

Imbalanced gp130-Dependent Signaling in Macrophages Alters Macrophage Colony-Stimulating Factor Responsiveness via Regulation of *c-fms* Expression

Brendan J. Jenkins,^{1*} Dianne Grail,¹ Melissa Inglese,¹ Cathy Quilici,¹
Steven Bozinovski,² Peter Wong,³ and Matthias Ernst¹

Ludwig Institute for Cancer Research, Colon Molecular and Cell Biology Laboratory,¹ The Walter and Eliza Hall Institute for Medical Research,³ and Pharmacology Department, University of Melbourne,² Parkville, Victoria, Australia

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The mechanisms by which interleukin-6 (IL-6) family cytokines, which utilize the common receptor signaling subunit gp130, influence monocyte/macrophage development remain unclear. Here we have utilized macrophages devoid of either gp130-dependent STAT1/3 (gp130^{ΔSTAT/ΔSTAT}) or extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein (MAP) kinase (gp130^{Y757F/Y757F}) activation to assess the individual contribution of each pathway to macrophage formation. While the inhibition by IL-6 of macrophage colony-stimulating factor (M-CSF)-induced colony formation observed in gp130^{wt/wt} mice was abolished in gp130^{ΔSTAT/ΔSTAT} mice, inhibition of macrophage colony formation was enhanced in gp130^{Y757F/Y757F} mice. In gp130^{ΔSTAT/ΔSTAT} bone marrow-derived macrophages (BMMs), both IL-6- and M-CSF-induced ERK1/2 tyrosine phosphorylation was enhanced. By contrast, tyrosine phosphorylation of ERK1/2 in response to M-CSF was reduced in gp130^{Y757F/Y757F} BMMs, and the pattern of ERK1/2 activation in gp130 mutant BMMs correlated with their opposing responsiveness to M-CSF-induced proliferation. When compared to the level of expression in gp130^{wt/wt} BMMs, *c-fms* expression was elevated in gp130^{ΔSTAT/ΔSTAT} BMMs but reduced in gp130^{Y757F/Y757F} BMMs. Finally, an ERK1/2 inhibitor suppressed M-CSF-induced BMM proliferation, and this result corresponded to a reduction in *c-fms* expression. Collectively, these results provide a functional and causal correlation between gp130-dependent ERK MAP kinase signaling and *c-fms* gene activation, a finding that provides a potential mechanism underlying the inhibition of M-CSF-dependent macrophage development by IL-6 family cytokines in mice.

The formation of macrophages involves the proliferation, differentiation, and functional maturation of multipotential hematopoietic progenitor cells from the bone marrow into monocytes and, ultimately, macrophages, a process that is primarily regulated by macrophage colony-stimulating factor (M-CSF, also known as CSF-1) (36). The central role that M-CSF plays in monocyte/macrophage development has been formally demonstrated by the severe depletion of macrophages in M-CSF-deficient osteopetrotic (op/op) mice (40) and, more recently, in mice subjected to targeted inactivation of the M-CSF receptor gene, *c-fms* (5). During mouse development, expression of *c-fms* is an early and robust marker of cells belonging to the macrophage lineage, and studies on the transcriptional regulation of *c-fms* have identified promoter elements underlying its lineage-specific activity (14, 15).

Members of the interleukin-6 (IL-6) cytokine family, in particular, IL-6, IL-11, and leukemia inhibitory factor (LIF), are multifunctional cytokines which also regulate the production of myelomonocytic cells (4, 27). In vitro studies with the M1 murine monocytic leukemia cell line revealed that IL-6 and LIF induce the terminal differentiation of these cells into mac-

rophages, suggesting that these factors promote macrophage development (25). However, the IL-6 family cytokines appear to act as both positive and negative regulators of the proliferation and differentiation of primary human and mouse macrophages (4, 17, 27), with recent evidence demonstrating that this may occur by regulating the expression of *c-fms* (3). IL-6 family cytokines share gp130 as a signaling receptor β (R β) subunit (19), and receptor binding of IL-6 or IL-11 leads to gp130 homodimerization (13, 29), whereas receptor binding of all other family members induces heterodimerization of gp130 with one of the highly related R β subunits, LIF-R β or oncostatin M-R β (10, 28). In all cases, β subunit dimerization triggers activation of two major signaling cascades, namely the SHP2/extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase (9) and the STAT1/3 (11) pathways. We have recently reported the generation of two strains of mice homozygous for knock-in mutations in gp130 which render gp130 incapable of activating either the STAT1/3 (in gp130^{ΔSTAT/ΔSTAT} mice) (6) or the SHP2/ERK MAP kinase (in gp130^{Y757F/Y757F} mice) (38) pathway. These mice display a wide variety of penetrant phenotypes, which in part mimic phenotypic pathologies observed in knockout mice lacking individual cytokines of the IL-6 family or their receptors, thereby genetically identifying the signaling pathway responsible for transducing a specific IL-6 family cytokine-mediated biological response. Surprisingly, the gp130^{ΔSTAT/ΔSTAT} and gp130^{Y757F/Y757F} mice also display novel phenotypes, which

* Corresponding author. Mailing address: Ludwig Institute for Cancer Research, Colon Molecular and Cell Biology Laboratory, P. O. Box 2008, Royal Melbourne Hospital, Victoria 3050, Australia. Phone: 61 3 9341 3155. Fax: 61 3 9341 3104. E-mail: Brendan.Jenkins@ludwig.edu.au.

most likely are a consequence of disturbing the otherwise normal balanced activation of these two signaling pathways (6, 38).

To dissect the contribution of each of the two gp130-dependent signaling cascades to the regulation of macrophage development by IL-6 family cytokines, we have studied macrophage populations derived from gp130^{ΔSTAT/ΔSTAT} and gp130^{Y757F/Y757F} mice. We report here that the absence of gp130-dependent SHP2/ERK MAP kinase activation in macrophage colony-forming cells (M-CFCs) from gp130^{Y757F/Y757F} mice enhanced the inhibition of M-CSF-induced macrophage colony formation by IL-6. In contrast, IL-6 does not inhibit M-CSF-induced macrophage colony formation by M-CFCs from gp130^{ΔSTAT/ΔSTAT} mice, where the absence of gp130-mediated STAT activation amplified signaling through the SHP2/ERK MAP kinase pathway. Similarly, bone marrow-derived macrophages (BMMs) from gp130^{ΔSTAT/ΔSTAT} and gp130^{Y757F/Y757F} mice also display opposing responsiveness to M-CSF-dependent proliferation and differentiation. Moreover, we provide evidence that the level of *c-fms* expression is directly related to the net output of ERK MAP kinase signaling emanating from gp130, thereby providing a molecular mechanism by which the IL-6 family cytokines modulate M-CSF-dependent macrophage development.

MATERIALS AND METHODS

Mice. The generation of mice homozygous for the gp130^{ΔSTAT} mutation (gp130^{ΔSTAT/ΔSTAT} mice) (6) and the gp130^{Y757F} mutation (gp130^{Y757F/Y757F} mice) (38) and of the compound gp130^{Y757F/Y757F}:IL-6^{-/-} mice (38) has been described previously. Experiments were conducted on adult mice 8 to 16 weeks of age and included wild-type littermate controls. Mice were housed under specific-pathogen-free conditions.

Cytokines and reagents. Recombinant murine M-CSF was a kind gift from Nic Nicola (Walter and Eliza Hall Institute, Melbourne, Australia). Recombinant human IL-6 and human IL-11 were generously provided by Glenn Begley (Amgen, Thousand Oaks, Calif.) and Lorraine Robb (Walter and Eliza Hall Institute), respectively. Commercially available antibodies were purchased as follows: the phospho-specific ERK1/2 antibody was purchased from Cell Signaling Technology (Beverly, Mass.), the ERK1 antibody was from Santa Cruz Biotechnology (Santa Cruz, Calif.), and the fluorescein isothiocyanate (FITC)-conjugated CD11b (Mac-1) antibody and streptavidin-phycoerythrin (PE) were purchased from Pharmingen (San Diego, Calif.). The *Fms* antibody was generously provided by Nicholas Wilson (University of Melbourne, Melbourne, Australia), and the biotinylated F4/80 antibody was a kind gift from Ken Harder (Ludwig Institute for Cancer Research, Melbourne, Australia). The MEK1 inhibitor U0216 and the p38 MAP kinase inhibitor SB203580 were obtained from Calbiochem (San Diego, Calif.).

