β common receptor inactivation attenuates myeloproliferative disease in *Nf1* mutant mice

Andrew Kim,[1] Kelly Morgan,[2] Diane E. Hasz,[2] Stephen M. Wiesner,[2] Jennifer O. Lauchle,[1] Jennifer L. Geurts,[2] Miechaleen D. Diers,[2] Doan T. Le,[1] Scott C. Kogan,[3,4] Luis F. Parada,[5] Kevin Shannon,[1,4] and David A. Largaespada[2]

[1]Department of Pediatrics, University of California San Francisco; [2]Department of Genetics, Cell Biology and Development, and Cancer Center, University of Minnesota, Minneapolis; [3]Department of Laboratory Medicine and [4]Cancer Center, University of California San Francisco; [5]Center for Developmental Biology and Kent Waldrep Foundation Center for Basic Neuroscience Research on Nerve Growth and Regeneration, University of Texas Southwestern Medical Center, Dallas

Neurofibromatosis type 1 (NF1) syndrome is caused by germline mutations in the *NF1* tumor suppressor, which encodes neurofibromin, a GTPase activating protein for Ras. Children with NF1 are predisposed to juvenile myelomonocytic leukemia (JMML) and lethally irradiated mice given transplants with homozygous *Nf1* mutant *(Nf1$^{-/-}$)* hematopoietic stem cells develop a fatal myeloproliferative disorder (MPD) that models JMML. We investigated the requirement for signaling through the GM-CSF receptor to initiate and sustain this MPD by generating *Nf1* mutant hematopoietic cells lacking the common β chain *(Beta c)* of the GM-CSF receptor. Mice reconstituted with *Nf1$^{-/-}$, beta c$^{-/-}$* stem cells did not develop evidence of MPD despite the presence of increased number of immature hematopoietic progenitors in the bone marrow. Interestingly, when the *Mx1-Cre* transgene was used to inactivate a conditional *Nf1* mutant allele in hematopoietic cells, concomitant loss of *beta c$^{-/-}$* reduced the severity of the MPD, but did not abrogate it. Whereas inhibiting GM-CSF signaling may be of therapeutic benefit in JMML, our data also demonstrate aberrant proliferation of *Nf1$^{-/-}$* myeloid progenitors that is independent of signaling through the GM-CSF receptor. (Blood. 2007;109: 1687-1691)

Introduction

Neurofibromatosis type 1 (NF1) is a common familial cancer syndrome that is associated with increased risk of specific neoplasms.[1] Children with NF1 are predisposed to juvenile myelomonocytic leukemia (JMML), a myeloproliferative disorder (MPD) characterized by leukocytosis, organomegaly with myeloid infiltration, hypersensitivity of myeloid progenitors to granulocyte-macrophage colony-stimulating factor (GM-CSF), and a poor prognosis.[2,3] *NF1* encodes neurofibromin, a GTPase-activating protein (GAP) that accelerates the hydrolysis of active GTP-bound Ras to inactive Ras-GDP.[4,5] NF1-associated tumors frequently demonstrate somatic loss of the normal *NF1* allele, and extensive data support the idea that *NF1* functions as a tumor suppressor by negatively regulating Ras signaling.[6]

Mice with a heterozygous germline *Nf1* mutation develop many of the tumors seen in persons with NF1, which show loss of the normal allele.[7] Homozygous *Nf1* inactivation is lethal at about E13[7,8]; however, fetal hematopoietic cells from these embryos are hypersensitive to GM-CSF in vitro and induce a JMML-like MPD when transplanted into irradiated recipient mice.[9,10] Recently, a conditional *Nf1* mutant allele and the interferon-inducible *Mx1-Cre* strain were harnessed to induce somatic *Nf1* inactivation in hematopoietic cells. Like recipients given transplants with *Nf1$^{-/-}$* fetal liver cells, these *Mx1-Cre, Nf1$^{flox/flox}$* mice consistently develop MPD.[11]

The GM-CSF receptor shares a common signaling β subunit (beta c) with the receptors for interleukins 3 and 5.[12] Unlike humans, the mouse has 2 homologous β subunits: beta c and $\beta_{IL\text{-}3}$. The GM-CSF and IL-5α subunits only pair with beta c, whereas IL-3α forms heterodimers with both beta c and $\beta_{IL\text{-}3}$.[13] Bone marrow cells from homozygous *beta c* mutant (*beta c$^{-/-}$*) mice respond to IL-3 but not GM-CSF. These mice maintain normal leukocyte counts.[14] We intercrossed *beta c* and *Nf1* mutant mice to determine the effects on progenitor populations and the MPD phenotype. We find that loss of beta c attenuates the severity of MPD in *Nf1* mutant mice. However, we also demonstrate that *Nf1* inactivation expands immature progenitors even in the absence of beta c.

