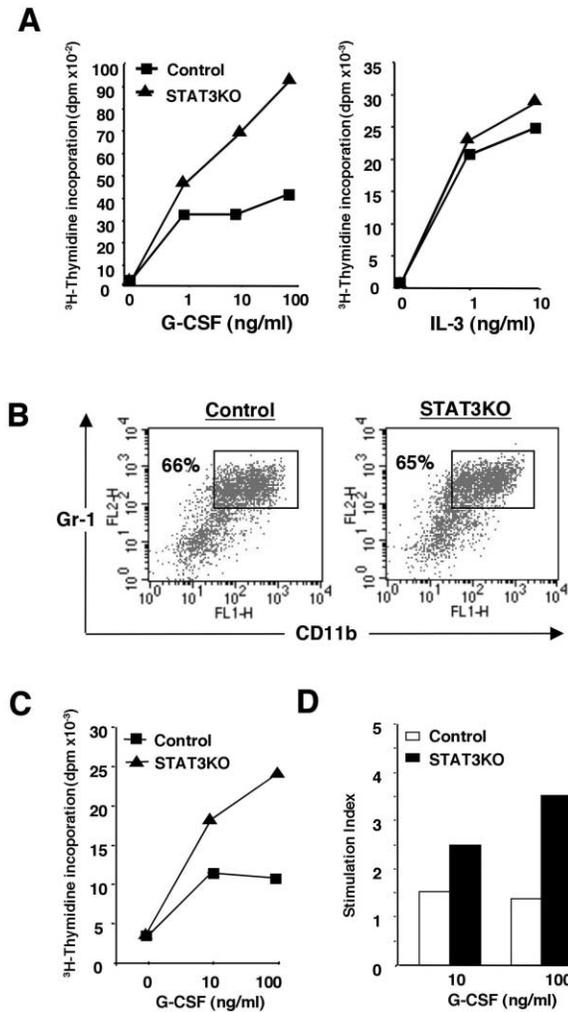


Figure 5. Enhanced G-CSF-Dependent Proliferation in the Absence of STAT3

(A) Enhanced proliferation of STAT3 null BM in response to G-CSF (left panel) but not to IL-3 (right panel). (B) Comparable numbers of immature granulocytes were fractionated from BM of control and STAT3 null mice 4 days after poly(I:C) treatment. (C) Enhanced proliferation of STAT3 null immature granulocytes in response to G-CSF, as measured by ³H-thymidine incorporation. (D) Dose-dependent enhancement of STAT3 null cell proliferation, measured by BrdU incorporation into Gr-1⁺ immature granulocytes.

proliferation in response to increasing G-CSF treatment by both ³H-thymidine incorporation (Figure 5C) and by BrdU incorporation into Gr-1⁺ cells (Figure 5D).

We considered what might underlie the increased granulocyte abundance in STAT3 null bone marrow and the G-CSF-dependent proliferation of STAT3 null granulocytes. One possibility might be increased expression of G-CSF or G-CSF receptors. However, no significant differences in G-CSF or G-CSFR mRNA levels were detected (data not shown). Alternatively, enhanced proliferation could be due to aberrant mitogenic signaling. G-CSF is known to activate STAT1 and STAT5 in addition to STAT3, each of which might be involved in regulating cell proliferation. To test for heightened signaling in the

