G-CSF Promotes Neuroblastoma Tumorigenicity and Metastasis via STAT3-Dependent Cancer Stem Cell Activation

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Abstract

Increasing evidence suggests that inflammatory cytokines play a critical role in tumor initiation and progression. A cancer stem cell (CSC)-like subpopulation in neuroblastoma is known to be marked by expression of the G-CSF receptor (G-CSFR). Here, we report on the mechanistic contributions of the G-CSF receptor in neuroblastoma CSCs. Specifically, we demonstrate that the receptor ligand G-CSF selectively activates STAT3 within neuroblastoma CSC subpopulations, promoting their expansion in vitro and in vivo. Exogenous G-CSF enhances tumor growth and metastasis in human xenograft and murine neuroblastoma tumor models. In response to G-CSF, STAT3 acts in a feed-forward loop to transcriptionally activate the G-CSFR and sustain neuroblastoma CSCs. Blockade of this G-CSF–STAT3 signaling loop with either anti-G-CSF antibody or STAT3 inhibitor depleted the CSC subpopulation within tumors, driving correlated tumor growth inhibition, decreased metastasis, and increased chemosensitivity. Taken together, our results define G-CSF as a CSC-activating factor in neuroblastoma, suggest a comprehensive reevaluation of the clinical use of G-CSF in these patients to support white blood cell counts, and suggest that direct targeting of the G-CSF–STAT3 signaling represents a novel therapeutic approach for neuroblastoma. Cancer Res; 75(12); 1–14. ©2015 AACR.

Introduction

Neuroblastoma accounts for 15% of all pediatric cancer-related mortality and remains a major clinical challenge. Curative therapy requires intensive chemotherapy, radiation, surgery, and biologic therapies, yet yields less than 50% success rate and imparts severe long-term side effects (1, 2). Neuroblastoma arises from the early embryonic neural crest, as this transient ectodermal population matures and undergoes a programmed ectoderm-to-mesodermal transition (EMT) to obtain their migratory mesenchymal-like phenotype (3). This process is, in part, controlled by STAT3 activation downstream of fibroblast growth factors (FGF), BMPs, and other neural crest specification factors (4, 5). Pro-oncogenic targets of STAT3 include genes involved in metastasis (MMP-2, MMP-9, vimentin), angiogenesis (VEGF-A), and inflammation (IL10, TGβ, COX-2; ref. 6). The proinflammatory cytokines IL6 and G-CSF activate STAT3 through phosphorylation via their respective cognate surface receptors. G-CSF is known to promote neuronal stem cell survival (7, 8). Accumulating evidence of similar downstream activation of STAT3 links inflammatory signals to tumor progression and spread (9, 10).

We previously defined the “stemness” of a neuroblastoma subpopulation isolated from primary tissues, xenografts, and cell lines, based on expression of the G-CSF receptor (G-CSFR; CD114; ref. 11). Understanding the biological effects of downstream signaling via this receptor on neuroblastoma tumorigenesis and metastasis is a critical question with important clinical implications due to the adjuvant role that ligand G-CSF serves in limiting chemotherapy-induced neutropenia in neuroblastoma patients. We therefore sought to comprehensively analyze the effects of G-CSF on neuroblastoma cancer stem cells (CSC) and how this alters overall tumor growth and metastasis. We also hypothesized that blocking the G-CSF–STAT3 signaling pathway can reduce the neuroblastoma CSCs’ effects and inhibit neuroblastoma tumorigenicity and metastasis.

Here, we demonstrate that G-CSF specifically activates STAT3 signaling in the receptor-positive CSC-like neuroblastoma subpopulation. G-CSF–STAT3 signaling promotes neuroblastoma colony formation, proliferation, tumor formation, and metastasis in both xenograft and allograft murine models of neuroblastoma. In contrast to the bulk CD114+ tumor population, the G-CSFR-positive (CD114+) cells are remarkably sensitive to STAT3...
inhibition. Blocking the G-CSF–STAT3 signaling axis either by anti-G-CSF antibody or by STAT3 inhibition reverses the protumorigenic effects of G-CSF and leads to tumor growth inhibition, decreased metastasis, and increased chemosensitivity. These effects strongly correlate with changes in the percentage of CD114 fluorescence

Our findings demonstrate that G-CSF acts as a CSC-specific growth factor for high-risk neuroblastoma. On the basis of these data, we propose a new therapeutic paradigm incorporating anti-G-CSF–STAT3 targeting for neuroblastoma CSCs combined with standard cytotoxic therapy to block neuroblastoma tumor growth, spread, and relapse. Our data also strongly advocate a reevaluation of the clinical utility of G-CSF as growth factor for neutrophil support in neuroblastoma patients.