Materials and methods

Mouse strains

The *Mx1-Cre*,[15] *Beta c* mutant,[14] *Nf1* mutant,[8] and conditional *Nf1* mutant[16] mice used in these studies have been described in detail.

Studies of *Nf1* mutant fetal liver cells

The experimental procedures for intercrossing mice, genotyping fetal tissues using the polymerase chain reaction (PCR), and adoptive transfer have been described.[9] The primer sequences and PCR conditions are available on request. Briefly, fetal livers from E12.5 *Nf1$^{-/-}$* embryos and *Nf1$^{-/-}$, beta c$^{-/-}$*; *Nf1$^{+/-}$, beta c$^{-/-}$*; and *Nf1$^{+/+}$, beta c$^{-/-}$* embryos were pooled and injected into congenic recipients (B6.SJL-PtprcaPep3b/BoyJ) that had been lethally irradiated with 900 rad. At 4 to 8 weeks after transplantation, bone marrow was harvested from primary recipients and intravenously injected into lethally irradiated secondary

Submitted May 30, 2006; accepted September 24, 2006. Prepublished online as *Blood* First Edition Paper, November 7, 2006; DOI 10.1182/blood-2006-05-025395.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

recipients (B6.SJL-PtprcaPep3b/BoyJ). Mice were housed in the University of Minnesota Research Animal Resources facilities and all procedures were approved by Institutional Animal Care and Use Committee.

Studies in *Mx1-Cre, Nf1$^{flox/flox}$, beta c* mutant mice

Mx1-Cre, Nf1$^{flox/flox}$ mice of all 3 beta c genotypes were generated by intercrossing. Pups that received a single intraperitoneal injection of polyinosinic-polycytidylic acid (pI-pC) at 3 to 5 days of age to activate Cre recombinase expression from the *Mx1* promoter were genotyped and monitored as described previously.[11] *Mx1-Cre, Nf1$^{flox/flox}$* mice were maintained in a sterile animal care facility under a protocol that was approved by the University of California San Francisco Committee on Animal Research.

Complete blood counts, flow cytometry, and pathological analysis

Blood counts were measured in Coulter ZBI (Beckman Coulter, Fullerton CA) or Hemavet 850 (CDC Technologies, Oxford, CT) instruments. Bone marrow and blood leukocytes were analyzed by flow cytometry on a FACSCalibur with data analysis via CELLQuest Pro software (BD Biosciences, Franklin Lakes, NJ). Tissue sections from control and mutant mice were stained with hematoxylin and eosin, visualized using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with either a 4×/0.10 numerical aperture (NA) or a 50×/0.90 NA objective. Images were photographed using a Nikon Coolpix 5000 digital camera.

Colony-forming unit assays

Colony-forming unit granulocyte-erythrocyte-megakaryocyte-macrophage (CFU-GEMM) assays were performed by seeding bone marrow (3×10^4) or spleen cells (1×10^5) in Methocult (M3434; StemCell Technologies, Vancouver, BC, Canada). Colonies were enumerated at day 7. Spleen colony-forming unit (CFU-S) assays were performed by injecting bone marrow cells (6×10^4) or splenocytes (2×10^5) intravenously into C57BL/6 recipients that were irradiated with 750 rad. Spleens were harvested from day 8 and fixed in Tellyesniczky solution (64% ethanol, 5% acetic acid, 2% formaldehyde) to enumerate surface colonies. Splenic CFU-GMs were enumerated from *Mx1-Cre, Nf1$^{flox/flox}$* and control mice in M3231 medium containing a saturating concentration of IL-3.

Statistical analysis

All graphical and statistical analyses were performed using StatView (SAS Institute, Cary, NC) software with statistical significance determined by ANOVA with Fisher post-hoc tests.

Results

beta c inactivation suppresses MPD in recipients of *Nf1* mutant fetal liver cells