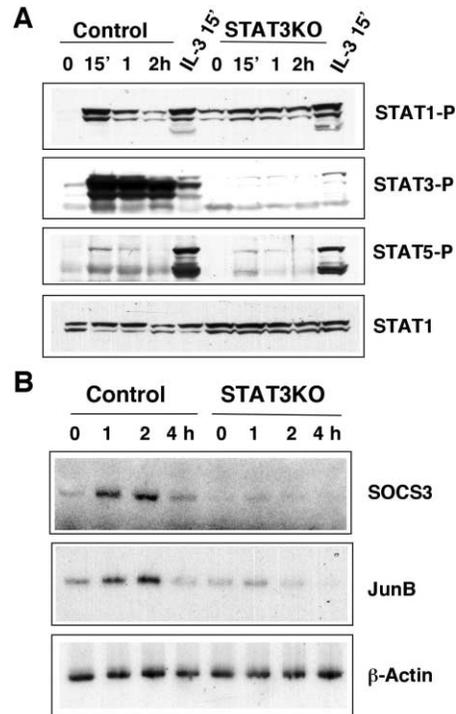


Figure 6. G-CSF Stimulates Prolonged STAT1 Phosphorylation but Impaired Gene Induction in STAT3 Null Cells

(A) BM cells were untreated or stimulated with 10 ng/ml G-CSF for 15 min, 1 hr, or 2 hr, or with 10 ng/ml IL-3 for 15 min and analyzed for phospho-STATs and STAT1 levels, as indicated. (B) Percoll-purified immature BM cells were stimulated with 10 ng/ml G-CSF and analyzed for SOCS3, JunB, and β-actin expression by RT-PCR.

absence of STAT3, activation of different STAT proteins was measured in response to G-CSF. Control cells displayed transient and high level activation of phospho-STAT3, with lower levels of phospho-STAT1 and barely detectable levels of phospho-STAT5, a protein that was much more sensitive to IL-3 treatment (Figure 6A). STAT3 null cells displayed essentially no phospho-STAT3, while both STAT1 and STAT5 were still phosphorylated. Interestingly, significant phospho-STAT1 was observed even under basal conditions, and its phosphorylation was more sustained, remaining significantly elevated 2 hr after G-CSF stimulation. Altered phosphorylation kinetics was not due to changes in STAT1 abundance, which remained approximately equal between control and STAT3 null cells (Figure 6A). Phosphorylation levels of STAT5 were comparable, regardless of genotype.

Prolonged activation of STAT1 might reflect sustained signaling in response to G-CSF. Important feedback inhibitors of JAK catalytic activity in response to cytokines are the SOCS proteins, and expression of SOCS3 is significantly induced in response to G-CSF (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Once expressed, SOCS3 is recruited to phosphorylated cytokine receptors and inhibits JAK catalytic activity (Krebs and Hilton, 2001). As shown in Figure 6B, SOCS3 was rapidly induced in G-CSF-treated control cells. By con-

trast, induction of SOCS3 was impaired in the absence of STAT3. Similarly, induction of other STAT3-inducible genes, such as JunB (Figure 6B) and Fc γ RI (data not shown), was decreased in response to G-CSF, consistent with the loss of STAT3. These results demonstrate that STAT3 is required for induction of SOCS3 in response to G-CSF and suggest that absence of SOCS3 negative feedback may allow prolonged JAK catalytic activity, resulting in enhanced signaling and heightened proliferation.

Discussion

STAT3 Is Dispensable for Granulocyte Proliferation and Differentiation

Normal development of the granulocyte lineage requires G-CSF signaling (Lieschke et al., 1994; Liu et al., 1997), and a major target of the G-CSFR is STAT3 (Figures 1A and 6A). Indeed, transgenic mice with a targeted mutation of G-CSF receptor (d715F) that abrogates STAT3 activation display severe neutropenia with an accumulation of immature myeloid precursors in their bone marrow. Expression of a constitutively active version of STAT3 rescues this phenotype, a condition that can also be mimicked by expression of a dominant-negative form of STAT3 in wild-type mice. Such data have led to the conclusion that STAT3 activation is required for normal G-CSF-dependent proliferation and granulocyte differentiation (McLemore et al., 2001). In this study, we have directly tested this notion through targeted deletion of STAT3 in hematopoietic progenitor cells in the bone marrow of transgenic mice. In contrast to previously reported results, we detected no impairment in granulocyte proliferation and terminal differentiation in the absence of STAT3. Rather, evidence was obtained that STAT3 is a negative regulator for G-CSF-mediated proliferation and homeostasis of granulocytes. In the absence of STAT3, enhanced G-CSF-dependent proliferation was observed in responsive progenitor cells, and mice lacking STAT3 in their hematopoietic precursors developed neutrophilia, as did wild-type mice transplanted with STAT3 null progenitors. Loss of STAT3 affected neither the frequency of granulocytic precursors nor their ability to differentiate into mature, functional effector cells. STAT3 null granulocytes displayed a respiratory oxidative burst, increased expression of surface markers, and the ability to phagocytose and kill bacteria. Therefore, STAT3 is dispensable for granulocyte proliferation, differentiation, and function but is required for homeostasis.

