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A truncation mutant of *Csf3r* cooperates with *PML-RARα* to induce acute myeloid leukemia in mice

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Abstract

Severe congenital neutropenia (SCN) is associated with a marked propensity to develop myelodysplasia (MDS) or acute myeloid leukemia (AML). Truncation mutations of *CSF3R*, encoding the G-CSF receptor (G-CSFR), are associated with the development of MDS/AML in SCN. However, a causal relationship between *CSF3R* mutations and leukemic transformation has not been established. Herein, we show that truncated G-CSFR cooperates with the *PML-RARα* oncogene to induce AML in mice. Expression of truncated G-CSFR significantly shortens the latency of AML in a G-CSF dependent fashion, and it is associated with a distinct AML presentation characterized by higher blast counts and more severe myelosuppression. Basal and G-CSF-induced STAT3, STAT5, and Erk1/2 phosphorylation were highly variable but similar in leukemic blasts expressing wild type and truncated G-CSFR. These data provide new evidence suggesting a causative role for *CSF3R* mutations in human AML.

Keywords

Acute myeloid leukemia; neutropenia; severe congenital neutropenia; myelodysplastic syndrome; granulocyte-colony stimulating factor (G-CSF); STAT3; STAT5

INTRODUCTION

A shared feature of many congenital and acquired bone marrow failure syndromes is a marked propensity to develop acute myeloid leukemia (AML) or a myelodysplastic syndrome (MDS). The cumulative risk of developing AML or MDS in patients with chronic bone marrow failure ranges from 2% to greater than 20%. With a few exceptions, the molecular basis for the increased risk of leukemic transformation is unknown. Severe congenital neutropenia (SCN) is a congenital bone marrow failure syndrome characterized by severe neutropenia present from birth and an arrest of myeloid differentiation at the promyelocyte/myelocyte stage. The cumulative risk of MDS/AML in patients with SCN after 10 years is 11–21%.[1,2] Treatment with granulocyte colony-stimulating factor (G-CSF) is effective in increasing neutrophil counts and preventing infections[3], and most patients

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now are treated lifelong with G-CSF. However, the quantity and duration of G-CSF treatment are correlated with an increased risk of MDS/AML.[1,2] This has sparked considerable debate on whether G-CSF treatment contributes to leukemic transformation in patients with SCN or other conditions requiring prolonged G-CSF treatment.

Nonsense mutations of *CSF3R* that truncate the distal cytoplasmic portion of the G-CSF receptor (G-CSFR) are present in approximately 40% of patients with SCN.[4] These mutations are acquired, heterozygous, and strongly associated with the development of AML/MDS.[4] Expression of mutant G-CSFR in myeloid cell lines results in enhanced proliferative and survival signals.[5] Transgenic mice carrying truncating mutations of *Csf3r* display a hyperproliferative response to G-CSF.[6,7] Moreover, a recent study showed that expression of mutant *Csf3r* in mice confers a strong clonal advantage at the hematopoietic stem cell (HSC) level that is dependent upon exogenous G-CSF.[8] However, *Csf3r* mutant mice do not develop MDS or AML; thus, a causal relationship between *CSF3R* mutations and leukemia has not been established.

The hyperproliferative response of the truncated G-CSFR is mediated, in least in part, by accentuated STAT5 activation. Compared with wild type G-CSFR, the amplitude and duration of STAT5 activation is increased in primary murine hematopoietic cells expressing truncated G-CSFR.[8–10] In contrast, STAT3 activation by the truncated G-CSF is modestly reduced.[8–10] STAT5 activation is a common feature of leukemias associated with activating mutations of tyrosine kinase genes, including *BCR-ABL*,[11] *JAK2*,[12] and *FLT3*.[13,14] STAT5 has been implicated in hematopoietic stem self-renewal.[15] Consistent with this observation, we recently showed that the clonal advantage of hematopoietic stem cells expressing truncated G-CSFR is dependent on STAT5 activation.[8] The importance of STAT5 activation in leukemic progression is further supported by studies showing that expression of constitutively active STAT5 results in a fatal myeloproliferative disease in mice.[16–18]