Nf1$^{+/-}$, beta c$^{-/-}$ compound mutant mice on an inbred C57BL/6 background were mated to generate E12.5 embryos (Figure 1A). Fetal livers were removed and made into single-cell suspensions while a portion of embryonic tissue was used to genotype the *Nf1* locus. *Nf1$^{-/-}$, beta c$^{-/-}$* and *Nf1$^{+/-}$, beta c$^{-/-}$* cells were pooled separately and 2×10^6 cells were injected intravenously into lethally irradiated congenic mice. The C57BL6/J recipients were syngenic to the donor mice, but expressed a variant allele of the CD45 cell surface antigen (CD45.1) to distinguish donor-derived from recipient hematopoietic cells by flow cytometry. To increase the number of recipient mice for subsequent analysis, the primary recipients were killed 4 to 8 weeks after adoptive transfer and 5 million bone marrow cells were injected into irradiated congenic CD45.1$^+$ hosts (Figure 1B). These secondary recipients were monitored for at least 13 months or until they developed overt MPD. Flow cytometric analysis of primary and secondary recipients demonstrated high levels of CD45.2 chimerism in recipient bone marrows, thereby confirming that they were derived from the transplanted fetal livers (Figure 2A). None of the recipients repopulated with *Nf1$^{-/-}$, beta c$^{-/-}$* or *Nf1$^{+/-}$, beta c−/−* cells showed leukocytosis, splenomegaly, or any other evidence of MPD (Figure 2B). Qualitative measurements of disease burden in mice with MPD correlate with increased numbers of mature myeloid lineage cells that express the surface markers Gr-1 and Mac-1. Animals from both cohorts exhibited 52% to 68% Gr-1/Mac-1 double-positive cells in the bone marrow and 12% to 20% in the peripheral blood. In addition, flow cytometric analysis of splenic cells revealed only 2% to 7% Gr-1/Mac-1 cells (data not shown). By contrast, recipients given transplants with C57BL/6 *Nf1$^{-/-}$* fetal liver cells develop leukocytosis and splenomegaly with myeloid infiltration 4 to 6 months after adoptive transfer in primary and secondary recipients.[17-19]

Bone marrow CFU-GEMMs and CFU-Ss are increased in recipients of *Nf1$^{-/-}$, beta c$^{-/-}$* fetal liver cells

Nf1$^{-/-}$ fetal liver cells form abnormal numbers of CFU-GM colonies in methylcellulose cultures containing low concentrations of GM-CSF.[9-11] The absolute number of immature progenitors is also elevated in the bone marrows of mice repopulated with *Nf1* mutant fetal liver cells.[20]

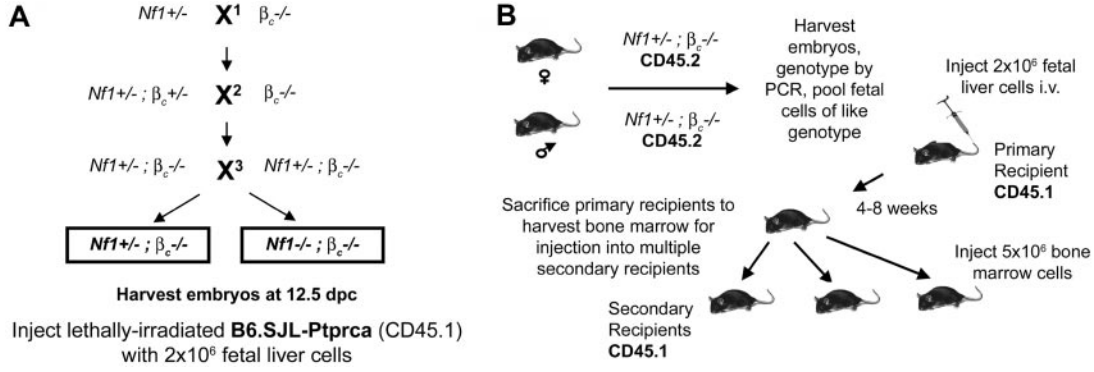

Figure 1. Experimental design of transplants. (A) Three classes of embryos result from the genetic crosses. X^1 is an outcross of *Nf1$^{+/-}$* to $\beta_c^{-/-}$ mice. This is to establish *Nf1* and β_c null alleles within the same animal. X^2 is another outcross to $\beta_c^{-/-}$ mice to homozygose the β_c allele in *Nf1$^{+/-}$*, $\beta_c^{-/-}$ mice. By intercrossing, *Nf1$^{+/-}$*, $\beta_c^{-/-}$ mice X^3, the 2 classes of embryos are generated. (B) To test the effect loss of β_c has on *Nf1$^{-/-}$*-induced MPD, *Nf1$^{+/-}$*, $\beta_c^{-/-}$ mice are intercrossed and fetal livers from embryos of the correct genotype are harvested. Lethally irradiated recipients are injected with 2 million fetal liver cells and allowed a 4- to 8-week recovery period. These primary recipients are then killed to provide bone marrow for secondary recipient transplantation.