Several possibilities may account for the discrepancy between functional impairments of STAT3 studied previously and the absence of STAT3 studied here. A trivial explanation could be that our mice express a low but sufficient level of STAT3. Although the deletion of STAT3 is not absolute in Mx-Cre mice, several lines of evidence preclude the possibility that mature neutrophils are derived from residual STAT3-containing precursors. First, little residual wild-type STAT3 allele was detected in mutant bone marrow (Figure 1A), and individual G-CSF-derived colonies from mutant progenitors demonstrated complete absence of STAT3 (Figure 3C). Similarly, protein immunoblots and DNA binding assays consistently

showed complete loss of STAT3 protein, and the bulk of autoreconstituted or transplant-derived neutrophils showed loss of STAT3 (Figure 4A). Finally, Western blot analysis of elicited peritoneal exudate cells, which consisted of highly enriched mature neutrophils, showed no STAT3 (Figure 4D).

Another consideration is the distinct differences between previous studies implying a requirement for STAT3 and the present gene-targeting approach. The mutant G-CSF receptor unable to activate STAT3 (d715F) is also severely truncated in its cytoplasmic domain, possibly complicating the phenotype of this mouse. In fact, the deleted G-CSFR induces a heightened proliferative response to G-CSF, resulting in prolonged activation of STAT1, STAT3, and STAT5 and impaired ligand-dependent receptor downregulation (Hermans et al., 1998). Similarly skewed responses have been observed with other mutant cytokine receptors. Forced expression of mutant gp130 proteins that contain only one of the critical tyrosine residues normally found in its cytoplasmic tail showed that different tyrosine residues contribute distinct signals to IL-6-mediated responses. Moreover, point mutant receptors behave differently from truncated mutant receptors (Schmitz et al., 2000). In the case of G-CSFR, the d715 receptor caused increased proliferation in response to G-CSF. Mutation of its single remaining tyrosine residue resulted in a loss of STAT5 phosphorylation, in addition to STAT3 (McLemore et al., 2001). While STAT5 is not required for granulopoiesis or G-CSF-dependent proliferation (Teglund et al., 1998), loss of STAT5 phosphorylation by the d715F receptor indicates that this mutation cannot be considered specific for STAT3. Consistent with this notion, it is probable that impaired granulopoiesis observed in mice expressing a dominant-negative STAT3 could reflect crossinhibition of other receptor-mediated signals beyond STAT3 activation that depend on the same recruitment site. Similar discrepancies between the effects of dominant-negative STAT proteins and the corresponding null phenotypes have been reported (Ihle, 2001).

STAT3 as a Negative Regulator of G-CSF-Mediated Granulopoiesis

The enhanced production of neutrophils in the absence of STAT3 is reminiscent of the phenotype derived from the G-CSFR mutant found in human severe congenital neutropenia (Hermans et al., 1998; McLemore et al., 1998). The hypergranulopoiesis of mutant mice bearing this receptor correlated with prolonged activation of STAT1, STAT3, and STAT5 in response to G-CSF treatment (Hermans et al., 1999). Interestingly, prolonged activation of STAT1 though not STAT5 was also observed in the absence of STAT3 following G-CSF stimulation. However, a requirement for STAT1 in G-CSF-mediated granulopoiesis is unlikely, given the normal proliferation, accumulation, and function of granulocytes in STAT1-deficient mice (Durbin et al., 1996; Meraz et al., 1996). Nonetheless, a critical interaction between STAT1 and STAT3 in G-CSF-dependent granulopoiesis cannot be excluded.