Activating mutations of receptor tyrosine kinases (e.g., *FLT3* and *KIT*), while present in approximately 30% of de novo AML, are rare in AML arising in the setting of SCN.[19] Since receptor tyrosine kinases and the G-CSFR activate similar signaling pathways, including STAT5, we hypothesized that G-CSFR truncation mutations may contribute to leukemic transformation by providing an ‘activated receptor tyrosine kinase signal’. SCN-associated AML is characterized by monosomy 7 and deletions of chromosome 5.[19,20] Chromosomal translocations or point mutations in transcription factors are uncommon.[19,20] Thus, in contrast to de novo AML where the initiating mutation (e.g., PML-RARα) is known in some cases, the initiating mutations in SCN-AML are largely unknown. Consequently, to test this hypothesis, we asked whether expression of truncated G-CSFR is able to cooperate with the PML-RARα oncogene, a well characterized initiating mutation for human promyelocytic leukemia, to induce AML in mice. This experimental approach has been used to demonstrate the transforming potential of activating mutations of *FLT3* and *NRAS*, which by themselves, are not sufficient to induce AML in mice.[21,22] We show that expression of truncated G-CSFR shortens the latency and alters the phenotype of AML in PML-RARα transgenic mice. Basal and G-CSF induced STAT5, STAT3, and Erk1/2 activation in leukemic blasts is highly variable but not affected by mutant G-CSFR expression. These data support a causative role for *CSF3R* mutations in leukemic progression in patients with SCN.

METHODS

Mice

d715 *Csf3r* mice backcrossed 10 generations onto a C57BL/6 background were generated as described previously.[7] Transgenic mice in which the PML-RARα oncogene was knocked-in to the Cathepsin G (*Ctsg*) locus were generated as described previously;[23] these mice also

were backcrossed at least 10 generation onto a C57BL/6 background. Mice were housed in a specific pathogen-free environment. All experiments were approved by the Washington University Animal Studies Committee.

G-CSF and pegylated-G-CSF administration

Human G-CSF was either given as a single subcutaneous dose (250 μg/kg) or twice daily for 7 days at 125 μg/kg. Pegylated G-CSF (1 mg/kg) was given every 4–5 days for 6 months. G-CSF and pegylated G-CSF were generous gifts from Amgen (Thousand Oaks, CA).

Bone marrow transplantation

A total of 5 million leukocytes harvested from the spleens of leukemic mice were injected into the tail vein of lethally irradiated wild type mice. Recipient mice were conditioned with 300 cGy from a ^{137}Cesium source at a rate of approximately 95 cGy/minute 24 hours prior to transplantation. Prophylactic antibiotics (Trimethoprim-Sulfamethoxazole, Alpharma, Baltimore, MD) were given during the initial two weeks after transplantation.

Assessment of STAT5, STAT3, and Erk1/2 phosphorylation

Spleen cells from leukemic mice were incubated with fluorescein isothiocyanate (FITC)-conjugated Gr-1 and allophycocyanin (APC)-conjugated c-Kit; as a control, bone marrow cells from healthy wild type mice were incubated with a cocktail of FITC-conjugated lineage markers (Gr-1, B220, Ter119, and CD3) and APC-conjugated c-Kit. In each case, cells were then incubated in BD Phosphflow Lyse/fix buffer (BD Biosciences) for 10 minutes and permeabilized in 100% methanol at 4° for 20 minutes cells. Cells were then incubated with phycoerythrin (PE)-conjugated anti-pSTAT5 (pY694), anti-pSTAT3 (pY705), or anti-pErk1/2 (pT202/pY204) at room temperature for 30 minutes. In some cases, cells from leukemic spleens were suspended in Opti-MEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and stimulated with G-CSF (100 ng/ml) for 30 minutes at 37 °C and then processed for STAT5, STAT3, and Erk1/2 phosphorylation. Cells were analyzed on a 2-laser, 5 color FACScan flow cytometer. All antibodies were purchased from BD Biosciences, unless otherwise indicated.

