Stat5 is critical for the development and maintenance of myeloproliferative neoplasm initiated by *Nf1* deficiency

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ABSTRACT

Juvenile myelomonocytic leukemia is a rare myeloproliferative neoplasm characterized by hyperactive RAS signaling. Neurofibromin1 (encoded by the *NF1* gene) is a negative regulator of RAS activation. Patients with neurofibromatosis type 1 harbor loss-of-function mutations in *NF1* and have a 200- to 500-fold increased risk of juvenile myelomonocytic leukemia. Leukemia cells from patients with juvenile myelomonocytic leukemia display hypersensitivity to certain cytokines, such as granulocyte-macrophage colony-stimulating factor. The granulocyte-macrophage colony-stimulating factor receptor utilizes pre-associated JAK2 to initiate signals after ligand binding. JAK2 subsequently activates STAT5, among other downstream effectors. Although STAT5 is gaining recognition as an important mediator of growth factor signaling in myeloid leukemias, the contribution of STAT5 to the development of hyperactive RAS-initiated myeloproliferative disease has not been well described. In this study, we investigated the consequence of STAT5 attenuation *via* genetic and pharmacological approaches in *Nf1*-deficient murine models of juvenile myelomonocytic leukemia. We found that homozygous *Stat5* deficiency extended the lifespan of *Nf1*-deficient mice and eliminated the development of myeloproliferative neoplasm associated with *Nf1* gene loss. Likewise, we found that JAK inhibition with ruxolitinib attenuated myeloproliferative neoplasm in *Nf1*-deficient mice. Finally, we found that primary cells from a patient with *KRAS*-mutant juvenile myelomonocytic leukemia displayed reduced colony formation in response to JAK2 inhibition. Our findings establish a central role for STAT5 activation in the pathogenesis of juvenile myelomonocytic leukemia and suggest that targeting this pathway may be of clinical utility in these patients.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloproliferative neoplasm (MPN) with no effective chemotherapy or targeted therapy options. Hematopoietic stem cell transplantation, with its considerable morbidity and morality burden, remains the only modality that can improve survival in patients

with this condition.[1,2] Nearly all patients (80-90%) harbor somatic or germline mutations that lead to hyperactive RAS signaling.[2-4] Recent deep sequencing efforts have discovered that some patients harbor two, co-occurring RAS-pathway activating mutations and that these compound mutations are associated with more aggressive disease,[3,4] underscoring the importance of hyperactive RAS in JMML. Neurofibromin, encoded by *NF1*, negatively regulates RAS activity.[5] Patients with inherited mutations of *NF1* have a 200- to 500-fold increased risk of developing JMML.[1] Mice harboring activated *Ras* genes or *Nf1* deficiency develop MPN that resembles human JMML.[6-14] Likewise, mice that harbor compound activating mutations that activate the RAS pathway also display a more aggressive JMML phenotype.[15] Notably, transplantation of *Nf1*-null fetal liver cells or somatic deletion of *Nf1* in the hematopoietic compartment results in progressive myeloid expansion.[9,10,16,17] Furthermore, induced pluripotent stem cells, generated from two patients with JMML, differentiated into myeloid cells with high proliferative capacity and enhanced basal ERK (a well-known mediator of RAS activation) and STAT5 activation.[18] Malignant cells from JMML patients and JMML mouse models display hypersensitivity to certain cytokines, in particular granulocyte-macrophage colony-stimulating factor (GM-CSF).[5,9,14,19] The absence of GM-CSF receptor signaling prevents the development of MPN in recipient mice receiving hematopoietic stem cells doubly deficient for *Nf1* and the GM-CSF receptor common β chain.[16] Similarly, in an $Nras^{G12D/+}$ model of MPN, β common chain deficiency did not prevent initiation of disease, but reduced splenomegaly and spontaneous colony formation and prolonged survival.[20] GM-CSF receptor signaling promotes proliferation and differentiation by activating a variety of signal transduction pathways including Janus kinase 2 - signal transducer and activator of transcription 5 (Jak2-Stat5) and Ras.[21,22]

Mek inhibitors to modulate RAS activation have had variable therapeutic efficacy in JMML models. Myeloid cells, derived from the induced pluripotent stem cells described above, displayed reduced GM-CSF independence in response to Mek inhibition. In an activated *Kras* model of MPN, Mek inhibition abrogated the disease.[23] In mouse models of *Nf1*-deficient or *Kras*-mutant MPN, Mek inhibition enhanced erythropoiesis and reduced spleen size, but failed to eradicate *Nf1*-deficient or *Kras*-mutant cells.[23,24] These studies support a central role of aberrant Raf/MEK/ERK signaling in the abnormal growth of JMML cells.

The importance of STAT5a/b activation in JAK2-mutant MPN has been well described. STAT5 is an important contributor to hematopoiesis and cancer.[25-28] Hyperphosphorylation of STAT5 in response to minimal concentrations of GM-CSF is a hallmark of JMML.[29] JAK2 mutations are common in other MPN, including 95% of cases of polycythemia vera and 50-60% of cases of primary myelofibrosis and essential thrombocythemia.[30] Treatment with the JAK2 inhibitor ruxolitinib improves the clinical parameters and symptoms associated with these disorders[31-34] and leads to a reduction of STAT5 activation in the cells of treated patients.[35] JAK2 inhibition reduces the viability of primary cells from patients with chronic myelomonocytic leukemia displaying hypersensitivity to GM-CSF signaling.[36] Likewise, *Stat5* deficiency abrogates disease in mouse models of $JAK2^{V617F}$ MPN.[37,38] These findings highlight the critical role of STAT5 signaling in JAK2-mutant and other MPN featuring hyperactive GM-CSF signaling.

The possible contribution of the JAK2/STAT5 pathway to MPN with hyperactive RAS signaling, such as JMML, has not been well described. JMML cells derived from NF1-deficient patients display differential STAT5 activation,[29] implicating this pathway in diseases with hyperactive RAS signaling. In a mouse model of $Nras^{G12D}$ CMML, a subset of cells developed hyperactive Erk and Stat5 activation in response to GM-CSF signaling.[39] Mek inhibition prolonged the life of 40% of CMML mice harboring $Nras^{G12D/G12D}$, while combined Mek inhibition with Jak2 inhibition abolished the disease in these mice.[40] These findings implicate STAT5 as a potential contributor to the pathogenesis of MPN with activated RAS. Since the therapeutic options in JMML are severely limited, identifying effective drug targets in this devastating disease of infancy is an important clinical priority.

To elucidate the contribution of the Jak2-Stat5a/b signaling pathway to MPN derived from loss of *Nf1*, we attenuated Stat5 signaling in *Nf1*-deficient mice using either a genetic *Stat5a/b* hypomorphic knockout[41,42] (which harbors a loss of both *Stat5a* and *Stat5b* genes) or pharmacological Jak2 inhibition with ruxolitinib.