More likely, however, is that enhanced proliferation of granulocytes in response to G-CSF and neutrophilia observed in mutant mice resulted from loss of negative

regulatory signals and that prolonged STAT1 activation is a symptom of that impaired negative feedback. The severe impairment of SOCS3 induction is consistent with this notion, since SOCS3 as an inducible suppressor of cytokine signaling is normally responsible for inhibition of receptor-associated JAK catalytic function. The promoter of SOCS3 contains a functional STAT3 binding element that is critical for LIF-mediated induction (Auernhammer et al., 1999). SOCS3 also negatively regulates IL-6 signaling, and antagonism of SOCS3 function can lead to development of colitis, due at least in part to hyperactivity of cytokine signals that target STAT3 (Suzuki et al., 2001).

It is interesting that a constitutively active form of STAT3 partially rescued the ability of progenitor cells from d715F mice to differentiate into neutrophils in response to G-CSF (McLemore et al., 2001). These data could be interpreted as showing that STAT3 is capable of driving G-CSF-dependent differentiation, even though STAT3 deletion showed that it is not necessary. It must be borne in mind, however, that the constitutively active STAT3 protein also displays oncogenic properties (Bromberg et al., 1999) and therefore cannot be considered a faithful mimic of normal STAT3 function. In fact, expression of constitutively active STAT3 in bone marrow progenitors led to increased cell numbers as well as increased differentiation (McLemore et al., 2001). In a similar fashion, constitutively active STAT5 is capable of causing myeloproliferative disease (Schwaller et al., 2000), even though it is not required for normal myeloproliferation or differentiation (Teglund et al., 1998). It is possible that STAT3 will be found to contribute to myeloid oncogenesis in spite of its dispensability for normal granulopoiesis.

Due to alternative splicing, STAT3 is expressed as two isoforms, STAT3 α and STAT3 β (Schaefer et al., 1997), both of which were functionally ablated by our targeting strategy. STAT3 β lacks a transactivation domain and antagonizes the activity of STAT3 α . Indeed, recent gene targeting of the STAT3 β locus documented a critical role for this isoform in recovery from endotoxic shock (Yoo et al., 2002), demonstrating its role as a negative regulator of gene expression. Therefore, it is possible that lack of STAT3 β function contributes to the loss of negative regulation responsible for the observed neutrophilia in STAT3 null mice. If so, STAT3 β would be a more general negative regulator than originally suggested, inhibiting gene expression beyond inhibition of STAT3 α .

STAT3 and Emergency Granulopoiesis

Under certain stressful situations, such as response to pathogen infection, rapid expansion of neutrophil populations can be achieved through a process known as emergency granulopoiesis that is accompanied by high levels of G-CSF (Basu et al., 2000; Lieschke et al., 1994; Ward et al., 1999). Emergency granulopoiesis demonstrates some of the complexity of molecular responses to G-CSF. For instance, STAT3 activation by G-CSFR can be achieved through both receptor tyrosine-dependent and -independent mechanisms, depending on G-CSF concentration. At saturating concentrations (100 ng/ml), STAT3 can be activated by a full-length G-CSFR

devoid of intracellular tyrosines (Ward et al., 1999). In contrast, activation of STAT3 by the truncated d715 receptor is completely blocked by loss of the single remaining tyrosine, even at high G-CSF concentrations. These results suggest that emergency granulopoiesis in response to high levels of G-CSF may be accomplished through an independent signaling pathway mediated by a non-phosphotyrosine-dependent mechanism involving the distal region of the G-CSFR. While STAT3 activation is a marker for both receptor tyrosine-dependent (low dose G-CSF) and receptor tyrosine-independent (high dose G-CSF) signaling pathways, it appears to be dispensable for both mechanisms of granulopoiesis. In fact, mice lacking STAT3 in the bone marrow develop severe neutrophilia in response to bacterial infection (data not shown).