Statistical Analysis

Statistical significance was determined either by a two-way ANOVA analysis or by a two-sided Student *t* test. Survival differences were assessed using the Log-rank (Mantel-Cox) test. All data represent the mean ± SEM, unless otherwise stated.

RESULTS

The *d715 Csf3r* can cooperate with *PML-RARa* to induce AML in mice in a G-CSF dependent fashion

Previous studies showed that activating mutations of *FLT3* or *KRAS* were able to cooperate with *PML-RARα* to induce leukemia in mice.[21,22] As a proof-of-principle experiment, we asked whether *Csf3r* truncation mutations also could cooperate with *PML-RARα* to induce leukemia. We previously described transgenic mice carrying a targeted mutation of their *Csf3r* gene (termed d715) that reproduces the *CSF3R* mutation found in a patient with SCN.[7] These mice (*Csf3r*$^{d715/d715}$) were crossed with transgenic mice expressing *PML-RARα*[23] and a tumor watch established (Table 1); all mice were backcrossed at least 10 generations onto a C57Bl/6 background. To simulate the high systemic level of G-CSF present in patients with SCN (either through increased endogenous production or exogenous administration), some of the mice were treated with G-CSF (pegylated human G-CSF, 1 mg/kg every 4–5 days) for 6 months starting at 6–10 weeks of age. Of note, the neutrophil

response to chronic G-CSF treatment was similar in all cohorts (data not shown). All mice were followed for at least 18 months.

As expected, none of the mice not containing the *PML-RARα* transgene developed AML (data not shown), confirming a previous study showing that expression of the d715 G-CSFR is not sufficient to induce AML in mice.[7] The cumulative incidence of AML in *PML-RARα* mice (with wild type G-CSFR) was approximately 40%, with a median survival of 541 days (Figure 1A). In the absence of G-CSF treatment, the presence of heterozygous or homozygous *Csf3r d715* mutation had no significant impact on leukemia penetrance or latency (Figure 1A). However, in mice treated with G-CSF, the presence of the d715 G-CSFR significantly shortened the latency to develop AML (Figure 1B and Table 2). Moreover, a non-significant trend to increase AML penetrance was observed (Table 2). These data demonstrate that the d715 G-CSFR is able to cooperate with *PML-RARα* to induce AML in mice in a G-CSF dependent fashion.

Expression of the d715 G-CSFR is associated with higher blast counts and more severe myelosuppression

We next compared the phenotype of AML arising in the setting of wild type or *d715 Csf3r*. We focused on mice with a history of G-CSF treatment, since changes in AML latency and penetrance were confined to these experimental cohorts. A similar degree of leukocytosis and splenomegaly were observed in all cohorts (Table 2). However, *PML-RARα* $Csf3r^{d715/d715}$ mice had more severe anemia and thrombocytopenia at presentation with a trend to decreased bone marrow cellularity. Moreover, a trend to an increase in the percentage of myeloblasts (defined as Gr-1+ Kit+ double positive cells)[23] in the bone marrow, blood and spleen was observed (Figure 2A and Table 2). Spleen cells from leukemic *PML-RARα* $Csf3r^{wt/wt}$ and *PML-RARα* $Csf3r^{d715/d715}$ mice were able to induce a fatal leukemia upon transplantation into sub-lethally irradiated syngenic hosts with a similar latency (Figure 2B). Collectively, these data show that expression of the d715 G-CSFR is associated with a distinct AML presentation characterized by higher blast counts and more severe myelosuppression.