Materials and Methods

Cell culture and reagents

Human neuroblastoma cell lines (NGP, SH-SY5Y, IMR-32, and CHLA-235) and mouse neuroblastoma cell line, NB975, were routinely maintained and cultured as described previously (11). For the in vitro assays performed in this study, cells were cultured routinely and then partially starved for at least 16 to 24 hours by culturing in medium supplemented with 1% FBS unless otherwise stated, before treating with drugs or cytokine. Primary antibodies anti-CD114-PE (554538), anti-CD56-APC (555318), anti-CD24- FITC (553261), and fluorochrome, isotype-matching mAbs for negative controls were purchased from BD Biosciences. DAPI (4’,6-diamidino-2-phenylindole; Invitrogen; D3571) staining was used to exclude dead cells in all flow cytometry analysis. LSRII 5-laser flow cytometer (BD Biosciences) was used to perform flow cytometry experiments followed by analysis using the FlowJo v. 8.7 (TreeStar). All flow cytometry assays were performed with antibody isotype controls. Static (S7947), etoposide (VP-16, E1383) from Sigma-Aldrich, and recombinant human G-CSF (BP000175-GD16) were purchased from Syd Labs. Anti-G-CSF antibody (MAB005) and matching isotype antibody control (MAB005) were purchased from R&D Systems.

Cell line validation and sources

NGP, IMR-32, and SH-SY5Y were obtained from the ATCC. CHLA-235 and the mouse neuroblastoma cell line NB975 were obtained from Dr. Metelitsa (Baylor College of Medicine, Houston, TX). All cell lines are validated for MYCN and CD56 expression under the following conditions: incubation at 50°C for 10 minutes and 40 cycles of 15 seconds for each of the DNA polymerase reactions (13). Bone marrow from bilateral femurs of engrafted mice was harvested in cold PBS at the time of necropsy. Bone marrow was mechanically disrupted using a 21-gauge needle and red blood cells were removed using lysis buffer (BD Pharm Lyse; 555899) followed by washing with cold PBS twice. Bone marrow samples from nude mice were used as control. Total RNA was extracted using the miRNeasy Mini Kit (217004; Qiagen) and residual DNA was removed by DNase treatment (79254; Qiagen) as specified by the manufacturer. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems) and real-time quantitative PCR was performed using TaqMan assay for human MYCN (assay ID: Hs03666850_s1; Applied Biosystems). Real-time quantitative PCR was performed in MicroAmp optical 96-well reaction plates using StepOnePlus Real-Time PCR Systems (Applied Biosystems) under the following conditions: incubation at 50°C for 2 minutes and 95°C for 10 minutes and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All assays were performed in triplicates with efficient controls. Metastatic incidence was calculated by dividing number of bone marrow MYCN+ mice with total number of mice in a treatment cohort.

Mice

Four- to 6-week-old female inbred athymic immunodeficient nude mice (Nu/Nu) were purchased from Taconic Biosciences and used for all xenograft studies. Heterozygous G-CSF−/− (B6;129P2-Csf3tm1Ard/J) mice were obtained from The Jackson Laboratory and housed in pathogen-free conditions. Mice were inbred and genotyped for colony maintenance. Mice were implanted using our previously described orthotopic xenograft model of neuroblastoma (12). Briefly, 1 × 106 neuroblastoma cells were surgically implanted in the sub-renal capsule of mice and tumor growth was monitored biweekly by bioluminescent imaging (IVIS Lumina XR System; Caliper Life Sciences).

Single cell assay

Stable enhanced green fluorescent protein (EGFP)–expressing NGP and SH-SY5Y cell lines were generated by transducing EFS-EGFP lentiviral vector (provided by Dr. Michael T. Lewis, Baylor College of Medicine) and flow sorting using MoFlo cell sorter (Beckman Coulter). Stably transduced cells were cultured and sorted again for CD114− and CD114+ subpopulation using anti-CD114 antibody. Single cells were aliquoted in wells of 96-well culture plate by serially diluting sorted cells with medium. Cells were cultured in medium supplemented with 1% FBS and untreated or treated with different doses of G-CSF for 28 days with weekly replenishing of cytokine. Cells and colonies were monitored, analyzed, and imaged using Olympus IX71 inverted fluorescence microscope with DP2-BSW image acquisition software (Olympus).

Cell-cycle analysis

Cell-cycle assay was performed using the APC BrdUrd Flow Kit (BD Biosciences) according to the manufacturer’s instructions and as described previously (11). Briefly, neuroblastoma cells were pulsed with 10 μmol/L bromodeoxyuridine (BrdUrd) for 1 hour followed by surface staining for CD114. DAPI was used for total DNA staining.

Metastatic incidence estimation

Metastatic incidence was estimated using a method described previously with modifications (13). Bone marrow from bilateral femurs of engrafted mice was harvested in cold PBS at the time of necropsy. Bone marrow was mechanically disrupted using a 21-gauge needle and red blood cells were removed using lysis buffer (BD Pharm Lyse; 555899) followed by washing with cold PBS twice. Bone marrow samples from nude mice were used as control. Total RNA was extracted using the miRNeasy Mini Kit (217004; Qiagen) and residual DNA was removed by DNase treatment (79254; Qiagen) as specified by the manufacturer. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems) and real-time quantitative PCR was performed using TaqMan assay for human MYCN (assay ID: Hs03666850_s1; Applied Biosystems). Real-time quantitative PCR was performed in MicroAmp optical 96-well reaction plates using StepOnePlus Real-Time PCR Systems (Applied Biosystems) under the following conditions: incubation at 50°C for 2 minutes and 95°C for 10 minutes and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All assays were performed in triplicates with efficient controls. Metastatic incidence was calculated by dividing number of bone marrow MYCN+ mice with total number of mice in a treatment cohort.