Hematopoietic progenitor cell assays. Clonal cultures of hematopoietic progenitor cells were assayed in semisolid agar containing 0.3% agar, Dulbecco's modified Eagle's medium (DMEM), and 20% fetal calf serum (FCS). Bone marrow cells were flushed from femurs in phosphate-buffered saline (PBS) containing 2% FCS, and cell counts revealed that the levels of cellularity (per femur) were comparable among the genotypes used in this study (data not shown). Briefly, triplicate cultures of either whole blood (1 μl) or unfractionated adult bone marrow cells (2.5 × 10⁴ to 5 × 10⁴) were stimulated with cytokines either individually or in multiple combinations at the indicated concentrations (see figure legends). For M-CSF stimulation of cultures containing either peripheral blood or bone marrow cells, maximal colony stimulation was achieved at a concentration of M-CSF of ≥5 ng/ml. Cultures were incubated for 7 days (bone marrow) or 14 days (blood) at 37°C in a humidified atmosphere of 10% CO₂ in air, and colonies containing 50 or more cells were enumerated by using a dissection microscope. Agar cultures were then fixed with 2.5% glutaraldehyde and sequentially stained with Luxol Fast Blue and hematoxylin.

Reconstitution experiments. One day prior to bone marrow transfer, gp130^{wt/wt} C57BL/6 Ly5.1-recipient mice were injected intraperitoneally with 300 μg of the CD4-depleting monoclonal antibody GK1.5 (kindly provided by Y. Zhang, Walter and Eliza Hall Institute). Flow cytometric analysis of peripheral blood from GK1.5-treated mice has previously demonstrated 98% depletion of

peripheral CD4⁺ T lymphocytes (22). Unfractionated bone marrow cells (5 × 10⁶) from Ly5.2 gp130^{wt/wt}, gp130^{ΔSTAT/ΔSTAT}, and gp130^{Y757F/Y757F} mice were then injected intravenously into lethally γ-irradiated (twice at 5.5 Gy) adult Ly5.1 recipients (4 or 5 per donor). Irradiated recipients were maintained on oral antibiotics (170 mg of enrofloxacin [Baytril] per liter; Bayer, Leverkusen, Germany). Flow cytometric analysis indicated that 89% ± 2.1% (mean ± standard deviation [SD]) of peripheral blood leukocytes obtained from chimeras expressed Ly5.2 donor cells 3 months posttransfer.

BMMs. Bone marrow cells were flushed from femurs into DMEM supplemented with 15% FCS, 20% L-cell conditioned (LC) media (as a source of M-CSF), 2 mM glutamax, and 0.075% sodium bicarbonate (Life Technologies, Rockville, Md.) and were cultured in flasks at 37°C with 10% CO₂. The following day, nonadherent cells were transferred into new flasks, and BMMs were used after 7 to 10 days of culture. Prior to experiments for cytokine stimulation, cells were washed in PBS and then starved in DMEM plus 1% FCS for 6 h before being stimulated.

Western blot analysis. Prior to lysis, BMMs were either grown continuously in LC media or were washed and starved of cytokine before being stimulated. All stimulations were performed at 37°C with M-CSF at a concentration of 20 ng/ml and IL-6 at a concentration of 100 ng/ml at specified times (see figure legends). Cells were lysed in ice-cold lysis buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% glycerol, 1% Triton X-100, 50 mM NaF, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 2 mM orthovanadate, and complete protease inhibitors [Roche Diagnostics Corporation, Indianapolis, Ind.]), and cell lysates were cleared of debris by centrifugation. Cell lysates were separated on sodium dodecyl sulfate-10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked in TBST (300 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.5% Tween 20) containing 1% bovine serum albumin and incubated with primary antibodies according to the manufacturer's recommendations; subsequently they were incubated with the appropriate secondary antibodies (anti-mouse or -rabbit) coupled to horseradish peroxidase (Bio-Rad, Hercules, Calif). Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.).

Northern blot analysis. Total RNA was extracted from BMMs and unfractionated bone marrow cells with an RNeasy kit (QIAGEN, Hilden, Germany). RNA (10 to 20 μg) was run on 1% agarose gels and transferred onto nitrocellulose, following which membranes were hybridized with a radiolabeled full-length *c-fms* cDNA probe (kindly provided by Nicholas Wilson, University of Melbourne). Membranes were stripped and reprobed with a radiolabeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (kindly provided by Megan Baldwin, Ludwig Institute for Cancer Research) to confirm equal loading and transfer of RNA samples.

Proliferation and cell viability assays. BMMs were washed, starved of LC media, and then harvested by scraping. Cells were then added to flat-bottom 96-well tissue culture plates at 2 × 10⁴ cells per well in triplicate containing 100 μl of DMEM supplemented with 15% FCS, M-CSF (20 ng/ml), and/or IL-6 (100 ng/ml) as indicated. In experiments that used MAP kinase inhibitors, SB203580 was used at a final concentration of 30 μM, and U0216 was used at a final concentration of 10 μM. To measure proliferation, after 2 days of culture at 37°C, 1 μCi of [³H]thymidine (ICN Biomedicals Australasia, Seven Hills, Australia) was pulsed for the last 3 h of culture, and [³H]thymidine incorporation was measured in a TopCountNXT microplate scintillation counter (Packard, Meriden, Conn.). To measure cell viability, after 4 days of culture at 37°C, 10 μl of MTT (dimethyl thiazole diphenyl tetrazolium bromide; Sigma, St. Louis, Mo.) at a concentration of 5 mg/ml was added to the wells and incubated for a further 4 h at 37°C. A total of 200 μl of isopropanol-0.04 N HCl was then added to each well, and absorbance was measured at 550 nm. Absorbance readings were normalized against those at day 0 for 2 × 10⁴ cells per well in triplicate.

Flow cytometry. BMMs grown continuously in LC medium were harvested and resuspended in ice-cold PBS plus 2% FCS (staining solution). For staining with the biotinylated F4/80 and FITC-conjugated CD11b (Mac-1) antibodies, cells were initially incubated in Fe-block (1:100 dilution) on ice for 30 min, following which they were incubated with antibodies (1:200 dilution) on ice for 30 min. Cells were then washed twice in staining solution and incubated with streptavidin-PE (1:500 dilution) for a further 30 min on ice. Stained cells were then washed and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

Transfections and reporter assays. RAW cells were maintained in DMEM containing 10% FCS. Cells were transiently cotransfected with 10 μg of the pGL3.5fms firefly luciferase reporter construct (14), 0.5 μg of the pRL-TK *Renilla* luciferase control reporter vector (Promega, Madison, Wis.), and 20 μg of either a hemagglutinin (HA)-tagged wild-type ERK1 construct (kindly provided by Ken Harder, Ludwig Institute for Cancer Research) or the granulocyte-