STAT3, STAT5, and Erk1/2 are not constitutively activated in leukemic blasts expressing the *d715 Csf3r*

Constitutive activation of STAT3, STAT5, and Erk1/2 has been detected in a subset of AML.[18,24–26] Moreover, sustained activation of these signaling molecules has been reported in myeloid cells expressing the *d715 Csf3r*.[7,9,27] Based on these observations, we hypothesized that excessive activation of one or more of these proteins by the d715 G-CSFR may have contributed to leukemic transformation. To test this hypothesis, we first isolated splenic cells from leukemic *PML-RARα* $Csf3r^{wt/wt}$ and *PML-RARα* $Csf3r^{d715/d715}$ mice (no mice were treated with G-CSF at the time these analyses were performed) and measured the basal level of phosphorylated STAT3, STAT5, and Erk1/2 in blasts (Gr-1+ Kit+) by flow cytometry (Figure 3). As a control, we also analyzed lineage- Kit+ cells from non-leukemic wild type mice. Considerable variability in the basal level of STAT3, STAT5, or Erk1/2 phosphorylation was observed. In *PML-RARα* $Csf3r^{wt/wt}$ blasts, constitutive activation of STAT3, STAT5, and Erk1/2 (as defined by a signal greater then the 95% confidence interval for control cells) was observed in 23.4%, 11.8%, and 26.7% of cases, respectively. Similar data were observed with *PML-RARα* $Csf3r^{d715/d715}$ blasts; constitutive activation of STAT3, STAT5, and Erk1/2 was observed in 22.7%, 14.8%, and 25.0% of samples, respectively.

We next stimulated leukemic cells with G-CSF in vitro and assessed STAT3, STAT5, and Erk1/2 activation using the phospho-flow assay. G-CSF stimulation induced STAT3 and STAT5 phosphorylation to a similar degree in *PML-RARα* $Csf3r^{wt/wt}$ and *PML-RARα*

Csf3r$^{d715/d715}$ leukemic blasts (Figure 4). G-CSF did not consistently induce Erk1/2 phosphorylation in either cell type. Together, these data suggest that, at least in fully transformed leukemic blasts, basal and G-CSF induced activation of STAT3, STAT5, and Erk1/2 is similar in cells expressing wild type or *d715 G-CSFR*.

DISCUSSION

The contribution of *CSF3R* truncation mutations to transformation to MDS/AML in patients with SCN is controversial. *CSF3R* mutations are strongly associated with the development of MDS/AML. Germeshausen et al. reported that the incidence of *CSF3R* truncation mutations was 78% (18/23) in individuals with SCN and monosomy 7, MDS, or AML, compared with 34% (43/125) in patients without MDS or AML.[4] In mice, expression of truncated G-CSFR confers a strong clonal advantage to HSCs that is dependent on G-CSF.[8] This is consistent with the observation, that in most (but not all) patients with SCN, the frequency of circulating cells containing mutant *CSF3R* increases over time.[4,28] Moreover, a recent study showed that *CSF3R* mutations are present in all hematopoietic lineages (including lymphocytes), suggesting that the clonal expansion in patients with SCN occurs at the HSC level.[29] On the other hand, *CSF3R* mutations are not sufficient to induce MDS/AML in humans or mice. The time course from detection of *CSF3R* mutations to leukemic transformation is quite variable and some patients with *CSF3R* mutations remain transformation free for years.[4,28] Moreover, transgenic mice expressing mutant *Csf3r* do not develop leukemia, despite prolonged treatment with G-CSF.[7,9]