Cell viability and soft agar assay

Cell viability assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (G3582; Promega) according to the manufacturer’s instructions. Briefly, 5,000 cells per well were plated into 96-well plate in 1% FBS supplemented medium and treated with indicated drugs for 24 hours followed by incubation with dye for 4 hours. The plates were analyzed by spectrophotometric absorbance at 490 nm using a microplate reader (DTX-800; Beckman Coulter) with Softmax Pro v6.2.2 software (Molecular Devices). The data were analyzed and IC50 were calculated using GraphPad Prism 6 (GraphPad Software).
Software). Colony formation soft agar assays were performed using standard conditions as described previously (14). Briefly, soft agar plates with bottom gel of medium supplemented with 10% FBS and 0.5% agar were set up followed by setting top gel of medium supplemented with 1% FBS and 0.3% agar. Neuroblastoma cells were plated with top agar and the plates were incubated for 15 days. Colonies were stained with thiazoyl blue tetrazolium bromide (M5655; Sigma-Aldrich) and visualized and counted using Versadoc (Bio-Rad; Quantity1 software). All assays were performed in triplicates and repeated twice with proper controls.

**Apoptosis assay**

Apoptosis in neuroblastoma cell lines was analyzed using APC Annexin V Apoptosis Detection Kit (88-8007; ebiosciences) according to the manufacturer's instructions. Briefly, cells were partially starved and treated with indicated doses of drugs for 2 hours followed by washing with cold PBS and stained with anti-CD114 antibody. Cells were then washed with cold PBS and resuspended in binding buffer followed by incubation with APC–Annexin V antibody for 15 minutes. Cells were washed and resuspended in binding buffer followed by analysis on LSRII flow cytometer. DAPI was used for analyzing dead and late apoptotic cells.

**Chromatin immunoprecipitation**

STAT3-binding sites on CSF3R 5'-untranslated region (5'-UTR) were analyzed by UCSC genome browser (www.genome.ucsc.edu) and confirmed by TransFac analysis (BioBase). Chromatin immunoprecipitation [ChIP] was performed on 0.5 × 10^6 flow-sorted CD114^+^ and CD114^-^ NGP cells using the ChIP-IT High Sensitivity Kit (Active Motif; 53040) according to the manufacturer's instructions. Samples were sonicated for 20 cycles of 30-second intervals in a Bioruptor UCD-200 sonicator (Diagenode). ChIP-grade anti-STAT3 (9132), anti-pSTAT3 (Y705; 9131; Cell Signaling Technology), and IgG control (12-370; EMD-Millipore) antibodies were used. Input was generated by purifying DNA from the sonicated lysates of each sample.

**ChIP-quantitative PCR**

Primers were designed for ChIP-quantitative PCR (ChIP-qPCR) using UCSC genome browser and Primer3 software (www.Sim- Gene.com) and are listed in Supplementary Table S3. Real-time PCR reactions were performed as described above using Power SYBR Green PCR master mix. Input and negative control IgG ChIP samples were also analyzed for each sample. The amount of genomic DNA precipitated with specific antibody was calculated in comparison with the total input DNA used for each immunoprecipitation and fold enrichment above background was calculated by normalizing against control IgG. The qPCR reactions were performed in triplicates for each sample with input and control IgG.

**Reporter assay**

The CSF3R 5'-UTR and promoter regions were amplified from genomic DNA isolated from NGP neuroblastoma cell line and cloned upstream of the EGFP gene by replacing existing promoter motifs in the lentiviral STAT3-EGFP reporter plasmid (11). The reporter lentiviral plasmids were packaged and NGP cells were transduced and further reporter assays were performed as described previously (11).

**Generation of stable STAT3 knockdown cell lines**

Lentiviral shRNA vectors pSIH1-puro-STAT3 (26596; Addgene) and pSIH1-puro-control (26597; Addgene; ref. 15) were used to transduce neuroblastoma cell lines as described previously (11). Seventy-two hours after transduction, cells were selected by media containing 1 μg/ml puromycin. Stably transduced cell lines were further verified for knockdown efficiencies by Western immunoblotting using STAT3 (4904; Cell Signaling Technology) antibody using protocol as described previously (14).

**Statistical analysis**

Data values for in vivo experiments are expressed as mean ± SEM and compared using the Mann–Whitney (two-tailed non-parametric analysis) test. The Fisher exact test was used to compare metastatic incidence between groups. The Student t test (two-tailed or one-tailed distribution with unequal variance) was applied to compare the results shown for in vitro experiments unless otherwise stated. Assays were performed in triplicates and repeated.