Methods

Mice

Animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota

A complex breeding scheme was established to generate animals of the appropriate genotypes (Figure 1A). The *Stat5a* and *Stat5b* alleles used in this study produce low amounts of an N-terminally deleted, partially functional form of their respective proteins.[42,43] Henceforth, *Stat5* refers to both *Stat5a* and *Stat5b* loci on mouse chromosome 11, with the status of both alleles indicated simultaneously as either + for both wild-type alleles or ΔN for the hypomorphic double knockout. The murine *Stat5* loci map approximately 15 cM away from the *Nf1* locus on chromosome 11. Therefore, two separate recombinant chromosomes were generated, one chromosome with the $Stat5^{\Delta N}$[44,45] combined with the $Nf1^{Fcr}$ (null) allele[46] and the other with *Stat5* combined with the $Nf1^{flox}$ allele.[47] Breeding was complicated because $Stat5^{\Delta N/\Delta N}$ females are infertile and $Stat5^{\Delta N/\Delta N}$ offspring often fail to thrive. The low ratio of useful animals per litter necessitated transplantation of donor bone marrow into histocompatible recipient animals.

Mx1-Cre transgenic animals (C57BL/6) were crossed with $Nf1^{flox}$ mice (C57BL/6) to generate $Nf1^{flox/+}$/*Mx1-Cre* animals. Separately, $Stat5^{\Delta N}$ mice on a C57BL/6 x 129/Sv background were crossed with $Nf1^{Fcr}$ mice (C57BL/6) to generate $Nf1^{Fcr/+}/Stat5^{\Delta N//+}$ animals and with $Nf1^{flox/+}$/*Mx1-Cre* animals to generate $Nf1^{flox/+}/Stat5^{\Delta N//+}$/*Mx1-Cre* animals. These animals were crossed to provide donor animals of the following genotypes: $Nf1^{flox/Fcr}/Stat5^{+/+}$/*Mx1-Cre*, $Nf1^{flox/+}/Stat5^{\Delta N//+}$/*Mx1-Cre*, $Nf1^{flox/Fcr}/Stat5^{\Delta N//+}$/*Mx1-Cre*, $Nf1^{flox/Fcr}/Stat5^{\Delta N/\Delta N}$/*Mx1-Cre* and $Nf1^{flox/+}/Stat5^{\Delta N/\Delta N}$/*Mx1-Cre* animals (Figure 1A).

$Stat5^{\Delta N/\Delta N}/Nf1$ heterozygous mice, whether with the $Nf1^{Fcr}$ or $Nf1^{flox}$ allele, had particularly poor health and frequently died by 6 to 8 weeks of age. Transplants involving these genotypes were, therefore, done with single donors, rather than donor cells pooled from multiple mice. Multiple transplants were performed to achieve adequate numbers of experimental transplant recipients.

For these and all of the other genotypes, all donor animals also carried the *Mx1-Cre* transgene.

Donor animals were F1 offspring from a cross of two strain backgrounds, C57BL/6 and 129/Sv, both of which express Ly5.2 (*Ptpcr*^{a}) on the surface of hematopoietic cells. Recipient animals were generated as F1 offspring from a cross between 129/Sv (Ly5.2, *Ptpcr*^{a}) and C57BL/6J (Ly5.1, *Ptpcr*^{b}) animals. The recipient offspring were therefore congenic at the *Ly5.1* locus, providing a mechanism by which to distinguish recipient Ly5.1^{+}Ly5.2^{+} cells from donor Ly5.1^{-} Ly5.2^{+} cells by immune staining for surface expression of Ly5.1 and Ly5.2.

Colony forming assays

Methylcellulose cultures were performed as previously described.[5] Briefly, peripheral blood mononuclear cells were isolated using Lymphoprep™ (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions and plated at 5x10^{3} cells/mL with MethoCult (Stemcell Technologies, Vancouver, BC, Canada), 100 U/mL penicillin G, 10 mg/mL streptomycin, and inhibitor. The inhibitors used were ruxolitinib at 0.4 μM, PD325901 at 13 μM (both from Selleckchem, Houston, TX, USA), or dimethylsulfoxide vehicle. Cultures were incubated at 37°C in 5% CO_2 and scored 7 days later. Experiments in each condition were performed in triplicate.

Results

Generation of Stat5, Nf1 double-knockout mice

Mice were bred to generate five experimental groups: wild-type at the *Stat5* loci and homozygous deficient at the *Nf1* locus (*Stat5*$^{+/+}$/*Nf1*$^{flox/Fcr}$), heterozygous at the *Stat5* loci and either heterozygous or homozygous deficient at the *Nf1* locus (*Stat5*$^{\Delta N/+}$/*Nf1*$^{+/Fcr}$ or *Stat5*$^{\Delta N/+}$/*Nf1*$^{flox/Fcr}$), and homozygous deficient at the *Stat5* loci and either heterozygous or homozygous deficient at the *Nf1* locus (*Stat5*$^{\Delta N/\Delta N}$/*Nf1*$^{+/Fcr}$ or *Stat5*$^{\Delta N/\Delta N}$/*Nf1*$^{flox/Fcr}$) (Figure 1A). All donor animals also carried the *Mx1-Cre* transgene. Bone marrow from mice from each of these groups was transplanted into histocompatible recipients. One week after the transplant, recipient animals were injected with polyinosinic-polycytidylic acid (pIpC) to induce expression of the interferon responsive *Mx1-Cre* transgene; this led to deletion of the *Nf1*^{flox} allele (*Nf1*$^{\Delta}$). Recipient animals heterozygous at the *Ptpcr* locus, *Ptpcr*$^{a/b}$, expressed both Ly5.1 and Ly5.2, and donor animals homozygous at the *Ptpcr* locus, *Ptpcr*$^{a/a}$, expressed only Ly5.2. This difference allowed us to identify cell origin by cell surface immune-staining. Recipient animals for all genotypes tested showed 70-90% engraftment 4 weeks after transplantation by flow cytometric