Activating mutations of receptor tyrosine kinase genes (*FLT3* and *KIT*) are present in approximately 30% of cases of de novo AML but are rare in SCN-associated AML.[19] Similar to mutant *Csf3r*, these mutations are not sufficient to induce AML in mice,[22,30] demonstrating that they are not leukemia-initiating mutations. However, these mutations are able to cooperate with initiating mutations, such as PML-RARα, to induce AML. There is strong evidence that *CSF3R* truncation mutations also are activating. The truncated G-CSFR transmits a hyperproliferative signal that is secondary, in part, to sustained STAT5 activation that, in turn, is related to impaired receptor internalization and the failure of the truncated G-CSFR to recruit and activate SOCS3 and SHP2.[31,32] Since the G-CSFR and receptor tyrosine kinases share many signaling pathways, these observations suggest that the mutant G-CSFR may provide the “activated tyrosine kinase signal” that is present in many cases of de novo AML. A previous study showed that activating mutations of FLT3 were able to cooperate with *PML-RARα* to induce leukemia in mice.[22] Likewise, in the present study, we show that expression of truncated G-CSFR cooperates with PML-RARα to induce AML in mice. Though to the best of our knowledge the t(15;17) translocation producing PML-RARα has not been reported in patients with SCN-associated MDS/AML, we suggest this “proof-of-principle” experiment provides strong evidence that *CSF3R* truncation mutations are leukemogenic. Interestingly, this cooperativity was dependent upon G-CSF administration. This is relevant to patients with SCN, since G-CSF levels are chronically elevated, either secondary to exogenous administration or through increased endogenous production (due to the severe neutropenia). In fact, the G-CSF dependence of the truncated G-CSFR provides an explanation for why *CSF3R* mutations are common in MDS/AML arising in the setting of SCN but are rare in de novo AML.

We observed considerable heterogeneity in baseline and G-CSF-induced STAT3, STAT5, and Erk1/2 phosphorylation in leukemic blasts. Constitutive STAT3 and STAT5 activation were observed in approximately 23% and 13% of cases, respectively, irrespective of G-CSFR mutation status. Though highly variable, similar data have been reported in primary human AML blasts. Constitutive activation of STAT3 has been observed in 20–44% of cases of AML and STAT5 activation in approximately 20% of cases.[24,26,33–35] Relevant to

this study, STAT3 activation has been observed in the majority (7 of 9 cases, 78%) of cases of M3-AML.[34,35] However, Spiekerman et al showed that expression of *PML-RARα* was not sufficient to induce STAT3 or STAT5 activation.[33] Contrary to expectations, enhanced STAT5 activation after G-CSF stimulation was not observed in leukemic blasts carrying mutant *Csf3r*, likely reflecting altered G-CSFR expression or signal transduction. In this regard, it is interesting to note that some cases of AML with activating mutations of *FLT3* also do not display constitutive STAT3 or STAT5 activation.[33] It is important to note that our analysis was confined to fully transformed leukemic blasts. It is possible that enhanced STAT5 and/or STAT3 activation contributed to leukemic transformation in preleukemic progenitors. It also is possible that mutant G-CSFR contributes to leukemic transformation in a STAT3 and STAT5 independent fashion. For example, similar to activating mutations of FLT3,[22] the mutant G-CSFR is associated with increased reactive oxygen species (ROS) production,[36] which has been implicated in oxidative DNA damage.[37]

In summary, our data show that *Csf3r* truncation mutations, similar to other mutations that activate receptor tyrosine kinase signaling pathways, cooperate with the *PML-RARα* oncogene to induce AML in mice. These data provide strong new evidence that truncating mutations of the *CSF3R* are leukemogenic.

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Nonstandard abbreviations

G-CSF	granulocyte colony-stimulating factor
G-CSFR	G-CSF receptor
HSC	hematopoietic stem cells
HSPC	hematopoietic stem/progenitor cells
AML	acute myeloid leukemia
MDS	myelodysplastic syndrome
LSK cells	lineage- Sca+ Kit+ cells

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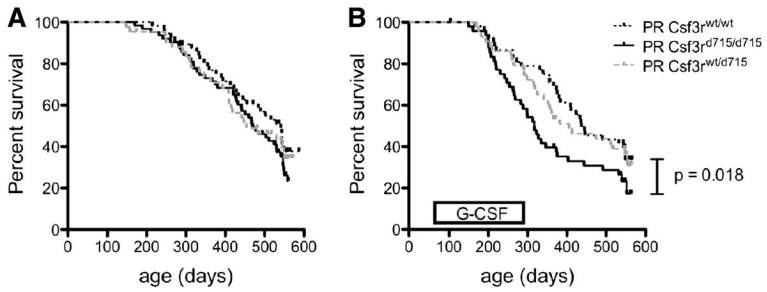