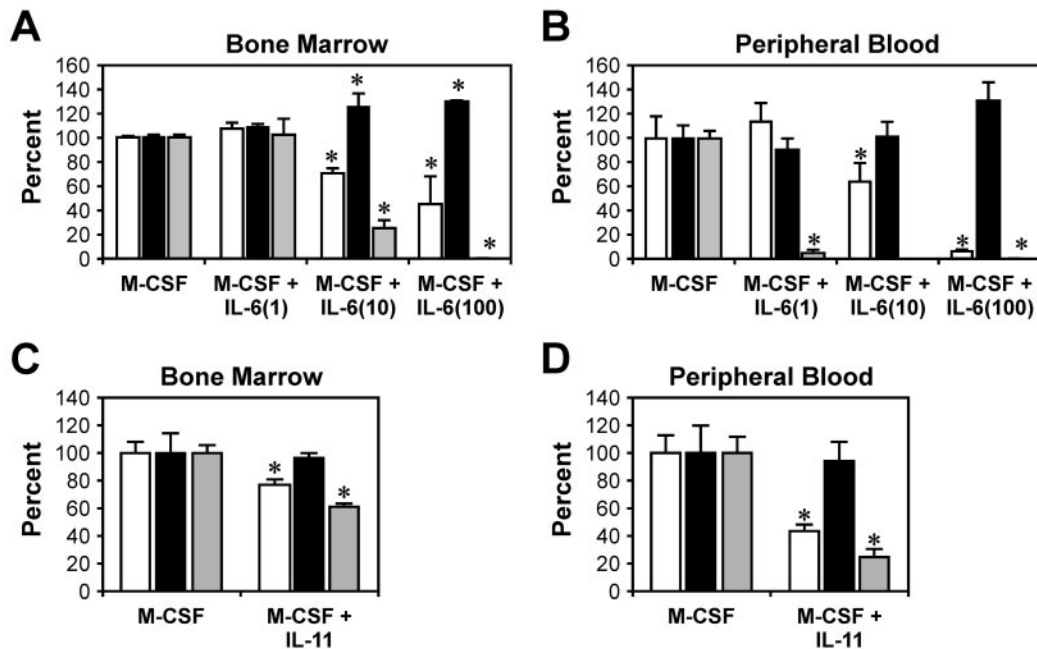


FIG. 1. Effects of IL-6 family cytokines on macrophage progenitor formation in $gp130^{\Delta STAT/\Delta STAT}$ mice (black bars), $gp130^{Y757F/Y757F}$ mice (grey bars) and wild-type littermates (white bars). In vitro macrophage colony formation of bone marrow cells (A and C) and peripheral blood (B and D) in semisolid agar containing M-CSF (10 ng/ml) and various concentrations of IL-6 (1 to 100 ng/ml) (A and B) or IL-11 (100 ng/ml) (C and D) is shown. In the presence of IL-6 or IL-11 alone, no colony formation was observed in cultures containing bone marrow or peripheral blood progenitors from mice of any genotype (data not shown). Data are presented as a percentage (\pm SD) of maximal stimulation achieved with 10 ng of M-CSF/ml alone and are representative of the results of 1 of at least 3 separate experiments. For each genotype, n was 3. *, $P < 0.05$ versus data from wild-type littermates.

CSF receptor (G-CSFR/ $gp130^{wt}$) or G-CSFR/ $gp130^{\Delta STAT}$ chimeric receptor constructs (7) by electroporation as previously described (14). For experiments with the receptor constructs, transfected cell cultures were incubated with G-CSF (100 ng/ml) in the absence or presence of U0216 (10 μ M) for 48 h prior to their lysis. Cell lysates were assayed for firefly luciferase activity and normalized against *Renilla* luciferase activity by the Dual-Luciferase Reporter assay system (Promega).

RESULTS

IL-6-induced suppression of macrophage colony formation is enhanced in the absence of gp130-dependent SHP2/ERK activation. Previous studies have indicated that activation of gp130 by IL-6 family cytokines inhibited M-CSF-dependent development of murine macrophage populations in vitro (4). In order to identify the signaling pathway responsible for transducing this activity, we initially examined the capacity of IL-6 to modulate M-CSF-dependent in vitro colony formation from bone marrow and peripheral blood M-CFCs derived from mice lacking either gp130-dependent STAT1/3 ($gp130^{\Delta STAT/\Delta STAT}$) or SHP2/ERK ($gp130^{Y757F/Y757F}$) activation. The frequencies of M-CSF-responsive M-CFCs in $gp130^{\Delta STAT/\Delta STAT}$ and $gp130^{Y757F/Y757F}$ bone marrow (94 ± 8 and 87 ± 13 M-CFCs/ 5×10^4 marrow cells, respectively) were similar to that in wild-type ($gp130^{wt/wt}$) bone marrow (92 ± 9 M-CFCs/ 5×10^4 marrow cells). In peripheral blood, there was a significant increase in the circulating concentration of M-CFCs in $gp130^{\Delta STAT/\Delta STAT}$ mice (341 ± 70 M-CFC/ μ l) compared to that in $gp130^{wt/wt}$ mice (217 ± 19 M-CFC/ μ l, $P < 0.05$) and $gp130^{Y757F/Y757F}$ mice (196 ± 37 M-CFC/ μ l, $P < 0.05$). The presence of IL-6 (100 ng/ml) inhibited M-CSF-stimulated mac-

rophage colony formation by $gp130^{wt/wt}$ M-CFCs, and peripheral blood M-CFCs were more sensitive to this inhibition than bone marrow M-CFCs (90 and 55% reduction in colony numbers, respectively) (Fig. 1A and B). However, IL-6 (100 ng/ml) did not suppress the proliferation of $gp130^{\Delta STAT/\Delta STAT}$ bone marrow and peripheral blood M-CFCs. Rather, IL-6 recruited additional M-CFCs to form colonies from $gp130^{\Delta STAT/\Delta STAT}$ bone marrow and peripheral blood (Fig. 1A and B). In contrast, macrophage colony formation by $gp130^{Y757F/Y757F}$ M-CFCs was completely abolished by IL-6 at a concentration of 100 ng/ml (Fig. 1A and B). Lower concentrations of IL-6 also led to a greater inhibition of macrophage colony formation by $gp130^{Y757F/Y757F}$ M-CFCs compared to that from $gp130^{wt/wt}$ M-CFCs. We also examined the role of IL-11 in regulating M-CSF-dependent macrophage colony formation since IL-11, like IL-6, signals exclusively via a gp130 homodimer. Although IL-11 reduced $gp130^{wt/wt}$ and $gp130^{Y757F/Y757F}$ macrophage colony formation (by 20 and 40% in bone marrow and by 55 and 76% in peripheral blood, respectively) (Fig. 1C and D), this inhibition was not as great as that seen with IL-6. Furthermore, IL-11 (100 ng/ml) had little or no effect on M-CSF-induced macrophage colony formation by $gp130^{\Delta STAT/\Delta STAT}$ bone marrow and peripheral blood M-CFCs.

Considering that these observations indicate a major role for IL-6 in suppressing macrophage colony formation in vitro, we hypothesized that the absence of IL-6 in $gp130^{wt/wt}$ and $gp130^{Y757F/Y757F}$ mice might result in an increase in M-CFC levels in vivo. Therefore, we enumerated the concentration of M-CFCs in peripheral blood of $gp130^{wt/wt}$ and IL-6-defi-

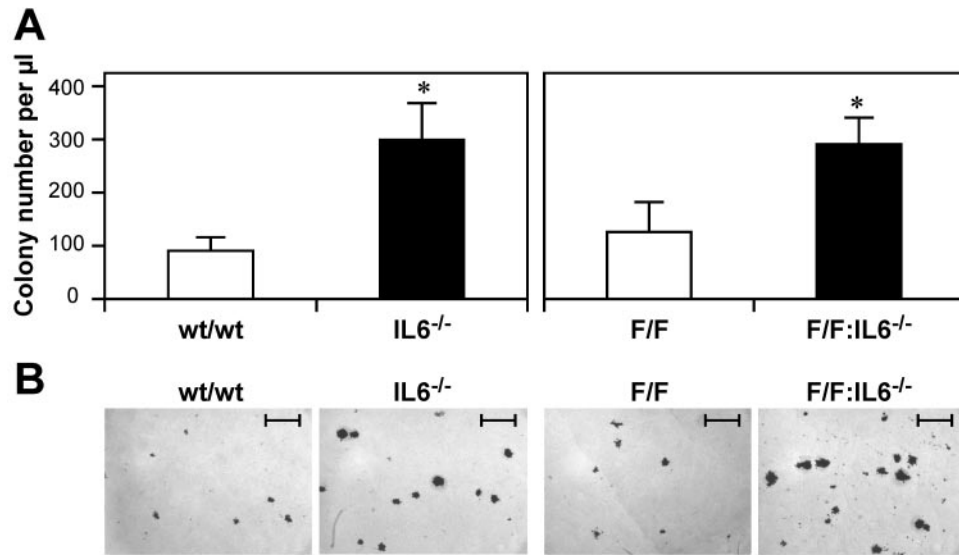


FIG. 2. Analysis of macrophage progenitors in the peripheral blood of wild-type (wt/wt) and gp130^{Y757F/Y757F} (F/F) mice on an IL-6-deficient background (IL-6^{-/-}). (A) In vitro macrophage colony formation of peripheral blood in semisolid agar containing M-CSF (10 ng/ml). Data are presented as the mean \pm SD from the results of 1 of at least 3 representative experiments. For each genotype, n was 4. *, $P < 0.05$ for IL-6^{-/-} and gp130^{Y757F/Y757F}:IL-6^{-/-} mice versus data from wild-type and gp130^{Y757F/Y757F} mice, respectively. (B) Photomicrograph of peripheral blood macrophage colonies from wild-type, IL-6^{-/-}, gp130^{Y757F/Y757F}, and gp130^{Y757F/Y757F}:IL-6^{-/-} mice. Size bars, 100 μm .