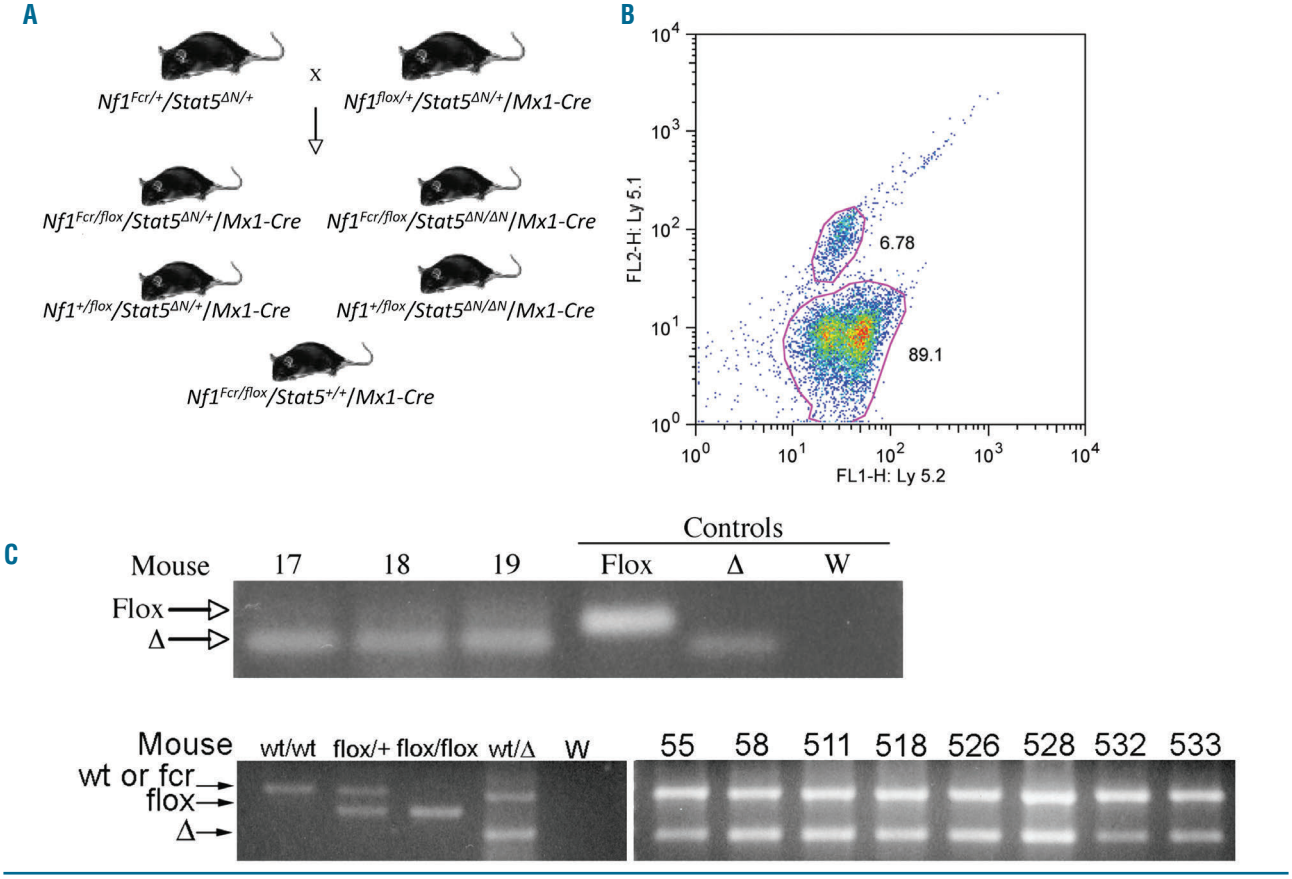
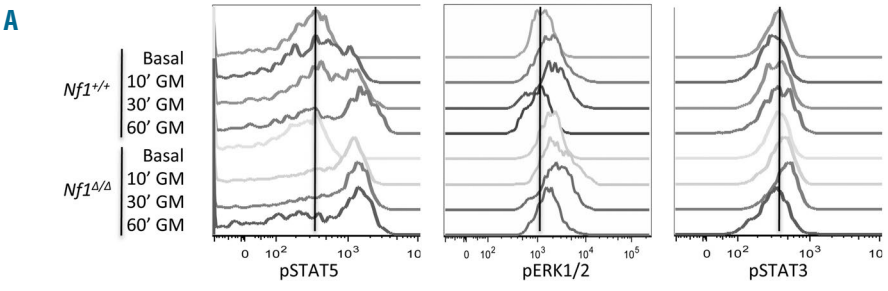


Figure 1. *Stat5/Nf1*-deficient bone marrow engrafts recipient animals. (A) Diagram depicting the breeding scheme to generate the five genetic backgrounds used in these studies. *Stat5*$^{\Delta N/+}$/*Nf1*$^{Fcr/+}$ were generated and crossed with *Stat5*$^{\Delta N/+}$/*Nf1*$^{Fcr/+}$/*Mx1-Cre* animals to generate the required genotypes. (B) Bone marrow was harvested from mice in each group and transplanted into syngeneic recipients. Four weeks after transplant and 2 weeks after induction of Cre recombinase, peripheral blood of recipient animals was immune-stained to measure the level of engraftment by Ly5.2^{+}/Ly5.1^{-} donor cells. Recipient mice showed greater than 70% engraftment by donor cells. Typical results are shown. (C) Eight weeks after transplantation, DNA was extracted from peripheral blood nucleated cells of recipient animals. Polymerase chain reaction analysis was performed on genomic DNA from each animal to determine the degree of deletion of the floxed *Nf1* allele. A band indicating deletion was detected in all animals from which adequate DNA was obtained. Typical results are shown for three animals. W: water; Δ: recombined flox allele.

analysis of circulating white blood cells (Figure 1B). Eight weeks after transplantation, peripheral blood was analyzed for deletion of the floxed *Nf1* allele (Figure 1C). In all animals from which adequate DNA was obtained, Cre-mediated recombination was detected in peripheral blood mononuclear cell DNA (*data not shown*).

Stat5 deficiency attenuates Nf1*-deficient myeloproliferative neoplasm*

To determine the potential contribution of Stat5 to the development of *Nf1*-deficient MPN, cells derived from $Nf1^{flox/flox}$ mice induced with pIpC to cause biallelic Nf1 deletion ($Nf1^{\Delta/\Delta}$) were used. Baseline levels of STAT5 phospho-



	Geometric Mean of Fluorescence (normalized for each experiment)					
	pSTAT5		pErk		pSTAT3	
	NF	WT	NF	WT	NF	WT
Basal	0.51	0.56	0.91	0.7	0.81	0.78
95% Confidence Interval	0.44-0.58	0.41-0.71	0.72-1.09	0.68-0.72	0.76-0.86	0.66-0.90

	Induction of Phosphorylation (magnitude of the difference of fluorescence from basal)					
	pSTAT5		pErk		pSTAT3	
	NF	WT	NF	WT	NF	WT
10' GM-CSF	1.09	0.7	0.69	0.47	0.26	0.18
95% Confidence Interval	0.93-1.25	0.46-0.94	0.19-1.19	0.43-0.51	0.21-0.31	0.04-0.32