Figure 1. The d715 Csf3r cooperates with PML-RARα to induce AML in mice in a G-CSF dependent fashion

(A). Kaplan Meier survival curves are shown for mice expressing *PML-RARα* (PR) and wild type *Csf3r*, heterozygous d715 *Csf3r*, or homozygous d715 *Csf3r*. A separate cohort of mice (B) was treated with pegylated human G-CSF (1 mg/kg every 4–5 days) for 6 months. There were 42–67 mice in each cohort. Significance was assessed using the Mantel-Cox test.

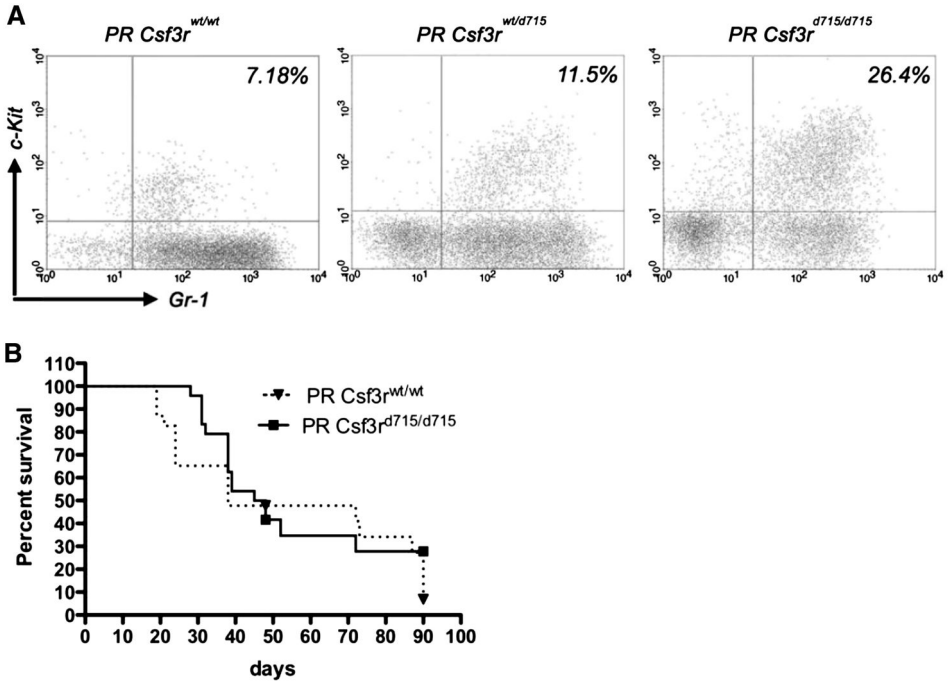

Figure 2. Phenotypic characterization of leukemic mice

(A) Peripheral blood cells from leukemic mice of the indicated genotype were analyzed by flow cytometry for Gr-1 and c-Kit expression. Leukemic blasts were identified as Gr-1+ Kit + cells. Data are representative of at least 3 independent experiments. (B) Spleen cells (1×10^6) from leukemic mice of the indicated genotype were transplanted into syngenic irradiated mice. Kaplan Meier survival curves are shown.