cient (IL-6^{-/-}) mice and of gp130^{Y757F/Y757F} and compound gp130^{Y757F/Y757F}:IL-6^{-/-} mice. As shown in Fig. 2A, we observed significant increases in the concentration of peripheral blood M-CFCs from IL-6^{-/-} mice compared to the level in gp130^{wt/wt} mice and from gp130^{Y757F/Y757F}:IL-6^{-/-} mice compared to the level in gp130^{Y757F/Y757F} mice. Furthermore, the size of M-CSF-responsive colonies was greater in peripheral blood cultures from IL-6^{-/-} and gp130^{Y757F/Y757F}:IL-6^{-/-} mice compared to the size of the colonies in cultures from gp130^{wt/wt} and gp130^{Y757F/Y757F} mice, respectively (Fig. 2B). Collectively, these observations suggest that the inhibitory actions of IL-6 and, to a lesser extent, IL-11 on macrophage colony formation by M-CFCs were reciprocally affected by the absence of either one of the two major intracellular gp130 signaling cascades.

Inhibition of macrophage colony formation by IL-6 is intrinsic to the hematopoietic progenitors and not conditioned by the hematopoietic microenvironment. We next examined whether the inhibitory action of IL-6 on macrophage progenitor formation was an intrinsic property of macrophage progenitors or was indirectly mediated by a conditioning effect of the stromal microenvironment. Accordingly, bone marrow cells from gp130^{wt/wt}, gp130 ^{Δ Δ} , and gp130^{Y757F/Y757F} mice were injected into lethally irradiated gp130^{wt/wt} recipient mice. Three months after transplantation, flow cytometric analysis of peripheral blood leukocytes in recipient mice indicated that mice had been reconstituted with between 77.9 and 97.3% donor cells (data not shown). Semisolid agar assays of peripheral blood M-CFCs from mice reconstituted with gp130^{wt/wt} and gp130^{Y757F/Y757F} bone marrow revealed that in the presence of IL-6, M-CSF-stimulated macrophage colony formation was inhibited by 88 and 100%, respectively (Fig. 3). In contrast, no inhibition by IL-6 on macrophage colony formation was observed for peripheral blood M-CFCs

from mice reconstituted with gp130 ^{Δ Δ} bone marrow. These results are consistent with the relative sensitivities of gp130 ^{Δ Δ} and gp130^{Y757F/Y757F} peripheral blood macrophage progenitors to IL-6 (Fig. 1B) and therefore support the notion that the inhibitory action of IL-6 is directly on the macrophage progenitors.

Altered response of gp130 mutant M-CFCs to M-CSF. The increase in macrophage colony size in cultures of M-CFCs

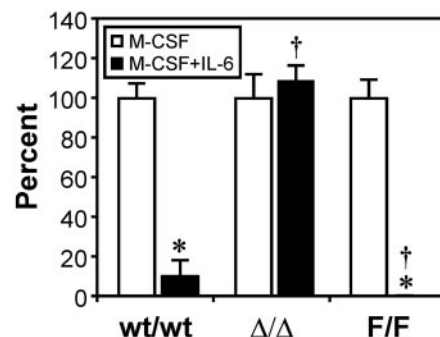


FIG. 3. Effects of IL-6 on macrophage progenitors in mice reconstituted with gp130 ^{Δ Δ} and gp130^{Y757F/Y757F} bone marrow. Wild-type recipient mice were reconstituted with bone marrow cells from gp130 ^{Δ Δ} ($\Delta\Delta$) mice, gp130^{Y757F/Y757F} (F/F) mice, and wild-type (wt/wt) littermates as described in Materials and Methods. In vitro macrophage colony formation in the presence of M-CSF (10 ng/ml) with or without IL-6 (100 ng/ml) was assessed on peripheral blood from recipient mice 3 months posttransplantation. For each group of recipient mice, n was 4. Data are presented as a percentage (\pm SD) of maximal stimulation achieved with 10 ng of M-CSF/ml alone. *, $P < 0.05$ versus data from stimulation with M-CSF alone within the same recipient group; †, $P < 0.05$ versus data from recipient mice reconstituted with wild-type bone marrow cells and stimulated with M-CSF and IL-6.

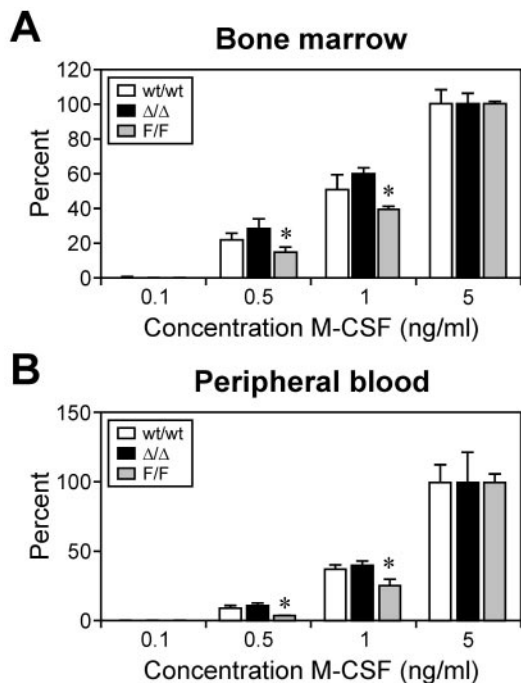


FIG. 4. Dose response of macrophage progenitor formation in $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ and $gp130^{Y757F/Y757F}$ mice. Cells from bone marrow (A) and peripheral blood (B) of $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ (Δ/Δ) mice, $gp130^{Y757F/Y757F}$ (F/F) mice, and wild-type (wt/wt) littermates were plated in semisolid agar containing various concentrations of M-CSF (0.1 to 5 ng/ml). Data are presented as a percentage (\pm SD) of maximal stimulation achieved with 5 ng of M-CSF/ml alone. *, $P < 0.05$ versus data from wild-type littermates at the corresponding concentration of M-CSF.

from mice lacking IL-6 (Fig. 2) suggested that the in vitro proliferative potential (in response to M-CSF) of M-CFCs was suppressed, at least in part, by basal levels of IL-6-driven gp130 signaling. Therefore, we next assessed the sensitivity of gp130-mutant M-CFCs to M-CSF in vitro, in which maximal colony formation was induced at 5 ng of M-CSF/ml. At lower concentrations of M-CSF (≤ 1 ng/ml), the size and number of macrophage colonies formed from $gp130^{Y757F/Y757F}$ bone marrow and peripheral blood M-CFCs were significantly reduced compared to those of colonies formed from $gp130^{\text{wt/wt}}$ and $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ bone marrow and peripheral blood M-CFCs (Fig. 4 and data not shown). Furthermore, in the presence of M-CSF alone, the proliferation of $gp130^{Y757F/Y757F}$ bone marrow macrophages was reduced compared to proliferation of $gp130^{\text{wt/wt}}$ macrophages, as measured by [^3H]thymidine incorporation (Fig. 5A). In contrast, the proliferation of $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ macrophages was greater. Consistent with the responsiveness of bone marrow M-CFCs to IL-6 (Fig. 1), the presence of IL-6 in M-CSF-containing cultures further inhibited the proliferation of $gp130^{Y757F/Y757F}$ macrophages but augmented the proliferation of $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ macrophages (Fig. 5A). Notably, cell viability, as measured by MTT assays (Fig. 5B), in M-CSF-containing cultures with or without IL-6 was comparable to the proliferation of the corresponding BMM cultures (Fig. 5A). Since viable cell number is the sum of proliferation and survival, we therefore reasoned that the opposing viability of

$gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ (increased) and $gp130^{Y757F/Y757F}$ (reduced) BMMs, with respect to $gp130^{\text{wt/wt}}$ BMMs, was due to differences in cell proliferation and not survival. Indeed, the phosphorylation and kinase activity levels of the serine and threonine kinase Akt, which plays a critical role in the survival of macrophages (24), among $gp130^{\text{wt/wt}}$ and $gp130$ mutant BMMs stimulated with either IL-6 or M-CSF were comparable (data not shown), suggesting that Akt-mediated survival signaling in macrophages was unaffected by the $gp130^{Y757F}$ and $gp130^{\Delta\text{STAT}}$ mutations.