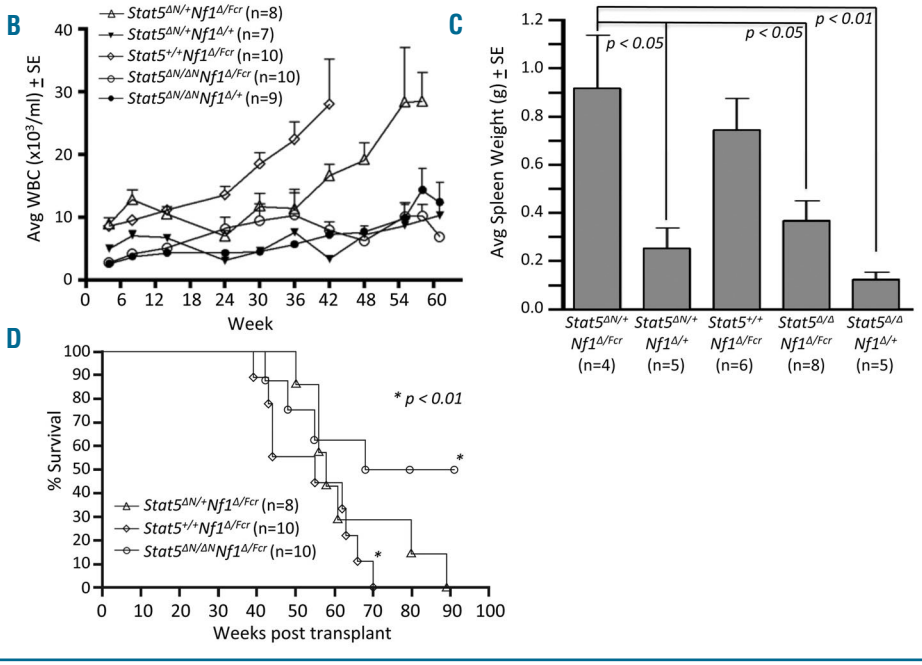


Figure 2. *Stat5* insufficiency alleviates MPN in *Nf1*-deficient mice. Bone marrow was harvested from donor mice, transplanted into syngeneic recipients, and allowed to engraft (as described in Figure 1). (A) Bone marrow was harvested from recipient animals [$Nf1^{\Delta/\Delta}$ (n=4) and $Nf1^{+/+}$ (n=3)], serum- and cytokine-starved, then stimulated with GM-CSF (10 ng/mL) for 10, 30 and 60 min. Levels of phosphorylated STAT5 (pSTAT5), ERK1/2 (pErk1/2), and STAT3 (pSTAT3) were measured using phospho-specific, intracellular flow cytometry of c-Kit$^+$/lineage$^-$ cells. A representative histogram is shown. Geometric mean of fluorescence is normalized for each experiment by dividing the geometric mean of the fluorescence of each sample by the average of the geometric mean of all the samples in each experiment. Induction of phosphorylation is reported as the fraction of basal levels. Induction of phosphorylation is calculated by subtracting the basal geometric mean of fluorescence from the geometric mean of fluorescence from each GM-CSF-stimulated sample; this difference is normalized for each sample by dividing by the basal geometric mean of fluorescence. (B) Peripheral blood from transplant recipients was collected every 6 weeks for the duration of experiments and with increased frequency in diseased animals. Total white blood cells counts (WBC) and peripheral blood smears (*data not shown*) were used to monitor the development of myeloproliferative disease in recipient mice. $P<0.01$, one way ANOVA followed by the Bartlett test for equal variance and the Tukey multiple comparison were performed for the comparison between $Stat5^{+/+}/Nf1^{\Delta N/Fcr}$ bone marrow and $Stat5^{\Delta N/+}/Nf1^{\Delta N/+}$, $Stat5^{\Delta N/\Delta N}/Nf1^{\Delta N/Fcr}$, and $Stat5^{\Delta N/\Delta N}/Nf1^{\Delta N/+}$ bone marrow. (C) Moribund animals were sacrificed and spleen weights assessed. ANOVA followed by the Tukey test for significant differences were performed. (D) Kaplan-Meier survival plot comparing overall survival of recipients with the indicated genetic background. n ≥ 7 recipients per group; Log-rank followed by chi square testing was performed. In all figure panels, error bars represent standard errors of the mean.