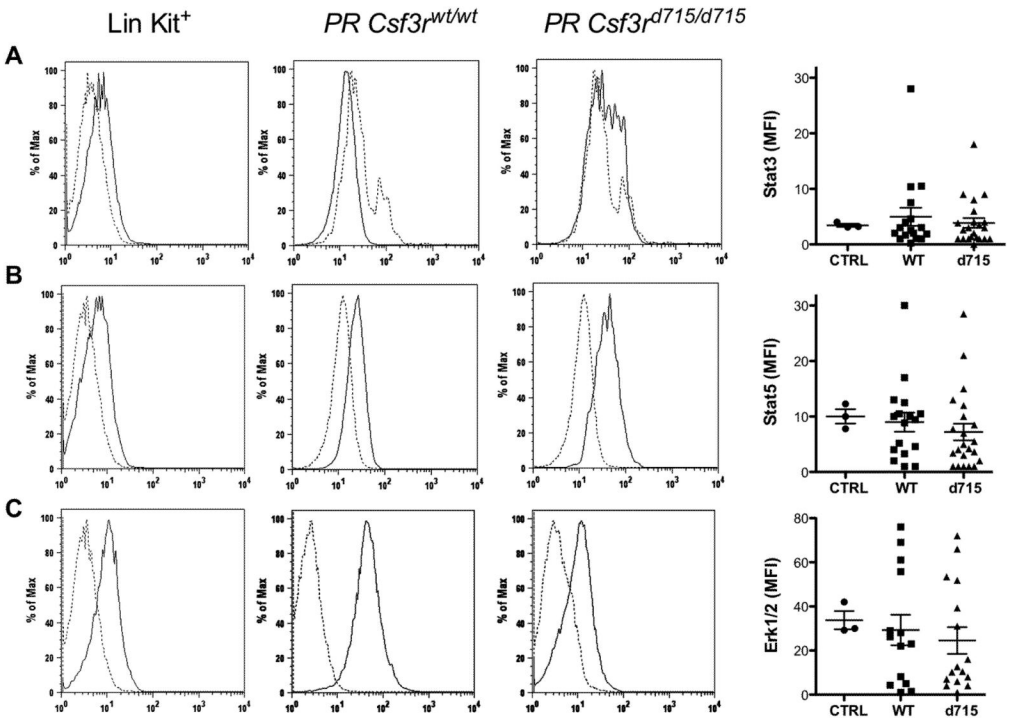

Figure 3. STAT3, STAT5, and Erk1/2 phosphorylation in leukemic cells

Spleen cells from leukemic mice of the indicated genotype were harvested and immediately analyzed for phosphorylated STAT3 (A), STAT5 (B), or Erk1/2 (C). Representative histograms showing staining for the indicated phosphorylated protein after gating on Gr-1+ Kit+ leukemic blasts (left 3 panels). As a control, bone marrow cells from healthy wild type mice were analyzed in the same fashion, gating on Lineage- Kit+ cells (Lin- Kit+). The right panel shows the median fluorescent intensity (MFI) after subtraction of the signal from the isotype control. Each data point represents a single mouse, and the mean ± SEM are shown. CTRL: lineage- Kit+ wildtype cells; WT: *PR Csf3r*$^{wt/wt}$ blasts; d715: *PR Csf3r*$^{d715/d715}$ blasts.