The M-CSF-induced macrophage differentiation program is characterized by a progressive decrease in proliferative capacity accompanied by morphological changes. Thus, we reasoned that the altered growth rates of $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ and $gp130^{Y757F/Y757F}$ BMMs in M-CSF might reflect different stages of macrophage differentiation compared to growth in $gp130^{\text{wt/wt}}$ BMMs. We therefore performed flow cytometric characterization of the BMM populations in the presence of M-CSF to assess their morphology by cell size (forward scatter) and granularity (side scatter), both of which increase during macrophage differentiation. Macrophages from $gp130^{Y757F/Y757F}$ cultures were smaller and less granular than those from $gp130^{\text{wt/wt}}$ and $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ cultures (Fig. 6A). The frequency of $gp130^{Y757F/Y757F}$ macrophages expressing the mature macrophage surface marker F4/80 was significantly lower than that of $gp130^{\text{wt/wt}}$ and $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ macrophages. In contrast, levels of expres-

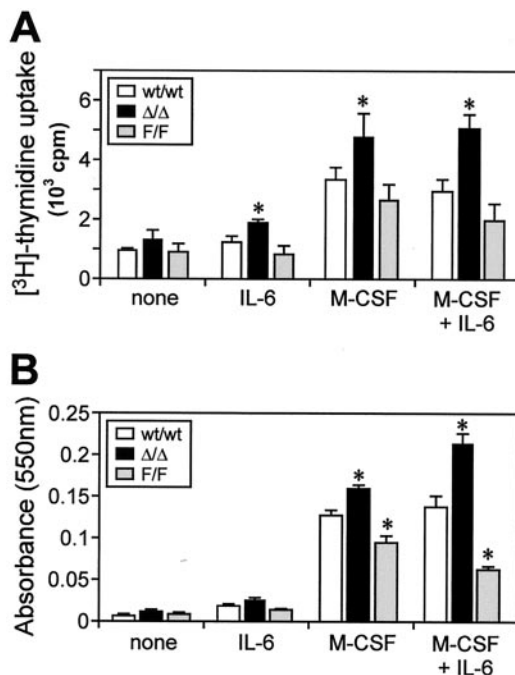


FIG. 5. Altered proliferation of gp130 mutant macrophages. BMMs from $gp130^{\Delta\text{STAT}/\Delta\text{STAT}}$ (Δ/Δ) mice, $gp130^{Y757F/Y757F}$ (F/F) mice, and wild-type (wt/wt) littermates were cultured in media containing either IL-6 (100 ng/ml), M-CSF (10 ng/ml), or both for 2 to 4 days, following which proliferation was measured by [^3H]thymidine incorporation assays (A), and cell viability was measured by MTT assays (B). Data are expressed as the mean \pm SD for each triplicate from the results of 1 of at least 3 representative experiments. *, $P < 0.05$ versus data from wild-type BMMs grown under the same conditions.

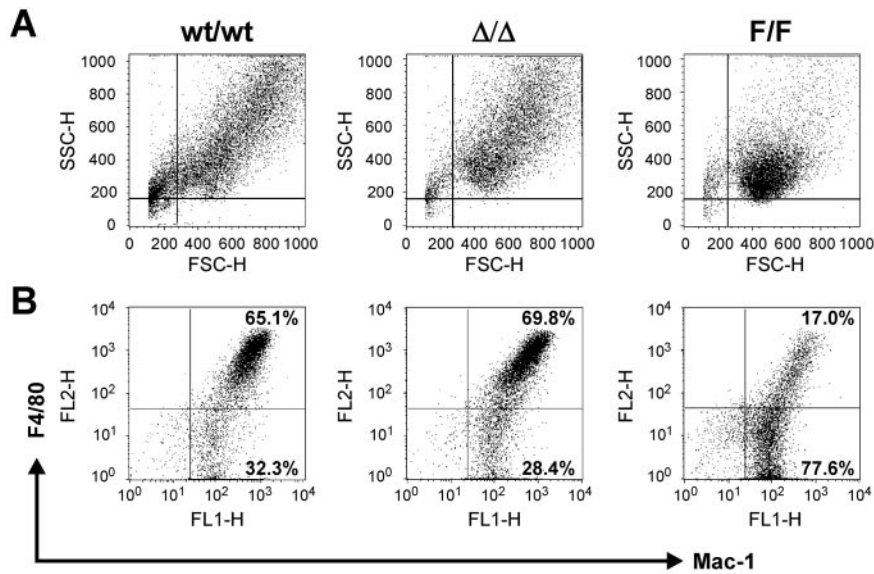


FIG. 6. Phenotypic analysis of gp130 mutant macrophages. (A and B) BMMs from gp130 ^{Δ STAT/ Δ STAT} (Δ/Δ) mice, gp130^{Y757F/Y757F} (F/F) mice, and wild-type (wt/wt) littermates were cultured in 20% LC medium, and cells were subjected to dual-color flow cytometric analysis. In panel A, the x axis represents forward scatter (cellular size) and the y axis represents side scatter (granularity). Macrophage populations are presented in the upper right quadrant. In panel B, the x axis represents FITC intensity (Mac-1), and the y axis represents PE intensity (F4/80).

sion of Mac-1, a myeloid cell-specific integrin, were similar among the three genotypes (Fig. 6B). Collectively, the above observations suggest that the absence of gp130-dependent SHP2/ERK MAP kinase activation in gp130^{Y757F/Y757F} BMMs leads to a reduction or delay in their M-CSF-induced proliferation and differentiation, whereas the absence of gp130-dependent STAT signaling in gp130 ^{Δ STAT/ Δ STAT} BMMs appears to augment their responsiveness to M-CSF.

Reciprocal negative regulation between gp130-dependent STAT3 and ERK MAP kinase signaling pathways correlates with the M-CSF-dependent growth responsiveness of gp130 mutant BMMs. One of the unexpected findings reported here was the failure of IL-6 to inhibit gp130 ^{Δ STAT/ Δ STAT} M-CSF-dependent macrophage colony formation and BMM proliferation, which was in contrast to the exaggerated IL-6-mediated inhibition observed on gp130^{Y757F/Y757F} macrophage colony formation and BMM proliferation (Fig. 1 and 5). These observations are reminiscent of the opposing capacities of gp130 mutant mice unable to elicit either gp130-dependent STAT3 or SHP2/ERK MAP kinase activation to mount an intestinal wound-healing response (38) or to balance the Th1 versus Th2 immune response (31). In both reports, the absence of either STAT3 binding or SHP2 binding to gp130 resulted in the enhanced IL-6-induced activation of the ERK MAP kinase and STAT3 pathways, respectively. To determine whether a similar reciprocal negative regulatory mechanism also occurred between the STAT3 and ERK MAP kinase pathways in gp130 mutant macrophages, we compared the levels of tyrosine phosphorylated ERK1/2 MAP kinase in gp130 ^{Δ STAT/ Δ STAT} BMMs and STAT3 in gp130^{Y757F/Y757F} BMMs to that in gp130^{wt/wt} cells. Indeed, as shown in Fig. 7, ERK1/2 tyrosine phosphorylation in gp130 ^{Δ STAT/ Δ STAT} BMMs was enhanced upon IL-6 stimulation compared to the level in gp130^{wt/wt} BMMs. Conversely, IL-6-induced STAT3 tyrosine phosphorylation was enhanced in gp130^{Y757F/Y757F} BMMs

compared to the level in gp130^{wt/wt} BMMs. As expected, no IL-6-induced STAT3 tyrosine phosphorylation and ERK1/2 tyrosine phosphorylation was detected in gp130 ^{Δ STAT/ Δ STAT} and gp130^{Y757F/Y757F} BMMs, respectively. Therefore, the reciprocal M-CSF responsiveness of gp130 mutant BMMs relative to gp130^{wt/wt} BMMs (i.e., reduced in gp130^{Y757F/Y757F} cells and enhanced in gp130 ^{Δ STAT/ Δ STAT} cells) correlates directly with the extent of ERK MAP kinase signaling and inversely with the degree of STAT3 activation. Together with previous observations in the liver and intestine of gp130 mutant mice (38), these data suggest that the reciprocal negative regulation between these two pathways may be of general