**Results**

G-CSF induces colony formation in CD114^+^ cells

To assess the differential responses of neuroblastoma subpopulations to G-CSF, we purified G-CSFR^+^ (CD114^+^) and receptor-negative (CD114^-^) subsets from the neuroblastoma cell lines SH-SY5Y and NGP using fluorescence activated cell sorting (FACS). Cell proliferation and colony formation from single cells were measured with and without G-CSF over 28 days. Treatment with G-CSF growth factor significantly increased the cell counts and colony counts generated from CD114^+^ subpopulation with minimal to no change in colony formation in response to G-CSF in the receptor-negative subpopulation (Fig. 1A and B). We note a difference in dose dependence between the NGP (MYCN-amplified) and SH-SY5Y (nonamplified) cell lines, possibly due to differences in feedback inhibition or cytokine receptor density (additional data in Supplementary Fig. S1). The NGP response fell off above 10 ng/mL while SH-SY5Y cells continued to respond to higher levels of G-CSF. Cell-cycle analysis with G-CSF treatment demonstrated a significant increase in S-phase population within the NGP CD114^+^ subset compared with control (Fig. 1C and D). In contrast, no significant changes in the cell-cycle phases of the CD114^-^ subpopulation were observed in response to G-CSF (Fig. 1C and D). These data correlated with increased activation of STAT3 as measured by increased pSTAT3 (Y705) levels in the CD114^+^ cells. No change in pSTAT3 was observed upon G-CSF treatment of CD114^-^ cells (Supplementary Fig. S2A). These in vitro data prompted a more detailed in vivo analysis of G-CSF on neuroblastoma tumor subpopulations, tumor growth, and metastasis.

G-CSF promotes tumorigenicity and metastasis in vivo

We next evaluated the impact of exogenous G-CSF on tumor growth in vivo. One million human neuroblastoma cells (NGP and SH-SY5Y) were xenotransplanted in athymic immunodeficient nude mice via injection under the renal capsule. This
previously well-established orthotopic model of neuroblastoma faithfully recapitulates the highly vascular and aggressive growth and metastatic patterns of human neuroblastoma (11, 12). Cohorts of mice were treated with either G-CSF or vehicle control daily for 21 days, starting the day after tumor cell implantation (Fig. 2A). For these studies, human recombinant G-CSF was administered at doses comparable to previous studies in murine tumor models, which takes into account the rapid metabolism of G-CSF in murine model systems (16, 17). Xenografts derived from the NGP cell line showed significant increase in tumor weights...
G-CSF promotes neuroblastoma tumorigenicity in vivo. A, schematic representation of experimental plan to analyze the effect of G-CSF on neuroblastoma in vivo. One million neuroblastoma cells were injected under the sub-renal capsule of mouse to develop orthotopic xenografts/allografts by using an orthotopic mouse model. Implanted mouse treated with exogenous G-CSF (i.p. injection, 250 μg/kg/d) or vehicle control (5% dextrose water) starting next day from implantation until day 21, followed by necropsy at day 28. B, NGP xenografts showing significant increase in tumor weights in response to G-CSF treatment compared with controls ($P = 0.019$), with relative increase in the percentage of CD114$^+$ cells in treatment cohorts ($P = 0.003$). Linear regression analysis showed a direct correlation between individual tumor mass and percentage of CD114$^+$ cells ($*\, control; \, &\, G$-CSF$-$treated mice). C, SH-SY5Y xenografts showed a significant increase in tumor weights in response to G-CSF treatment compared with controls ($P = 0.038$), with relative increase in the percentage of CD114$^+$ cells ($P < 0.05$). Linear regression analysis showed a direct correlation between individual tumor mass and percentage of CD114$^+$ cells ($*\, control; \, &\, G$-CSF$-$treated mice). D, CSF3$^{-/-}$ mice allografted with murine neuroblastoma cell line NB975 showed a significant increase in tumor weights in response to G-CSF treatment compared with controls ($P = 0.04$) and increased the percentage of CD114$^+$ cells ($P = 0.01$). Linear regression analysis showed a direct correlation between individual tumor mass and percentage of CD114$^+$ cells ($*\, control; \, &\, G$-CSF$-$treated mice). G-CSF treatment significantly increased the metastatic incidence ($P = 0.016$; Mann–Whitney test; $*\, P < 0.05; \, **\, P < 0.01$). E, NGP xenografts treated with anti-G-CSF antibody (i.p. injection, 100 μg/kg/d) showed a significant decrease in tumor mass in contrast to exogenous G-CSF ($P = 0.0001$) and isotype control antibody treatment ($P = 0.04$). Treatment plan for all cohorts was followed similar to as shown in A. Tumor flow cytometry analysis showed a significant decrease in total percentage of CD114$^+$ cells in anti-G-CSF antibody cohort in contrast to G-CSF ($P = 0.003$) and control antibody ($P = 0.03$) cohorts. Metastatic incidence was significantly decreased in anti-G-CSF antibody cohort in comparison with G-CSF treatment ($P = 0.04$; Mann–Whitney test; $*\, P < 0.05; \, **\, P < 0.01; \, ***\, P < 0.001$).
with G-CSF treatment. Notably G-CSF treatment also increased the percentage of CD114+ cells in xenografts by 3-fold in comparison with controls. We also observed a significant direct correlation between tumor mass and percentage of CD114+ cells within the tumors (Fig. 2B). Similar results were observed for SH-SY5Y xenografts, where G-CSF treatment substantially increased tumor weight with a corresponding increase in CD114+ percentage compared with vehicle-treated controls. Again, we found a highly significant correlation between tumor mass and CD114+ percentage (Fig. 2C). Immunohistochemical analysis also showed a significant increase in pSTAT3 (Y705) staining in tumor xenografts treated with G-CSF in comparison with controls (Supplementary Fig. S5).