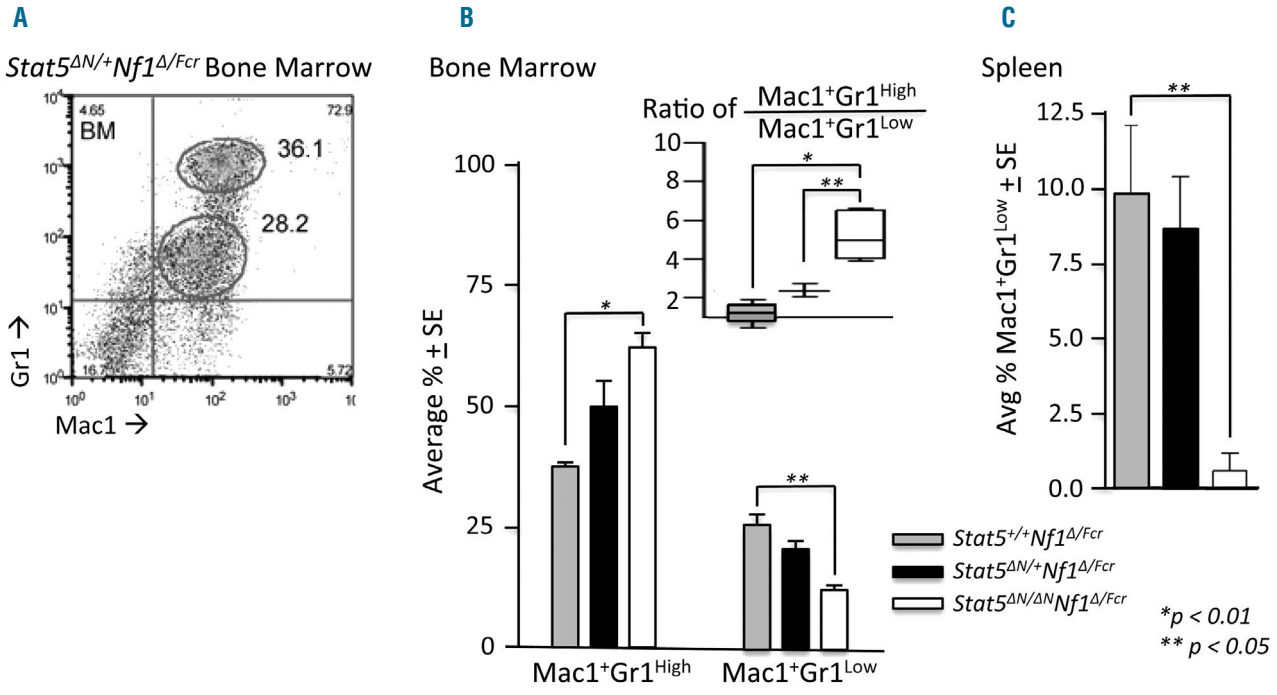


Figure 3. STAT5 insufficiency reverses immature myeloid expansion in *Nf1*-deficient mice. Surface immunophenotyping was performed on bone marrow and spleen mononuclear cells. (A) Representative immunophenotyping flow cytometry plot of *Nf1*-deficient bone marrow. (B) The proportion of cells from each immunophenotypic compartment in the bone marrow is indicated for each genotype. The inset shows the ratio of Mac1^{+}Gr1^{High} to Mac1^{+}Gr1^{Low} cells for each genotype. Two-way ANOVA followed by the Bonferroni post-test for significance was used for the main panel, and one way ANOVA followed by the Tukey multiple comparison test was used for the inset Mac1^{+}Gr1$^{High/Low}$ ratio. (C) The proportion of Mac1^{+}Gr1^{Low} cells in the spleen is indicated for each genotype. ANOVA followed by the Tukey test was used to determine significant differences. In all figure panels, n = 10 for *Stat5*$^{+/+}$/ *Nf1*$^{\Delta N/Fcr}$, n = 4 for *Stat5*$^{\Delta N/+}$/ *Nf1*$^{\Delta N/Fcr}$, and n = 6 for *Stat5*$^{\Delta N/\Delta N}$/ *Nf1*$^{\Delta N/Fcr}$; error bars represent standard errors of the mean.

rylation were comparable between *Nf1*$^{\Delta/\Delta}$ and *Nf1*$^{+/+}$ controls. GM-CSF-stimulation of cKit^{+} lineage$^{-/Low}$ populations (enriched for stem cells and progenitor cells) from these mice led to increased levels of STAT5 phosphorylation, a measure of STAT5 activation, in both populations. Notably, *Nf1*$^{\Delta/\Delta}$ cells achieved maximal levels of STAT5 phosphorylation within 10 min of stimulation, while *Nf1*$^{+/+}$ populations took 60 min to achieve comparable levels of STAT5 activation (Figure 2A). A more rapid response to cytokines in *Nf1*$^{\Delta/\Delta}$ cells tightly correlates with the hypersensitivity to GM-CSF observed in the hematopoietic compartment of these *Nf1*-mutant mice[10] and recapitulates the cytokine hyperresponsiveness described in other studies of MPN.[5,9,14,48-50] In contrast, these cells displayed a trend toward elevated levels of phosphorylated ERK (pERK) under basal conditions, without significant differences in induction of pERK. Phosphorylated STAT3 levels were similar between the two genotypes; GM-CSF stimulation did not significantly increase STAT3 phosphorylation in either group. Transplantation of *Stat5*$^{+/+}$/*Nf1*$^{\Delta/Fcr}$ bone marrow in this study resulted in MPN similar to that found in previous studies as assessed by elevated white blood cell counts and spleen weights[9,51] (Figure 2B,C and *Online Supplementary Tables S1-S5*). Hemoglobin concentration did not vary significantly by genotype and remained within the normal range (*Online Supplementary Figure S1A*). The platelet count remained in the low-normal range for all genotypes for the first year after transplantation (*Online Supplementary Figure S1B*), likely reflecting low-level radiation-induced bone marrow toxicity. After 1 year after transplantation, *Stat5*$^{\Delta N/\Delta N}$/ *Nf1*$^{\Delta/+}$ animals developed platelet counts that were significantly higher than those of the animals with other genotypes, but well within the normal range. Since these animals had an intact *Nf1* allele, this phenotype likely reflects the effect of isolated Stat5 deficiency on the platelet count. *Stat5*$^{+/+}$/*Nf1*$^{\Delta/Fcr}$ animals succumbed to MPN at a median of 55 weeks after transplantation (Figure 2D and *Online Supplementary Figure S1C*). In contrast, animals with a single, intact *Nf1* allele (*Nf1*$^{\Delta/+}$) did not develop MPN, as assessed by white blood cell counts and spleen weights (Figure 2B,C). Animals that received bone marrow harboring a single, intact *Stat5* allele and homozygous *Nf1* deficiency (*Stat5*$^{\Delta/+}$/*Nf1*$^{\Delta/Fcr}$) also developed MPN. These *Stat5*$^{\Delta/+}$/*Nf1*$^{\Delta/Fcr}$ recipients had a comparable median survival to that of *Stat5*$^{+/+}$/*Nf1*$^{\Delta/Fcr}$ recipients (58 *versus* 55 weeks) and comparable spleen sizes (average spleen 1.03 g *versus* 0.75 g, *P*=0.25) but did display a delay in the development of MPN as measured by peripheral white blood cell counts (Figure 2B). In contrast, recipients of *Nf1*$^{\Delta/Fcr}$ bone marrow lacking both copies of wild-type *Stat5* (*Stat5*$^{\Delta/\Delta}$/ *Nf1*$^{\Delta/Fcr}$) did not develop MPN (as determined by white blood cell counts and spleen weights) and had a prolonged median survival of 79 weeks. The experiment was terminated 80 weeks after the transplants. Three *Stat5*$^{\Delta/\Delta}$/ *Nf1*$^{\Delta/Fcr}$ animals died prior to this time point with no obvious cause of death but their premature death may be attributed to the complications of radiation exposure. The survival of animals of all geno-