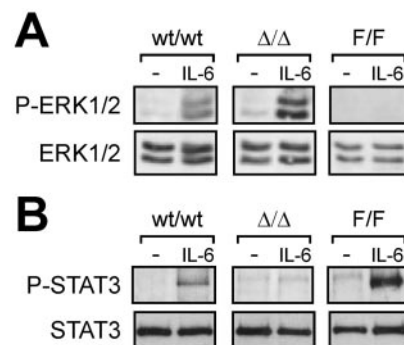


FIG. 7. Reciprocal negative regulation between gp130-dependent STAT3 and ERK MAP kinase activation in gp130 mutant macrophages. BMMs from gp130 ^{Δ STAT/ Δ STAT} (Δ/Δ) mice, gp130^{Y757F/Y757F} (F/F) mice, and wild-type (wt/wt) littermates were starved (-) and then stimulated with IL-6 for 5 min. Cell lysates were run out on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and then immunoblotted with either anti-phospho-ERK1/2 (A) and anti-phospho-STAT3 (B) antibodies (upper panels) or anti-ERK1/2 (A) and anti-STAT3 (B) antibodies (lower panels), as indicated.

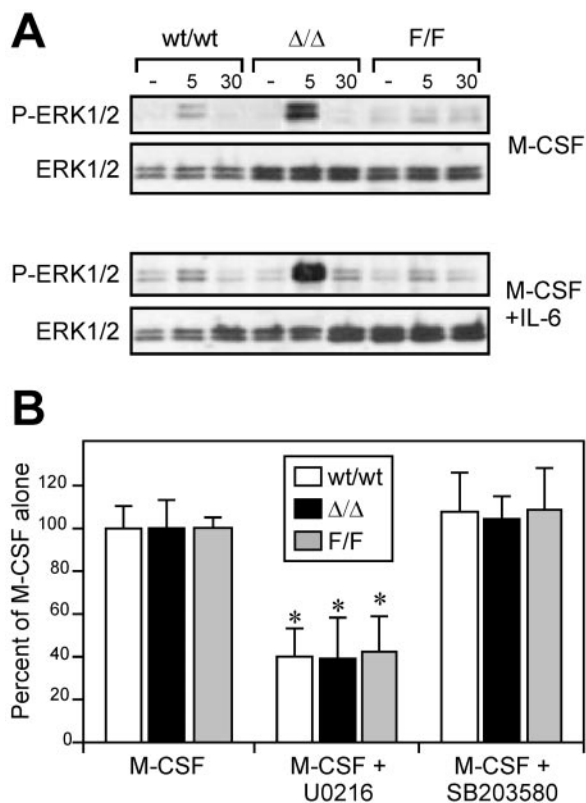


FIG. 8. Altered M-CSF-dependent ERK MAP kinase activation in gp130 mutant macrophages. (A) BMMs from gp130^{ΔSTAT/ΔSTAT} (Δ/Δ) mice, gp130^{Y757F/Y757F} (F/F) mice, and wild-type (wt/wt) littermates were starved (-) and then stimulated with M-CSF or M-CSF plus IL-6 for the times (minutes) indicated. Cell lysates were immunoblotted with either an anti-phospho-ERK1/2 antibody or an anti-ERK1/2 antibody, as indicated. (B) BMMs were grown in M-CSF (10 ng/ml) in the presence of the ERK1/2 inhibitor U0216 or the p38 MAP kinase inhibitor SB203580 for 2 days, following which proliferation was measured by a [³H]thymidine incorporation assay. Proliferation is presented as a percentage ± SD of maximal stimulation achieved with M-CSF alone. *, P < 0.05 versus data from BMMs within the same genotype grown in M-CSF alone.

importance for the biological response of cells to gp130-dependent intracellular signals.

Altered M-CSF-dependent responsiveness of gp130 mutant BMMs correlates with differential regulation of *c-fms* expression. Activation of the Ras/MAP kinase pathway has been suggested to play an important role in M-CSF-induced mitogenesis (1, 23). Since the proliferation of gp130^{Y757F/Y757F} and gp130^{ΔSTAT/ΔSTAT} BMMs in response to M-CSF was reduced and elevated, respectively, compared to that of gp130^{wt/wt} BMMs (Fig. 5A), we examined whether alterations in gp130-dependent ERK MAP kinase signaling affected M-CSF-induced activation of ERK MAP kinase. Compared to levels in gp130^{wt/wt} BMMs, M-CSF-induced ERK1/2 tyrosine phosphorylation was enhanced in gp130^{ΔSTAT/ΔSTAT} BMMs and reduced in gp130^{Y757F/Y757F} BMMs (Fig. 8A). Consistent with a further increase in the proliferation of gp130^{ΔSTAT/ΔSTAT} macrophages by the presence of IL-6 in M-CSF-containing cultures (Fig. 5A), we detected an increase in the level of ERK1/2 tyrosine phosphorylation in gp130^{ΔSTAT/ΔSTAT} BMMs stimulated with M-CSF plus IL-6 compared to the level with

M-CSF alone (Fig. 8A). By contrast, no alteration was detected in the level of M-CSF-induced ERK1/2 tyrosine phosphorylation in gp130^{wt/wt} and gp130^{Y757F/Y757F} BMMs by the presence of IL-6. This observation may reflect the limited sensitivity of the Western blot analysis, since IL-6-induced ERK1/2 tyrosine phosphorylation was weaker in gp130^{wt/wt} and gp130^{Y757F/Y757F} BMMs than the strong phosphorylation in gp130^{ΔSTAT/ΔSTAT} BMMs (Fig. 7A). Nonetheless, the importance of ERK MAP kinase activation for M-CSF-driven proliferative signaling was confirmed in experiments assessing the proliferation of gp130^{wt/wt} and gp130 mutant BMMs in the presence of the ERK1/2 MAP kinase-specific inhibitor U0216, in which proliferation of BMMs from all three genotypes was inhibited by approximately 40% (Fig. 8B). In contrast, the addition of the p38 MAP kinase inhibitor SB203580 had no effect on M-CSF-stimulated macrophage proliferation.

We next assessed the expression levels of *c-fms* in gp130^{ΔSTAT/ΔSTAT} and gp130^{Y757F/Y757F} BMMs in order to explore whether the altered M-CSF-induced ERK1/2 MAP kinase activation in gp130 mutant macrophages was due to corresponding alterations in *c-fms* expression (Fig. 9). Western blot analysis revealed a higher abundance of Fms protein in gp130^{ΔSTAT/ΔSTAT} BMMs than in gp130^{wt/wt} BMMs grown in medium containing M-CSF (Fig. 9A, LC). Furthermore, the presence of IL-6 in culture medium led to a marked increase in the amount of Fms protein in gp130^{ΔSTAT/ΔSTAT} BMMs but not in gp130^{wt/wt} BMMs (Fig. 9A, compare panels LC and LC plus IL-6). Fms expression in gp130^{Y757F/Y757F} BMMs grown

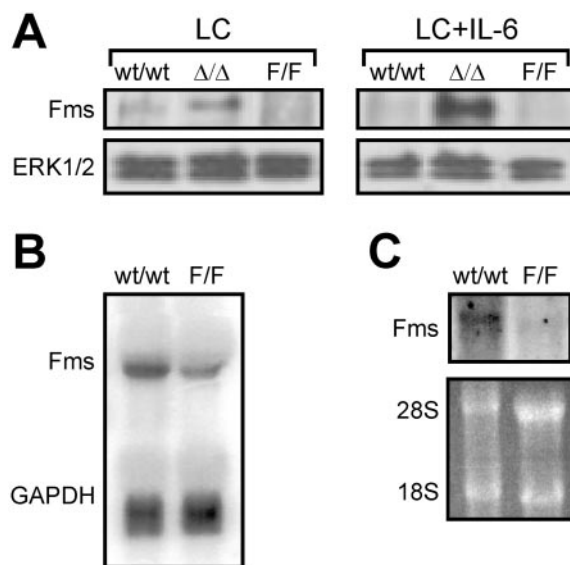


FIG. 9. Altered expression of *c-fms* in gp130 mutant macrophages. (A) BMMs were grown in 20% LC media in the absence or presence of IL-6 (100 ng/ml) as indicated. Cell lysates were run out on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and then immunoblotted with an anti-Fms antibody (top panels) or anti-ERK1/2 antibody (bottom panels). (B) Ten micrograms of total RNA from BMMs grown continuously in 20% LC media was hybridized with a radiolabeled full-length *c-fms* cDNA fragment. The membrane was stripped and re-probed for expression of the *GAPDH* gene. (C) Twenty micrograms of total RNA from unfractionated bone marrow was hybridized with the *c-fms* cDNA probe. Ethidium bromide staining revealed similar amounts of RNA analyzed.