We next examined the effect of G-CSF on bone marrow metastasis (a common site of neuroblastoma metastasis) by detecting human MYCN mRNA in bone marrow of xenotransplanted mice using qPCR. The MYCN oncone is a well-characterized driver of both MYCN-amplified and nonamplified neuroblastoma that is not expressed in normal adult tissues (18). Results have shown that administration of exogenous G-CSF significantly increased the incidence of bone marrow metastasis by 2-fold for the MYCN-amplified NGP xenografts in contrast to vehicle-treated controls. MYCN nonamplified SH-SY5Y xenografts, which express lower but readily detectable levels of MYCN, also showed a similar trend of increase in bone marrow metastasis in response to G-CSF (Fig. 2B and C).

G-CSF may be produced by both tumor cells and the stromal cells within the tumor microenvironment (19). To evaluate the contribution of stroma-derived G-CSF, we used G-CSF knockout mice (CSF3−/−). We engrafted a syngeneic murine neuroblastoma tumor cell line, NB975, which is derived from transgenic TH-MYC neuroblastoma tumors and is previously shown to contain a small CD114+ subpopulation analogous to human tumors (11). We found that while stromal G-CSF was not required for tumor engraftment, tumor growth and metastasis were markedly enhanced in the presence of exogenous G-CSF (Fig. 2D). Treatment with exogenous G-CSF significantly increased tumor weight with a correlated increase in percentage of G-CSFR+ positive cells within these tumors, derived from murine neuroblastoma cells in CSF3−/− mice. Here again, exogenous G-CSF significantly increased metastasis compared with controls (Fig. 2D). These data demonstrate, in both xenogeneic (immunodeficient) and syngeneic murine (immunocompetent) neuroblastoma mouse models, that exogenous G-CSF significantly promotes tumor growth and metastasis. These results are also consistent with our early data defining the increased tumorigenicity and stem cell-like features of CD114+ neuroblastoma subpopulations found in primary human biopsy specimens and in all neuroblastoma cell lines and xenografts analyzed to date (11). They also agree with increased downstream pSTAT3 levels in response to G-CSF both in vitro and in vivo (Supplementary Figs. S2 and S5).

Neuroblastoma cell lines and tumors produce elevated levels of G-CSF in comparison with respective controls, as demonstrated by ELISA assay (Supplementary Fig. S3). Other studies suggest that the tumor-derived or endogenous G-CSF also influences the tumor microenvironment and metastatic potential (19). Therefore, we evaluated the effect of direct inhibition of endogenous or tumor-derived G-CSF on neuroblastoma xenografts. Here, NGP neuroblastoma xenografts were treated with anti-G-CSF antibody or isotype-matched control antibody, starting the day after tumor cell implantation and tumor growth was compared with treatment with G-CSF in an independent experiment (Fig. 2E). Remarkably, we found significant reduction in tumor growth, metastasis, and percentage of CD114+ cells after treatment with anti-G-CSF antibody. Taken together, these in vivo data support our hypotheses that tumor-derived G-CSF is vital for neuroblastoma CSC maintenance and that exogenous G-CSF promotes the expansion of neuroblastoma CSCs, which leads to overall tumor proliferation and metastasis.

**STAT3 activation is critical for G-CSF–G-CSFR functions in neuroblastoma**

In neutrophil and myeloid lineages, STAT3 transcriptional activation is the primary consequence of G-CSFR ligation. We therefore evaluated the specificity of the G-CSF–STAT3 pathway using a small-molecule inhibitor (Stattic; Sigma), which blocks STAT3 dimerization and prevents subsequent STAT3-dependent transcriptional responses (20). Cellular proliferation of multiple neuroblastoma cell lines (i.e., NGP, SH-SY5Y, IMR-32, and CHLA-255) was markedly inhibited by the STAT3 inhibitor at low μmol/L levels (P < 0.0001; Fig. 3A; Supplementary Table S1). Colony formation in soft agar was also reduced more than 80% in all tested cell lines with similarly low doses (Fig. 3B and C). Interestingly, when apoptosis in response to STAT3 inhibition was evaluated on FACS-purified neuroblastoma subpopulations from NGP and SH-SY5Y cell lines, we observed differential sensitivity of the CD114+ and CD114− subpopulations. CD114+ cells were highly sensitive to STAT3 inhibition, whereas the CD114− cells were relatively resistant (Fig. 3D). BrdUrd-based cell-cycle analysis further validated these findings, demonstrating that STAT3 inhibition selectively increased the sub-G1/G0, G1 population (apoptotic cells) and decreased the S-phase percentage within the CD114+ subpopulation. STAT3 inhibition also blocks the cell-cycle progression within the CD114− subset at G1–S checkpoint and increases the population of cells in G1-phase (Fig. 3E and F). Intracellular staining of pSTAT3 (Y705) by phospho-flow assay in the NGP neuroblastoma cell line shows that Stat3 regulates the percentage of CD114+ cells and levels of pSTAT3 (Y705) in a dose-dependent manner either alone or combined with G-CSF or with anti-G-CSF antibody (Supplementary Fig. S2B and S2C). We also found that anti-G-CSF antibody neutralizes the effects of G-CSF (Supplementary Fig. S2C).