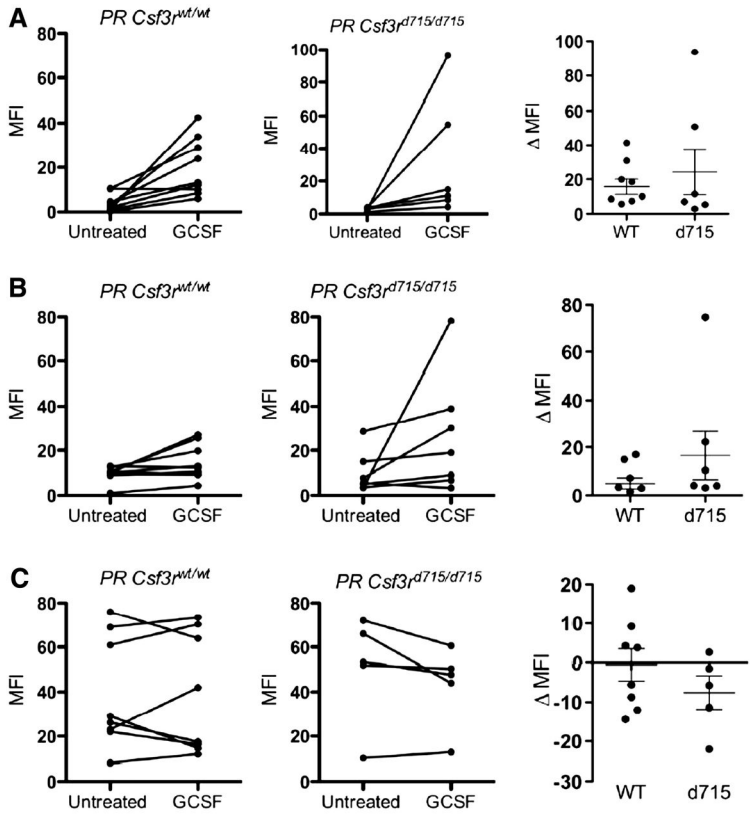

Figure 4. G-CSF induced STAT3, STAT5, and Erk1/2 activation in leukemic cells

Splenic cells from leukemic mice of the indicated genotype were stimulated in vitro with G-CSF (100 ng/ml) for 15 minutes, and phosphorylation of Stat 3 (A), STAT5 (B), or Erk1/2 (C) was measured. Each point represents data from an individual mouse. Data are gated on Gr-1$^+$ Kit$^+$ leukemic blasts. The difference in signal between untreated and G-CSF treated samples (ΔMFI) is shown in the right panel. The mean ± SEM is shown. WT: PR *Csf3r*$^{wt/wt}$ blasts; d715: PR *Csf3r*$^{d715/d715}$ blasts.

Table 1

Tumor watch

PML-RARα	*Csf3R*	G-CSF Rx*	N
No	WT	No	19
No	d715	No	45
No	WT	Yes	12
No	d715	Yes	47
Yes	WT	No	57
Yes	WT/d715	No	46
Yes	d715	No	67
Yes	WT	Yes	54
Yes	WT/d715	Yes	42
Yes	d715	Yes	50

WT/d175 refers to *Csf3r* $^{WT/d715}$ mice

d715 refers to *Csf3rd715*$^{/d715}$ mice

*Pegylated human G-CSF (1 mg/kg) was given subcutaneously every 4–5 days for 6 months

Table 2

AML parameters

Parameter	PR Csf3r$^{WT/WT}$	PR Csf3r$^{d715/WT}$	PR Csf3r$^{d715/d715}$	^ P value
Median Survival	437 days	406 days	317 days	0.013
* Cumulative incidence of AML	61.4%	65.1%	77.1%	0.089
# Blood				
WBC(× 10^{-3}/μl)	81.6 ± 59.2	88.7 ± 37.8	62.8 ± 45.9	0.808
RBC (× 10^{-6}/μl)	6.7 ±1.3	4.8 ± 0.9	1.6 ± 0.7	0.013
Platelets (× 10^{-3}/μI)	527± 133	535 ± 115	188 ± 62	0.052
% Blasts	5.16 ± 1.2	7.93 ± 2.9	22.6 ± 9.8%	0.195
# Bone marrow				
Cells per femure (× 10^{-6})	18.2 ± 4.6	24.1 ± 4.1	11.9 ± 3.1	0.276
% Blasts	20.7 ± 5.3	26.1 ± 7.9	38.2 ± 16.7	0.381
# Spleen				
Weight (grams)	1.32 ± 0.33	1.20 ± 0.17	0.94 ± 0.12	0.235
% Blasts	25.4 ± 4.7	20.2 ± 8.3	34.3 ± 7.3	0.37

All mice had a history of G-CSF treatment

^P value (t-test) comparing *PR Csf3r$^{WT/WT}$* with *PR Csf3rd$^{715/d715}$*

*Cumulative incidence of AML @ 560 days

#Data based on the analysis of morbidly ill mice: n = 3–6 PR *Csf3r$^{WT/WT}$*, *4–8 PR* Csf3rdWT/d715, and 4–9 PR *Csf3r$^{d715/d715}$* mice.