Emergency granulopoiesis can also be independent of G-CSF, presumably through the action of other hematopoietic cytokines. For instance, G-CSF-deficient mice develop neutrophilia during *Candida* infections (Basu et al., 2000). Although IL-6 is an independent regulator of granulopoiesis and is increased in the serum of *Candida*-infected, G-CSF-deficient mice, this cytokine was also dispensable for the observed neutrophilia since neutrophilia developed in infected mice deficient for both IL-6 and G-CSF (Basu et al., 2000). Although both IL-6 and G-CSF are efficient activators of STAT3, it is intriguing that neutrophilia can develop in the absence of all three of these molecules. It would be interesting if emergency, G-CSF-independent granulopoiesis was somehow caused by antagonism of the normally inhibitory function of STAT3, such as inhibition of SOCS protein expression.

Experimental Procedures

Animals

Generation of a conditional STAT3 allele in ES cells has been previously described (Raz et al., 1999). Mice were generated by injection of heterozygous targeted ES cells into blastocysts at the transgenic mouse facility of Albert Einstein College of Medicine by standard methods. Interbreeding with transgenic Mx-Cre mice (Kuhn et al., 1995) generated littermate mice that were homozygous for the conditional STAT3 allele (STAT3^{fl}) with or without the Mx-Cre transgene. Induction of Cre and subsequent deletion of STAT3 was accomplished by two successive intraperitoneal injections separated by 7 days with 100 μ g poly(I:C) per mouse (Radtke et al., 1999) unless otherwise specified and comparisons were made between Mx-Cre-positive and Mx-Cre-negative STAT3^{fl} littermates that had been similarly treated with poly(I:C). Neomycin (2 mg/ml) was added to drinking water to eliminate bacterial infection and intestinal flora. All work with animals conformed to guidelines approved by the Institutional Animal Care and Use Committee of the New York University School of Medicine.

Hematology

Peripheral blood or in vitro differentiated bone marrow (BM) was prepared by blood smear or cytospin centrifugation and stained with Wright-Giemsa. Differential cell counts were scored visually on coded samples. For in vitro differentiation, BM collected from femurs was cultured in RPMI-1640 medium containing 20% fetal bovine serum and 10 ng/ml recombinant murine G-CSF (PeproTech).

Autoreconstitution and Colony Formation

Age 6- to 8-week-old mice (five mice per group) were injected with 100 μ g poly(I:C) on days 1 and 7. 5-Fluorouracil (300 mg/kg) was injected on day 10 followed by subcutaneous injection of BSA or G-CSF (10 μ g/kg) daily for 7 days. BM cells were prepared 4 hr after

the last injection. For colony formation assays, 4×10^4 BM cells were incubated in complete medium (MethoCult M3434, Stem Cell Technology) containing 0.9% methylcellulose in Iscove's modified Dulbecco's media, 1% BSA, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 15% fetal bovine serum, 10 μ g/ml bovine pancreatic insulin, 200 μ g/ml human transferrin, 10 ng/ml recombinant murine IL-3, 10 ng/ml recombinant human IL-6, 50 ng/ml recombinant murine stem cell factor, and 3 U/ml recombinant human EPO. For granulocyte-specific colonies, 2×10^5 BM cells were incubated in medium (MethoCult M3234) containing the same components except with 1, 10, or 100 ng/ml of recombinant murine G-CSF as the only cytokine, followed by incubation for 7–10 days.