**STAT3 inhibition inhibits neuroblastoma tumorigenicity and sensitizes neuroblastoma to chemotherapy**

We next evaluated the hypothesis that dual treatment with a genotoxic chemotherapy agent combined with the STAT3 inhibitor would have a combined inhibitory effect on neuroblastoma tumorigenesis by targeting both tumor subpopulations as proposed for other CSC models (21, 22). Addition of the STAT3 inhibitor markedly sensitizes different neuroblastoma cell lines to etoposide (VP-16, a topoisomerase inhibitor used extensively in neuroblastoma treatment regimens) as determined by MTS proliferation assays (Fig. 4A and Supplementary Table S1). We further tested the efficacy of this combination in vivo using orthotopic xenografts of NGP and SH-SY5Y according to the treatment regimen outlined in Fig. 4B. For both cell lines, the STAT3 inhibitor alone significantly reduces tumor burden with an overall decrease in total percentage of CD114+ cells and metastatic incidence.
As expected, etoposide alone decreases the tumor burden but does not significantly reduce the CD114+ population or metastasis. In fact, genotoxic chemotherapy appears to modestly increase the relative CD114+ percentage in the xenografts. This observation is consistent with the enrichment of CD114+ subpopulations demonstrated previously in patient biopsies examined before and after intensive chemotherapy and correlates with increased pSTAT3 staining in tumors treated with etoposide (Supplementary Fig. S5; ref. 11). Combination treatment of the STAT3 inhibitor with etoposide further reduced tumor burden, and decreased metastatic incidence compared with etoposide alone or controls, and partially reversed the chemotherapy-induced increase in CD114 percentage in the tumors (Fig. 4C and D). Furthermore, immunohistochemical analysis showed an increase of cleaved caspase-3 (apoptosis marker) staining in tumor xenografts treated with Stattic and Combo in comparison with controls (Supplementary Fig. S4). These tumor responses correlated with decreased pSTAT3 (Y705) staining in tumor xenografts treated with Stattic and Combo in comparison with controls or etoposide alone (Supplementary Fig. S5). Taken together, these preclinical in vitro and in vivo data support the
STAT3 inhibition sensitizes neuroblastoma to chemotherapy. A, cell viability assay of different neuroblastoma cell lines (NGP, SH-SY5Y, IMR-32, and CHLA-255) were tested by the MTS assay in response to etoposide alone and in combination of 0.5 and 1.0 μmol/L Stattic treatment for 24 hours. Experiments were repeated twice with six replicates for each condition and are represented as mean ± SD. B, schematic representation of experimental plan to analyze the in vivo effect of STAT3 inhibitor and dual treatment strategy on neuroblastoma. An orthotopic mouse model was used to develop xenografts of neuroblastoma NGP and SH-SY5Y cell lines (implanted 1 × 10⁶ cells) and treated with either etoposide (12 mg/kg/d, i.p. injection, three times/week) and Stattic (25 mg/kg/d, i.p. injection, 5 days/week) alone or combined. Implanted mouse were treated 2 weeks after implantation for 2 weeks. Vehicle (DMSO) was used as a control in all experiments. C, NGP xenografts show significant decrease in tumor weights in response to Stattic treatment (P = 0.03), etoposide treatment, and combo treatment (P = 0.001) in comparison with controls. Combo treatment also significantly reduced tumor burden in comparison with etoposide alone (P = 0.008; Mann–Whitney test; ***, P < 0.01; ***, P < 0.001). The percentage of CD114⁺ cells determined by flow cytometry analysis on individual tumors is shown as mean ± SEM. Detection of human MYCN mRNA by qPCR in bone marrow of xenotransplanted mice showed a trend of reduction with Stattic (P = ns).

D, SH-SY5Y xenografts show significant decrease in tumor weights in response to Stattic treatment (P = 0.02), etoposide treatment, and combo treatment (P = 0.0001) in comparison with controls. Combo treatment also significantly reduced tumor burden in comparison with etoposide (P = 0.02) and Stattic alone (P = 0.0002; Mann–Whitney test; ***, P < 0.01; ***, P < 0.001). The percentage of CD114⁺ cells determined by flow cytometry analysis on individual tumors is shown as mean ± SEM. Detection of human MYCN mRNA by qPCR in bone marrow of xenotransplanted mice showed a trend of reduction of metastasis with Stattic (P = ns).
clinical testing of a dual treatment strategy of combining STAT3 inhibition (targeting the G-CSFR-positive CSC-like subpopulation) with standard cytotoxic chemotherapy (targeting the bulk tumor) for high-risk neuroblastoma.

STAT3 responsive genes are differentially regulated by G-CSF in neuroblastoma subpopulations

Transcriptional targets of STAT3 include genes and micro-RNAs controlling stemness and differentiation (6, 23, 24). STAT3 opposes p53-mediated effects on cell cycle, apoptosis, and inflammation through direct and indirect mechanisms (9, 25, 26). To evaluate the STAT3-specific molecular responses downstream of G-CSF signaling in the CD114+ subpopulation, we used a low-density qPCR array to profile JAK–STAT3 pathway genes (Fig. 5A). We found that G-CSF treatment strongly modulated 38 STAT3 pathway genes and that STAT3 inhibition reversed this effect (Fig. 5B). Subsequently, the expression pattern of key genes was independently validated by qPCR, confirming specific regulation of STAT3 targets via G-CSF–G-CSFR ligation in CD114+ cells as compared with CD114− cells that are relatively unresponsive to G-CSF, and moderately responsive to STAT3 inhibition (Fig. 5C and D). Scatter plot analysis further demonstrated that G-CSF treatment upregulates the key STAT3 pathway genes, whereas STAT3 inhibition reverses this effect (Supplementary Fig. S6A–S6C). The Ingenuity Pathway Analyses show that upregulation of STAT3 in CD114+ cells directly and indirectly inhibit TP53 (Supplementary Fig. S6D). Gene Ontology analysis demonstrates the
expected association of STAT3 activation with genes involved in proliferation, stemness, and antiapoptotic responses (Supplemental Table S2; ref. 27).