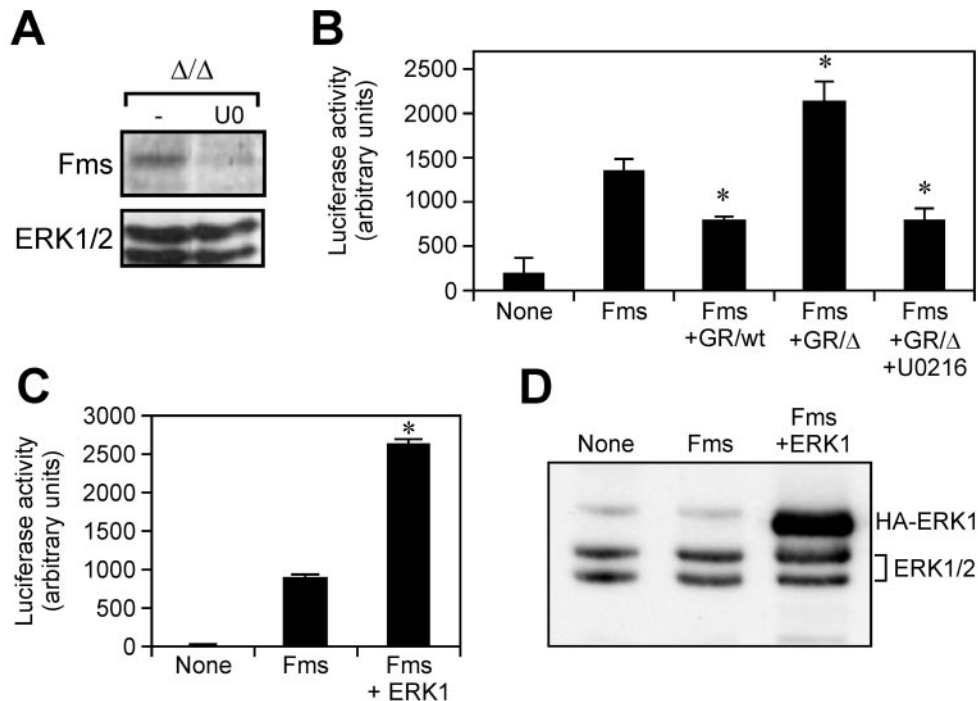


FIG. 10. Regulation of the *c-fms* promoter by gp130-dependent ERK1/2 MAP kinase activation. (A) BMMs were grown in 20% LC media in the absence or presence of the ERK1/2 inhibitor U0216 for 1 h, and cell lysates were subjected to Western blot analysis as described in the legend of Fig. 9A. (B and C) Transcriptional activation of the *c-fms*-luciferase reporter construct upon transient cotransfection with the G-CSFR/gp130^{wt} (GR/wt) or G-CSFR/gp130 ^{Δ STAT} (GR/ Δ) chimeric receptor constructs (B) and HA-tagged wild-type ERK1 construct (C) in RAW cells. In panel B, transfected cells were incubated with G-CSF (100 ng/ml) in the absence or presence of the ERK1/2 MAP kinase inhibitor U0216 for 48 h and assayed for luciferase activity. The data presented were corrected for transfection efficiency by reference to the activity of *Renilla* luciferase driven by the thymidine kinase promoter in a cotransfected plasmid. *, $P < 0.05$ versus basal *c-fms*-luciferase activity in cells transfected with the *c-fms*-luciferase reporter construct alone (Fms). (D) Cell lysates from transfected RAW cells were run out on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and then immunoblotted with an anti-ERK1/2 antibody; overexpressed, HA-tagged ERK1 (HA-ERK1; upper band) runs above the endogenous ERK1/2 (middle and lower bands).

in medium containing M-CSF with or without IL-6 was below the level of detection by Western blot analysis (Fig. 9A). However, Northern blot analysis indicated that *c-fms* was expressed, albeit at a lower level, in gp130^{Y757F/Y757F} BMMs compared to gp130^{wt/wt} BMMs (Fig. 9B). Similarly, the amount of *c-fms* RNA transcript in the bone marrow compartment of gp130^{Y757F/Y757F} mice was also severalfold lower than in bone marrow from gp130^{wt/wt} mice (Fig. 9C), confirming that the reduced expression of *c-fms* in cultured gp130^{Y757F/Y757F} BMM populations was not the result of their ex vivo expansion. Thus, the data presented in Fig. 8 and 9 correlate with alterations in the M-CSF-dependent proliferative response of gp130 mutant macrophages with the level of ERK MAP kinase activation, which in turn corresponds with the differential expression of *c-fms*. In addition, our observations further support the notion that ERK1/2 are the primary MAP kinases promoting M-CSF-dependent mitogenesis.

The *c-fms* gene is a direct target of the gp130-dependent SHP2/ERK MAP kinase pathway. The reciprocal negative regulation among the two pathways emanating from gp130 provides a unifying explanation for the opposing biological effects of IL-6 on gp130 ^{Δ STAT/ Δ STAT} and gp130^{Y757F/Y757F} BMMs. Our observation in Fig. 9A that the presence of IL-6 in gp130 ^{Δ STAT/ Δ STAT} BMM cultures increased Fms expression suggested that the altered capacity of IL-6 to modulate the

M-CSF responsiveness of gp130 mutant macrophages could correlate directly with the extent of gp130-dependent ERK MAP kinase activation, since gp130-dependent STAT activation is abolished in gp130 ^{Δ STAT/ Δ STAT} mice. In support of this notion, addition of the ERK MAP kinase inhibitor U0216 to cultured gp130 ^{Δ STAT/ Δ STAT} BMMs led to a severalfold reduction in the level of Fms protein (Fig. 10A), which suggests that the relative overexpression of Fms in gp130 ^{Δ STAT/ Δ STAT} BMMs compared to gp130^{wt/wt} BMMs was due to enhanced activation of the ERK MAP kinase pathway.

In order to test directly whether gp130-dependent ERK1/2 activation enhanced *c-fms* promoter activity, we assayed the transcriptional activation of a *c-fms* promoter-luciferase reporter construct (14) following its transient transfection in RAW macrophage cells, which also expressed either a chimeric G-CSFR/gp130^{wt} or G-CSFR/gp130 ^{Δ STAT} receptor construct (7). In unstimulated RAW cells, the 3.5-kb proximal *c-fms* promoter showed a high level of basal activity (Fig. 10B), which was consistent with previous findings (14). However, *c-fms*-luciferase activity was reduced by 40% upon ligand-induced activation of the G-CSFR/gp130^{wt} receptor. In contrast, *c-fms* reporter activity was elevated by 55% upon activation of the G-CSFR/gp130 ^{Δ STAT} receptor. Moreover, the presence of the ERK1/2 MAP kinase inhibitor U0216 resulted in a threefold reduction in promoter activity upon G-CSFR/gp130 ^{Δ STAT} re-

ceptor activation. A role for ERK MAP kinase in the transcriptional activation of the *c-fms* promoter was further demonstrated upon transient cotransfection of cells with constructs for the *c-fms* promoter-luciferase reporter and wild-type ERK1, overexpression of which has been shown to enhance gene expression (32). As shown in Fig. 10C and 10D, overexpression of wild-type ERK1 increased *c-fms* reporter activity by 2.5-fold relative to that in cells transfected with the *c-fms* promoter-luciferase reporter alone. Therefore, we propose that the increased M-CSF responsiveness of BMMs following activation of the gp130^{ΔSTAT} receptor is attributable to enhanced ERK MAP kinase activation which is the direct consequence of the incapacity of the gp130^{ΔSTAT} receptor to activate the STAT pathway. Moreover, our data presented here identify a unique mechanism whereby the differential regulation of the *c-fms* gene is dependent upon the level of gp130-dependent ERK1/2 MAP kinase activation.