Thioglycollate Challenge and Respiratory Burst Assay

Age-matched STAT3^{fl/fl} and Mx-Cre:STAT3^{fl/fl} mice were treated with poly(I:C) followed by i.p. injection of 2 ml 3% TGA. Peritoneal exudate cells were harvested after 4 hr by PBS lavage. Granulocytes were enumerated following staining with anti-CD11b-FITC and anti-Gr-1-PE (Caltag) by FACS analysis. Dihydrorhodamine 123 (DHR) oxidation assay was performed as described (Vowells et al., 1995). In brief, 1×10^5 cells were incubated with 100 μ M DHR and 1000 U/ml catalase in 100 μ l of medium at 37°C for 5 min. One hundred microliters of medium containing 200 ng PMA was added and incubated for another 20 min. Cells were washed with cold PBS twice, resuspended in PBS containing 1% paraformaldehyde, and analyzed by FACS.

BM Transplantation

4×10^6 STAT3^{fl/fl} BM cells were injected intravenously into lethally irradiated (950 rad) wild-type recipients. Five weeks later, peritoneal exudate and BM cells were prepared for FACS analysis, Wright-Giemsa staining, genomic PCR, and Western blotting.

PCR and RT-PCR Assays

Mice, tissues, and cells were genotyped by PCR, using primer sets that distinguished STAT3, STAT3-flox, and STAT3-deleted alleles, as previously described (Raz et al., 1999). To genotype single hematopoietic colonies grown in methylcellulose, colonies were resuspended in 5 μ l of buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, and 200 μ g/ml proteinase K; incubated at 55°C for 2 hr and then at 95°C for 10 min; and subjected to 40 cycles of PCR.

RT-PCR was performed as previously described (Lee et al., 2000) using total RNA prepared from four to six million cells. Cycle number and template dilutions were empirically chosen to insure that reactions remained within a linear range.

Sequences of PCR primers are available on request.

Proliferation Assay

BM cells were seeded at 2×10^5 cells per well and incubated in the absence or presence of 1, 10, and 100 ng/ml recombinant murine G-CSF or 1 and 10 ng/ml recombinant murine IL-3. 0.5 μ Ci ³H-thymidine was added after 48 hr and incubated for a further 16 hr prior to harvest. To enrich immature granulocyte precursors, BM was fractionated by Percoll step-gradient ($\rho = 1.065$ and $\rho = 1.08$), and cells located at the 1.065 interface were recovered for FACS and proliferation analysis by ³H-thymidine and BrdU incorporation. For BrdU incorporation, cells treated with G-CSF for 24 hr were incubated with 10 μ M BrdU for 1 hr, followed by FACS staining for Gr-1 and BrdU. Stimulation index was calculated as the ratio of Gr-1⁺/BrdU⁺ cells with and without G-CSF.

Electrophoretic Mobility Shift Assay (EMSA) and Western Blots

EMSA and Western blots were performed as described (Lee et al., 1999). Nuclear extracts or total cell extracts were prepared from five million BM cells following treatment with 10 ng/ml G-CSF. Nuclear extracts were tested by EMSA using a radiolabeled GAS probe in the absence or presence of anti-STAT1 or anti-STAT3 antibodies (Zymed). Total cell extracts were examined by Western blot using anti-STAT and anti-phospho-STAT antibodies (Zymed and Cell Signaling).

Phagocytosis and Bactericidal Assays

Peritoneal granulocytes were harvested by lavage from thioglycollate-treated mice, washed, and resuspended in medium containing 5% FCS and lacking antibiotics. *Listeria monocytogenes* labeled with 5 μ M CFSE was mixed with 2×10^6 peritoneal exudate cells and incubated at 37°C for 30 min. Subsequently, extracellular bacteria were killed by incubation with 50 μ g/ml gentamycin. Phagocytic activity was assessed by FACS on paraformaldehyde-fixed cells. To measure bactericidal activity, engulfed bacteria were released by lysing eukaryotic cells in water, and surviving bacteria were enumerated by serial dilution.

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