G-CSFR is a direct transcriptional target of STAT3 in neuroblastoma

Our findings suggest that G-CSF increases both STAT3 activation and percentage of CD114+ neuroblastoma cells. We hypothesized that STAT3 may directly regulate expression of the gene coding for CD114 (CSF3R) in neuroblastoma as was previously demonstrated for murine neutrophil precursors (28). First, we analyzed CSF3R mRNA expression by qPCR in FACS-isolated subpopulations from multiple neuroblastoma cell lines. There is a significant increase in expression in response to exogenous G-CSF in CD114+ cells with no increase in CD114− tumor cells. This increase in CSF3R mRNA was blocked by STAT3 inhibition, either alone or combined with G-CSF treatment, suggesting that pSTAT3 directly regulates CSF3R expression in CD114+ cells (Fig. 6A).

Next, we analyzed the CSF3R 5′-UTR (3.8 kb) and promoter (2.5 kb) regions for putative STAT3-binding sites and found a canonical STAT3-binding site TTCCCGTAA in the 5′-UTR, located 200 bp (S) upstream from the translation start site (Fig. 6B). This increase in CSF3R mRNA was blocked by STAT3 inhibition, either alone or combined with G-CSF treatment, suggesting that pSTAT3 directly regulates CSF3R expression in CD114+ cells (Fig. 6A).
Figure 7.
Model representing positive feedback loop of the G-CSF–STAT3 signaling axis in neuroblastoma CSCs. Schematic representation of conclusions drawn from this study showing upregulation of the JAK–STAT3 pathway in response to binding of G-CSF to G-CSFR. Active STAT3 translocate to nucleus and upregulate the expression of key target genes that are known to be involved in tumorigenicity, metastasis, and drug resistance. CSF3R (gene coding for G-CSFR) is also a direct transcriptional target of STAT3. Binding of STAT3 on CSF3R promoter further upregulates surface expression of the G-CSFR (CD114), making a feedback loop mechanism for tumor progression in neuroblastoma. STAT3 inhibition and anti-G-CSF antibody are shown as blocking this positive feedback loop and therefore decrease the CSC functions in neuroblastoma.

Discussion
G-CSF is an inflammatory cytokine and neutrophil growth factor expressed from multiple cell types. G-CSF activates pSTAT3, a transcription factor with essential functions for induced pluripotent stem cells (iPSCs) reprogramming and for maintenance of ESC (embryonic stem cell) pluripotency (29–31). Here, we demonstrate a novel function of G-CSF promoting the proliferation and metastasis of neuroblastoma. This primarily occurs through the G-CSF–dependent activation of pSTAT3 signaling in CD114+ CSC-like tumor subpopulation. In addition, this tumorigenic population is highly sensitive to blockade of G-CSF signaling, as well as to STAT3 inhibition. Our data confirm that G-CSF is (i) an important growth factor for neuroblastoma, (ii) markedly affects tumor metastasis, and (iii) suggest that inhibition of the G-CSF–STAT3 signaling axis may be an effective therapeutic approach.

Our results demonstrate a direct effect of G-CSF, promoting the expansion and activity of the G-CSF–positive CSC-like subpopulation in both xenogeneic and syngeneic neuroblastoma murine tumor models. Previous studies of the effect of G-CSF on other murine tumor models demonstrated pro-tumorigenic effects through indirect immune-mediated processes. In breast and lung carcinoma models, exogenous or tumor-derived G-CSF promotes expansion of myeloid-derived suppressor cell (MDSC) subsets within the circulation and tumor microenvironment (19, 32). These MDSCs, in turn, secrete proangiogenic factors and downregulate proapoptotic TRAIL and MPO, leading to tumor progression and metastatic spread to the lung (33, 34). These results are consistent with our findings that G-CSF promotes overall tumorigenicity and

The reporter shows significant increase in percentage of CD114+/GFP+ cells in contrast to untreated or reporter driven by promoter region (Fig. 6B). Finally, ChIP-qPCR confirmed direct binding of STAT3 and pSTAT3 (Y705) to the STAT3 transcriptional response element (S) upstream of the translational start site for CSF3R in neuroblastoma tumor subpopulations (Fig. 6C). Importantly, we demonstrate that G-CSF treatment, which we have shown activates pSTAT3, significantly enhanced pSTAT3 binding in CD114+ cells. Stattic treatment dramatically reduced both STAT3 and pSTAT3 binding to the CSF3R-binding site. Minimal STAT3 or pSTAT3 enrichment was observed in the CD114+ tumor population (Fig. 6C). Consistent with STAT3 transcriptional activation of the G-CSFR and inhibition by Stattic, STAT3 knockdown with shRNA reduced the percentage of CD114+ cells in different neuroblastoma cell lines tested (Supplementary Fig. S7). These data confirm that in neuroblastoma, STAT3 directly regulates CD114 expression in response to G-CSF treatment in CD114+ receptor–positive cells.