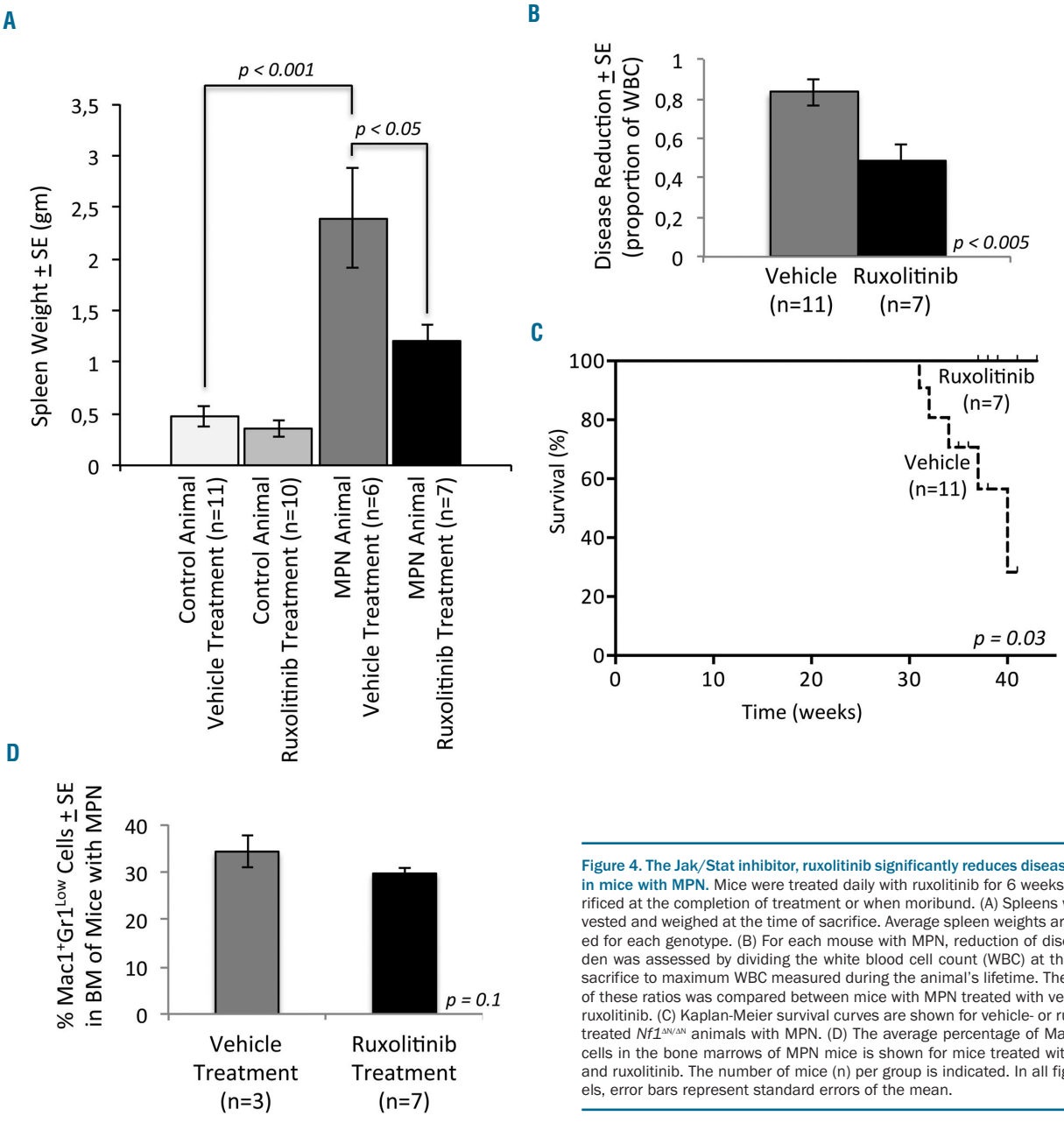


Figure 4. The Jak/Stat inhibitor, ruxolitinib significantly reduces disease burden in mice with MPN. Mice were treated daily with ruxolitinib for 6 weeks and sacrificed at the completion of treatment or when moribund. (A) Spleens were harvested and weighed at the time of sacrifice. Average spleen weights are indicated for each genotype. (B) For each mouse with MPN, reduction of disease burden was assessed by dividing the white blood cell count (WBC) at the time of sacrifice to maximum WBC measured during the animal's lifetime. The average of these ratios was compared between mice with MPN treated with vehicle and ruxolitinib. (C) Kaplan-Meier survival curves are shown for vehicle- or ruxolitinib-treated $Nf1^{\Delta N/\Delta N}$ animals with MPN. (D) The average percentage of $Mac1^{+}Gr1^{Low}$ cells in the bone marrows of MPN mice is shown for mice treated with vehicle and ruxolitinib. The number of mice (n) per group is indicated. In all figure panels, error bars represent standard errors of the mean.

types along with a comprehensive table of their clinical status is shown in the *Online Supplementary Material* (*Online Supplementary Figure S1C* and *Online Supplementary Table S1*). Chimerism was measured *via* Ly5.1/5.2 mismatching for all animals at the termination of the experiment and necropsy. All surviving $Stat5^{\Delta N/\Delta N}$ animals were sufficiently reconstituted with donor hematopoietic cells (average % donor cells ± SE in bone marrow = 83.3 ± 13.4 and 73.1 ± 16.2 for $Stat5^{\Delta N/\Delta N}Nf1^{\Delta Fcr}$ and $Stat5^{\Delta N/\Delta N}Nf1^{\Delta/+}$mice, respectively), yet did not develop disease. Only one animal showed chimerism with less than 60% donor cells (49.7%). Thus, the failure to observe MPN in $Stat5^{\Delta N/\Delta N}$ recipients could not be attributed to engraftment failure. These data show that the absence of any wild-type STAT5 abrogates the development of NF1-deficient MPN and demonstrate that STAT5 activity is a critical contributor to NF1-deficient MPN.

Stat5 *deficiency reverses myeloid precursor accumulation characteristic of myeloproliferative neoplasm*

MPN in $Stat5^{\Delta N/+}/Nf1^{\Delta Fcr}$ animals was typical of MPN associated with $Stat5^{+/+}/Nf1^{-/-}$ animals and did not differ significantly from that reported by other investigators[10] in terms of absolute leukocytosis, splenomegaly, and morphology (Figure 2 and *Online Supplementary Figure S1*). Furthermore, as has been described in other models of *Nf1*-deficient MPN,[10] homozygous *Nf1*-deficient animals displayed an expansion of immature myeloid precursors ($Mac1^{+}Gr1^{Low}$ double-positive cells) in the bone marrow (Figure 3A,B), spleen (Figure 3A,C), and peripheral blood

(*data not shown*). Homozygous *Stat5* deficiency reversed this expansion (Figure 3). Interestingly, mice with *Stat5* heterozygous, *Nf1*-deficient MPN displayed an expansion of the Mac1^{+}Gr1^{Low} (immature precursors) compartment that was intermediate between the expansion of this compartment in *Stat5*$^{\Delta N/\Delta N}$ and *Stat5*$^{+/+}$ animals. This intermediate phenotype suggests that haploinsufficiency at the *Stat5* locus may abrogate MPN as well.