DISCUSSION

We report here the use of mice deficient in gp130-dependent activation of either STAT1/3 (gp130^{ΔSTAT/ΔSTAT}) or SHP2/ERK MAP kinase (gp130^{Y757F/Y757F}) to demonstrate that the capacity of IL-6 family cytokines to inhibit M-CSF-dependent proliferation and differentiation of hematopoietic cells is inversely related to their ability to activate gp130-dependent ERK MAP kinase signaling. Hence, IL-6 and, to a lesser extent, IL-11 exhibited greater inhibition of macrophage colony formation (in response to M-CSF) from peripheral blood and bone marrow M-CFCs from gp130^{Y757F/Y757F} mice than from gp130^{wt/wt} mice. By contrast, in gp130^{ΔSTAT/ΔSTAT} mice, where gp130-dependent ERK MAP kinase activation was enhanced, IL-6 and IL-11 failed to inhibit macrophage colony formation by M-CFCs but, rather, augmented colony formation. Similarly, the presence of IL-6 in liquid cultures enhanced the proliferation of gp130^{ΔSTAT/ΔSTAT} BMMs in response to M-CSF, whereas M-CSF-stimulated proliferation of gp130^{Y757F/Y757F} BMMs was reduced. In all cases, the inhibition by IL-11 was less pronounced than inhibition by IL-6, an observation which is consistent with that made by Clutterbuck et al. (4), and may reflect reduced surface expression of IL-11 receptor α (IL-11R α) compared to expression of IL-6R α on macrophage progenitor cells. We also note that peripheral blood M-CFCs from gp130^{wt/wt} and gp130^{Y757F/Y757F} mice were more sensitive than bone marrow M-CFCs to IL-6-induced inhibition of macrophage colony formation. The inhibitory effect of IL-6 has been previously shown to be inversely proportional to the proliferative capacity of the macrophage progenitor, in which case peripheral blood progenitors produce smaller colonies (i.e., have lower proliferative potential) than bone marrow progenitors and are more sensitive to IL-6 inhibition (4). Although the molecular explanation for this differential IL-6 sensitivity is unknown, it most likely reflects a reprogramming of the transcriptional machinery within a progenitor cell during differentiation or maturation, which is associated with a reduced proliferative capacity, to a growth inhibitory response by IL-6.

In gp130^{ΔSTAT/ΔSTAT} and gp130^{Y757F/Y757F} BMMs, we found that functional elimination of one signaling pathway resulted in enhanced activation of the remaining pathway in

response to IL-6. This observation is consistent with the emerging theme that the gp130-dependent SHP2/ERK MAP kinase and STAT1/3 pathways negatively regulate each other (31, 38). We have previously shown that the absence of gp130-dependent STAT1/3 signaling causes up-regulation of IL-6-induced SHP2 and ERK1/2 tyrosine phosphorylation due to impaired STAT-mediated induction of SOCS1 and SOCS3, two key negative regulators of gp130 signaling (6). Conversely, the gp130^{Y757F} substitution mutation removes the phosphotyrosine residue that simultaneously provides a binding site for SHP2 and SOCS3, and therefore disrupts the capacity of SOCS3 to negatively regulate gp130-dependent signaling (35) and of SHP2 to dephosphorylate critical tyrosine residues in the gp130-JAK signaling complex (18). Therefore, the collective loss of these negative feedback mechanisms may augment JAK activity and subsequent STAT3 activation in gp130^{Y757F/Y757F} mice. Importantly, this reciprocal negative regulation between the STAT and ERK MAP kinase pathways highlights how the overall balance of positive and negative signaling cascades can mediate specific biological responses to IL-6 family cytokines. Indeed, recent examples include the findings that mucosal wound healing (38) and the Th1 versus Th2 immune response (31) are influenced by the balance of gp130-dependent SHP2/ERK MAP kinase and STAT3 activation.

The other major finding uncovered by our study presented here is the regulation of *c-fms* gene expression by IL-6 family cytokines via the gp130-dependent ERK MAP kinase pathway. The *c-fms* promoter contains several conserved Ets/AP-1 growth factor response elements (15), and the synergistic actions of the Ras pathway and Ets family transcription factors to induce expression of M-CSF-responsive genes have been demonstrated (12, 41). Furthermore, activation of the Ras/ERK MAP kinase pathway is necessary for both Ets-2 gene expression and protein activation (8), which in turn positively regulates Ras-dependent proliferation in response to M-CSF (20, 21). The Ras pathway has been shown to be a signaling component downstream of Fms that is necessary for M-CSF-driven mitogenesis (2, 21), which is also supported by our observation here that the ERK1/2 MAP kinase-specific inhibitor U0216 reduced M-CSF-dependent proliferation of BMMs. However, here we provide compelling evidence for a situation where the Ras-MAP kinase pathway (activated via gp130) can also be functionally upstream of Fms, thereby providing a link by which IL-6 family cytokines suppress the responsiveness of macrophages to M-CSF. Thus, we speculate that a reduction in gp130-dependent ERK1/2 MAP kinase signaling, as is the case for gp130^{Y757F/Y757F} macrophages, might suppress the activity of Ets family members, and therefore the level of *c-fms* expression, which in turn leads to a reduced biological response to M-CSF-induced signaling. Detailed analysis of the ability of gp130-dependent ERK MAP kinase signals to induce the expression of downstream effectors of M-CSF-stimulated proliferative signaling, such as Ets-2 and AP-1, and induce *c-fms* promoter activity in the absence of specific (i.e., Ets/AP-1) binding sites, will ultimately test the validity of this model.

Several recent studies have highlighted the importance of STAT3 signaling during maturation and activation of macrophages. A study by Takeda et al. (37) revealed that IL-6 not only failed to inhibit the M-CSF-driven proliferation of

STAT3-deficient BMMs but, rather, enhanced their proliferation. Notably, this observation correlates with our data presented here for gp130^{ΔSTAT/ΔSTAT} macrophages and is therefore consistent with our interpretation that exaggerated gp130-dependent ERK MAP kinase activation, as a consequence of an absence in STAT3 signaling, enhances these macrophage activities. In contrast, it has also been reported that constitutive activation of STAT3 in macrophages inhibited their proliferation (30). While these reports provide compelling evidence for a STAT3-specific role in mediating antiproliferative effects on macrophages, they provide little evidence as to how altering the activation status of STAT3 elicits these actions. In light of our observations here, we propose that altering the level of STAT3 activation, for example by gene-specific inhibition or expression of a constitutively active STAT3, may affect target gene regulation indirectly by unbalancing the net output of complex signaling networks. Specifically, we identify here that, at least in the context of gp130 activation, the signaling output from the ERK MAP kinase cascade is inversely related to the extent of STAT3 activation.

The inhibition of M-CSF-stimulated macrophage formation by IL-6 family cytokines contrasts with the synergistic actions of this cytokine family with other hematopoietic growth factors, such as stem cell factor and IL-3, on promoting the proliferation and differentiation of multipotential (immature) hematopoietic progenitors (16, 39). Furthermore, IL-6 promotes the formation of macrophages in cultures containing granulocyte-M-CSF (17, 27) and has also been shown to enhance the expansion of primitive macrophage-committed (pre-CFU-M) progenitors (26). These observations suggest differences in the transcriptional programming of immature and committed (i.e., CFU-M) hematopoietic progenitors to respond to gp130 signaling, with the latter primed for inhibition by IL-6 family cytokines. Indeed, such a notion is supported by recent observations by Clutterbuck et al. (4) on the preferential inhibitory action of IL-6 on macrophage progenitors displaying a reduced proliferative capacity, which itself is a hallmark of committed cells undergoing differentiation and maturation. Although a molecular mechanism underlying these opposing effects remains to be elucidated, in both situations it is likely that the effect of IL-6 is via the regulation of *c-fms* expression (3). In this regard, it is therefore not surprising that the human and mouse *c-fms* promoter regions contain binding sites for transcription factors that mediate opposing effects on the transcriptional regulation of *c-fms* (33). With the recent generation of mice expressing a *c-fms*-enhanced green fluorescent protein reporter gene within the monocyte/macrophage lineage throughout development (34), the tools are now in place for further in vivo analyses of the transcriptional machinery controlling both negative and positive regulation of *c-fms* during macrophage development.

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