Taken together, these data establish a positive feedback loop between G-CSF binding to its receptor (CD114) and STAT3-mediated upregulation of this receptor in CD114+ CSC-like neuroblastoma subpopulations (Fig. 7). Blockade of the G-CSF–STAT3 signaling pathway, either by pSTAT3 inhibition or by anti-G-CSF antibody, specifically downregulates expression of the G-CSFR or limits pSTAT3 signaling events that are involved in neural crest development and in CSC-driven neuroblastoma tumorigenesis. Our data also suggest that STAT3–mediated antiapoptotic pathways likely promote the survival and expansion of the CD114+ tumorigenic subpopulation (e.g., the observed marked sensitivity of CD114+ cells to pSTAT3 inhibitors compared with CD114– cells (Fig. 3D). Therefore, we propose that a G-CSF–G-CSFR–STAT3 feedback loop influences the survival and metastatic behavior of the neuroblastoma CSC subpopulation and represents an important CSC-specific therapeutic target.
metastasis. We demonstrated for the first time a direct effect of G-CSF in expanding a unique neuroblastoma CSC subpopulation (<1% of total neuroblastoma cells) to promote neuroblastoma tumorigenicity and metastasis. Our data also demonstrate maintenance of a positive feedback loop between G-CSFR and STAT3-mediated transcription of the G-CSFR gene (CSF3R). Thus, G-CSF–mediated STAT3 signaling actively maintains and increases surface expression of the G-CSFR (CD114) in this tumorigenic subpopulation to further enhance G-CSF–derived neuroblastoma tumorigenicity and metastasis. Neutralizing G-CSF or inhibiting STAT3 can disrupt this positive feedback loop, leading to a marked reduction in the neuroblastoma CSC subpopulation. This correlated with decreased tumor growth, decreased metastasis, and increased chemosensitivity. Therefore, G-CSF–mediated activation of STAT3 significantly influences the survival and stem cell–like behavior of the receptor–positive subpopulation. Conversely, inhibition of STAT3 or blockade of G-CSF signaling represents a novel strategy for CSC–specific therapy for neuroblastoma. Previous studies demonstrated the role of STAT3 in IL6-mediated drug resistance in neuroblastoma (35) and showed that STAT3 inhibition sensitizes colorectal cancer and nasopharyngeal carcinoma to both radiotherapy and chemotherapy (36, 37).

Increasing evidence suggests that STAT3-mediated inflammatory processes are critical for tumorigenesis (38–40). In addition, downstream effectors of chronic inflammation, such as TNFα and IL10, and other inflammatory cytokines, such as G-CSF and IL6, can activate immune responses that indirectly promote cellular proliferation and limit apoptosis (41). Specifically, Akt, Wnt/β-catenin, and the STAT3 signaling pathways are all linked to tumor promotion and survival of malignant stem cell populations (42–44). These findings further support the novel concept that aggressive developmental malignancies, such as neuroblastoma, co-opt immune-mediated inflammatory signaling pathways to drive progression and metastasis as suggested for colorectal, pancreatic, and hepatic cancers (45). Importantly, we note that administration of anti-G-CSF antibody, which inhibits signaling, led to decreased tumor growth and metastatic spread, whereas exogenous G-CSF induces expansion of the entire tumor mass, of which, only a small percentage is G-CSFR–positive (<1% typically). In addition, in vitro experiments confirmed that CD114+ subpopulation was at least 5 to 10 times more sensitive to STAT3 inhibition than CD114– bulk tumor cells. Doses of Statitc that had minimal effect on the bulk tumor population strongly induced apoptosis in the <1% CSC population and led to marked growth inhibition of the entire cell line both in vitro and in vivo (Figs. 3 and 4).

Because we demonstrate that G-CSF signaling is restricted to the CSC-like receptor–positive cells, these observations suggest that CD114+ cells provide a paracrine stimulus to the bulk tumor population that indirectly promotes proliferation and survival. Alternatively, tumor expansion may be secondary to the noted expansion of the small CD114+ population and subsequent increase in daughter cell production. In either case, we note that tumor growth and tumor response to anti-G-CSF antibody or STAT3 inhibitor appears to correlate well to changes in the percentage of CD114+ subpopulation within the tumor. Inhibition of the G-CSF–STAT3 pathway by anti-G-CSF or G-CSFR antibodies, or blockade of STAT3 signaling, may well represent a novel neo-adjuvant to standard chemotherapy. In addition to STAT3 inhibition, Janus-activated kinase (JAK) inhibition may also provide therapeutic efficacy by blocking phosphorylation of STAT3. Both JAK and STAT3 inhibitors are under clinical development (46). Novel nongenotoxic therapeutic strategies for infants and children with neuroblastoma are urgently needed. These might include combining STAT3 blockade with transcriptional inhibition of MYCN (e.g., with CDK7; ref. 47) or BRD4 (48).

Finally, G-CSF is currently extensively used in pediatric and adult oncology as an adjuvant to chemotherapy to limit therapy-related neutropenia (49). In high-risk neuroblastoma, it is typically administered after each cycle of chemotherapy to reduce treatment-related toxicity although there is very limited evidence that this improves overall survival (50). Our data argue for a reconsideration of G-CSF use in neuroblastoma, as well as a careful reevaluation of the risk-benefit ratio for G-CSF and other proinflammatory cytokines in cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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