The Jak2 inhibitor, ruxolitinib, diminishes myeloproliferative neoplasm

Next, we investigated whether pharmacological inhibition of the Jak/Stat pathway abrogates MPN initiated by *Nf1* deficiency. We used ruxolitinib because Jak inhibition with ruxolitinib has been shown to attenuate Stat5 activation.[52] We analyzed the bone marrow and spleen compartments of *Mx1-Cre, Nf1*$^{flox/flox}$ animals.[10] Treatment with pIpC homozygously ablates the *Nf1* locus (*Nf1*$^{\Delta/\Delta}$) in the hematopoietic compartment of these mice leading to JMML-like MPN.[10] These mice were treated with pIpC at 2 months of age and aged for an additional 6 months to allow MPN to develop before treatment with ruxolitinib. The mice were treated twice daily with ruxolitinib for 6 weeks. Complete blood counts were obtained weekly throughout the treatment period. At the completion of treatment, mice were sacrificed and bone marrow and spleens were harvested. *Nf1*-deficient animals developed MPN characterized by splenomegaly, leukocytosis, and anemia (Figure 4A, *Online Supplementary Figure S2A,B*, *Online Supplementary Tables S6-S9*). Platelet counts remained within the normal range but were higher in *Nf1*-deficient, vehicle-treated animals than in animals retaining a wild-type copy of the *Nf1* allele (*Online Supplementary Figure S2C*). Ruxolitinib treatment attenuated MPN in mice, as evidenced by reduced spleen size (Figure 4A). White blood cell counts varied significantly among the mice with MPN (*Online Supplementary Figure S2A*). Ruxolitinib therapy reduced the white blood cell count by 50% in these MPN animals (Figure 4B). Ruxolitinib treatment was also associated with worsening anemia in animals with MPN (as has been described in clinical trials with this agent) but did not reduce the hemoglobin concentration of animals without MPN (*Online Supplementary Figure S2B*). Additionally, ruxolitinib was associated with a reduction in platelet count that was more pronounced in animals with MPN (*Online Supplementary Figure S2C*). All of the ruxolitinib-treated *Nf1*$^{\Delta/\Delta}$ mice survived until completion of the experiment. In contrast, 5/13 (38%) of vehicle-treated *Nf1*$^{\Delta/\Delta}$ mice succumbed to MPN during the treatment course (Figure 4C). Although this experiment was not designed to detect the effect of ruxolitinib on survival, the difference that we observed is statistically significant (P<0.05). Since the mice were sacrificed at completion of therapy, the difference in survival between the two treatment groups likely under-estimates the effect of ruxolitinib on survival. In accordance with our findings in *STAT5*$^{\Delta/\Delta}$ mice, Jak/Stat inhibition with ruxolitinib tended to reduce the percentage of Mac1^{+}Gr1^{Low} cells in the bone marrow of mice with *Nf1*$^{\Delta/\Delta}$ MPN, although this trend was not statistically significant (Figure 4D). These data demonstrate that a targeted inhibitor of Jak/Stat signaling is efficacious in attenuating the clinical features of *Nf1*-deficient MPN in mice.

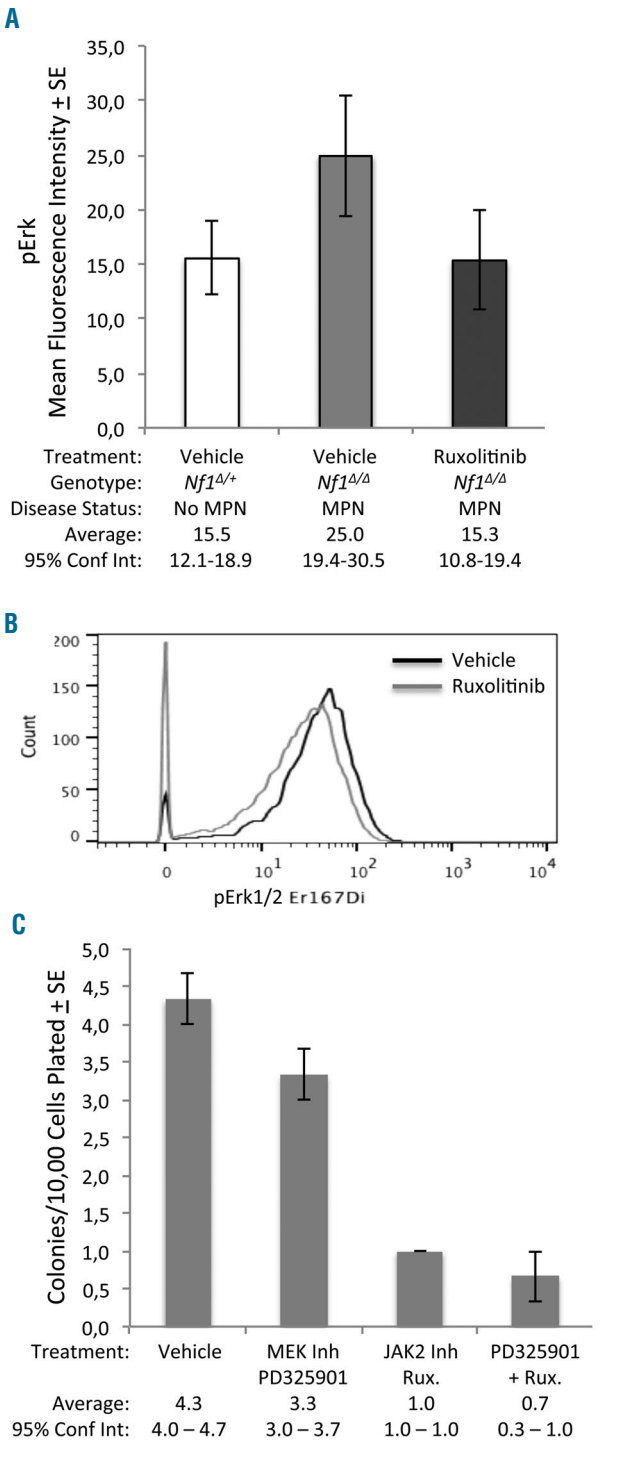


Figure 5. Jak/Stat inhibition inhibits ERK phosphorylation and colony formation in RAS-activated MPN. (A) Intracellular, phospho-specific flow cytometry was performed on bone marrow cells from vehicle-treated *Nf1*$^{\Delta/+}$ control mice (n=12), vehicle-treated *Nf1*$^{\Delta/\Delta}$ MPN mice (n=3), and ruxolitinib-treated *Nf1*$^{\Delta/\Delta}$ MPN mice (n=7). Average mean fluorescence intensity representing levels of phospho-Erk are shown for each cohort. (B) Primary bone marrow mononuclear cells from a patient with *KRAS*-mutant (*KRAS*^{G13D}) JMML were incubated with vehicle or ruxolitinib (4 μm) for 30 min before fixation and permeabilization. Levels of phospho-Erk were assessed by mass cytometry. (C) Peripheral blood mononuclear cells from a patient with KRAS-mutant JMML were plated in methylcellulose containing MEK inhibitor, JAK inhibitor, both inhibitors, or vehicle. Each condition was plated in three replicates. Colony formation was scored after 7 days. In all figure panels, error bars represent standard errors of the mean. 95% confidence intervals are indicated.

Stat5 activity is implicated in maintaining signaling through the Ras/Nf1 pathway.

Bone marrow cells of *Nf1*-deficient (*Mx1-Cre*, $Nf1^{\Delta/\Delta}$) mice with MPN displayed hyperactive Erk signaling in comparison to bone marrow from *Mx1-Cre*, $Nf1^{\Delta/+}$ controls with no MPN (Figure 5A). Ruxolitinib treatment, which inhibits the Jak/Stat pathway, reduced levels of phosphorylated Erk in *Nf1*-deficient bone marrow (Figure 5A). This finding, along with our data from $Stat5^{\Delta N/\Delta N}/Nf1^{\Delta/Cr}$ mice (Figures 2 and 3), suggests that sustained Stat5 signaling may be required to maintain hyperactive Mek/Erk signaling conferred by *Nf1* deficiency. Indeed, mononuclear cells of mice treated with ruxolitinib showed a trend to reduced pSTAT5 induction in response to *in vitro* GM-CSF stimulation, although this trend was not statistically significant (*Online Supplementary Figure S2D*). To investigate whether the STAT5 and RAS pathways are similarly inter-connected in human MPN, we studied a patient with JMML harboring a *KRAS* mutation ($KRAS^{G13D}$). As in our murine model, ruxolitinib treatment led to reduced levels of pErk in bone marrow mononuclear cells from this patient (Figure 5B). Treatment with a MEK inhibitor (PD325901) or a JAK inhibitor (ruxolitinib) led to a decrease in colony formation in methylcellulose by peripheral blood mononuclear cells from this patient (Figure 5C). Notably, JAK inhibition had a more profound effect on colony formation than had MEK inhibition. Simultaneous treatment with both inhibitors gave results similar to those with inhibition of JAK alone. These data suggest that active JAK/STAT is required for the proliferative phenotype of MPN with hyperactive RAS.

Discussion

In this study, we used genetic and pharmacological approaches to demonstrate the importance of Stat5 in the pathogenesis of MPN initiated by *Nf1* inactivation. We showed that MPN in *Nf1*-deficient, *Stat5* hypomorphic mice is significantly diminished, leading to prolonged survival, improvement in blood count indices, and reduced spleen size in comparison to *Nf1*-deficient mice with intact *Stat5* genes. Similarly, *Nf1*-deficient mice treated with ruxolitinib, an inhibitor of Jak/Stat signaling, had attenuated MPN with reduced white blood cell counts and smaller spleens. Both approaches tended to reverse the $Mac1^{+}Gr^{Low}$ immature myeloid cell accumulation seen in *Nf1*-deficient MPN bone marrow. We showed that ruxolitinib treatment diminished Erk signaling in these mice and in the bone marrow of a *KRAS*-mutated JMML patient. Ruxolitinib treatment also inhibited colony formation of primary cells from this JMML patient. Our ruxolitinib data implicate the Jak/Stat pathway in the pathogenesis of MPN but do not rule out effects of other STAT in the phenotype we observed. However, the ruxolitinib data, together with the data from our genetic Stat5-deficient model, suggest that STAT5 can modulate RAS-activated MAPK pathway activity.

The *Stat5* alleles utilized in these experiments express an N-terminally deleted form of *Stat5* that retains partial Stat5 function.[42,43] Nevertheless, we showed that attenuation of Stat5 with retention of residual Stat5 function was sufficient to alleviate MPN in our genetic model. In a mouse model of MPN mediated by *Mpl* mutation, which leads to tonic activation of the Jak2/Stat5 pathway, conditional genetic ablation of *Jak2* (*via* floxed alleles) was sufficient to induce complete remission of MPN, while ruxolitinib treatment of these *Mpl* mutant mice could only attenuate the disease.[53] This study indicates that ruxolitinib does not completely inhibit Jak2 signaling, a finding that is consistent with clinical trials that show that ruxolitinib improves the clinical parameters of MPN but does not cure the disease.[32-34,54] Likewise, incomplete inactivation of Stat5 with ruxolitinib alleviated many features of MPN in our model. Our data indicate a reduction of STAT5 activity, as is clinically attainable with ruxolitinib, may be sufficient to alleviate disease.

Activation of JAK-STAT and RAS signaling are both common features of myeloid leukemias.[30,55,56] Previous work has demonstrated that MEK inhibition can attenuate myeloid neoplasia but is insufficient to cure this disease in either mouse models or human patients.[23,56-58] $Kras^{G12D}$ myeloid cells remain hypersensitive to cytokines (according to colony-forming assays) despite MEK inhibition.[23] Jak inhibition abrogates GM-CSF-dependent ERK phosphorylation in $Kras^{G12D}$ myeloid cells.[59] These results indicate that other pathways contribute to disease in leukemias with hyperactive RAS signaling. Our work suggests activation of the STAT5 pathway may provide these critical signals in leukemia. Two recent reports from independent groups show that activated NRAS directs self-renewal in hematopoietic[60] and leukemia stem cells.[61] Gene expression analyses by both of these groups revealed that oncogenic NRAS led to activation of Stat5-mediated gene transcription and confirmed a relationship between RAS and STAT5 activity.

Early T-precursor acute lymphoblastic leukemia (ETP ALL) is a treatment-resistant, fatal leukemia with a mutational and gene expression signature comparable to that of poor-risk acute myeloid leukemia.[62-65] Like acute myeloid leukemia, most ETP ALL harbor mutations that activate RAS and RAS pathway components.[63,65,66] *JAK1* and *JAK3* are also commonly mutated in ETP ALL.[65,66] Recently, activated STAT5 (phospho-STAT5) levels were found to be elevated in all ETP-ALL cases tested. This elevation was not related to *JAK* mutational status but to surface levels of interleukin-7 receptor (a receptor known to activate RAS, JAK/STAT, and PI3K signaling[67,68]). Intriguingly, ruxolitinib treatment of ETP-ALL xenografts led to profound reduction in disease, independently of *JAK* mutational status.[67] Analogous to our work, this study also demonstrated the efficacy of STAT5 inhibition in leukemia with hyperactive RAS signaling.

Despite the considerable strides in the development of targeted therapies for treating myeloid neoplasms, there are no chemotherapy or targeted treatment options that have been shown to improve outcomes in JMML. As >90% of JMML patients exhibit activated RAS signaling, our data suggest that combination therapy with RAS-pathway inhibitors and ruxolitinib may be an effective, rational therapeutic strategy in this disease. MEK inhibition in mice with Nf1-deficient or Ras-activated MPN led to improvements in disease parameters but failed to eradicate leukemia cells.[24,40] Likewise, MEK inhibition in early phase clinical trials in acute myelogenous leukemia has yielded largely disappointing results.[57] In contrast, combined MEK and JAK/STAT inhibition in an $NRAS^{G12D/G12D}$ model of MPN significantly improved survival of these mice.[40] Our JMML patient harbors a *KRAS* mutation, yet ruxolitinib was more effective than MEK inhibition at controlling colony formation of the patient's cells. These data provide a rationale for

clinical trials combining ruxolitinib with RAS-pathway inhibitors to control activated RAS myeloid neoplasia.

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