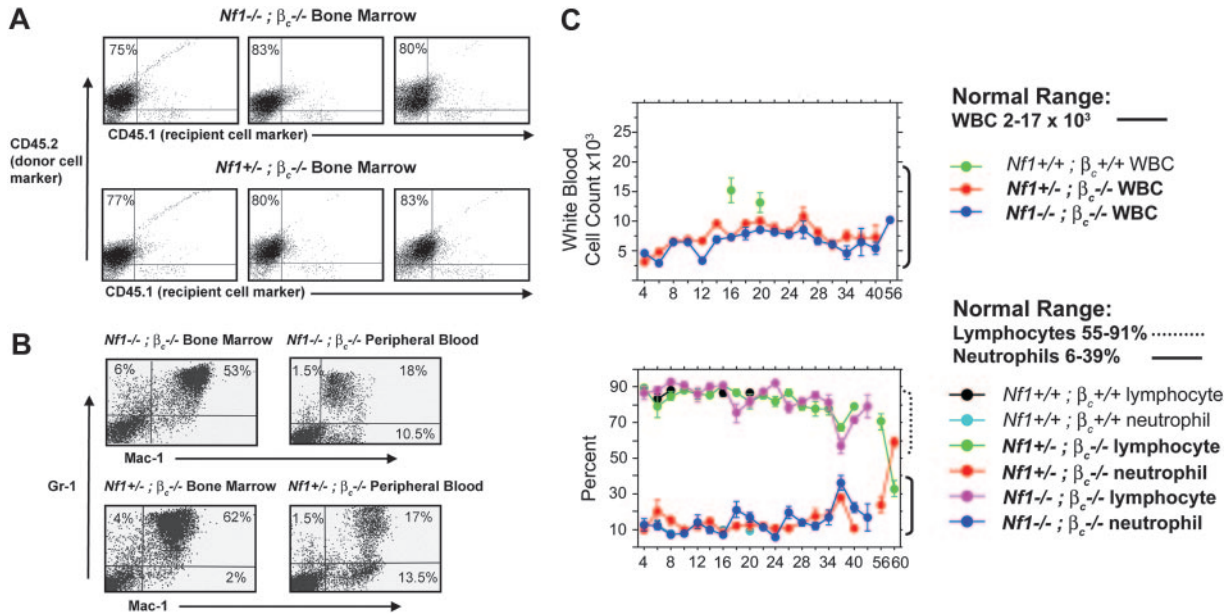

Figure 2. Engraftment but lack of MPD in *Nf1*$^{-/-}$, *beta c*$^{-/-}$ recipients. (A) Bone marrow from 3 representative irradiated mice given transplants with *Nf1*$^{-/-}$, *beta c*$^{-/-}$ (top panel) and *Nf1*$^{+/-}$, *beta c*$^{-/-}$ (bottom panel) cells was mostly comprised of cells expressing donor CD45.2, with only a few cells staining positive for CD45.1. Recipients that do not receive donor cells do not stain for the donor-cell marker (data not shown). (B) Flow cytometric analysis of 2 representative mice. Bone marrow and peripheral blood stained for the cell-surface markers Gr-1 and Mac-1, markers commonly associated with mature granulocytes. Similar staining profiles were seen in all recipients. Ranges of double-positive Gr-1/Mac-1 cells in both cohorts, bone marrow 52% to 68% and peripheral blood 12% to 20%. (C) Peripheral-blood analysis monitored over 46 weeks *Nf1*$^{+/-}$, *beta c*$^{-/-}$ and *Nf1*$^{-/-}$, *beta c*$^{-/-}$ secondary recipients. Animals that display symptoms of MPD would show drastic increases in total WBC counts outside of the range indicated by the bar. Left graph illustrates peripheral-blood analysis monitored over 56 weeks *Nf1*$^{+/-}$, *beta c*$^{-/-}$ and *Nf1*$^{-/-}$, *beta c*$^{-/-}$ secondary recipients. Upper lines are lymphocyte percentages and lower lines are neutrophil percentages. Animals that display symptoms of MPD would show drastic increases in neutrophil percentages and reductions in lymphocytes, with values outside of the normal ranges indicated by the dotted (lymphocyte) and solid (neutrophil) bars. Four data points included for *Nf1*$^{+/+}$, *beta c*$^{+/+}$ primary recipients to illustrate normal values.

Based on these data, we investigated progenitor colony growth in recipients of transplants with *Nf1*$^{-/-}$, *beta c*$^{-/-}$ and *Nf1*$^{+/-}$, *beta c*$^{-/-}$ fetal liver cells. Whereas splenocytes from *Nf1*$^{-/-}$, *beta c*$^{-/-}$ and *Nf1*$^{+/-}$, *beta c*$^{-/-}$ recipients yielded similar numbers of progenitor colonies, bone marrow cells from *Nf1*$^{-/-}$, *bta c*$^{-/-}$ recipients contained significantly more CFU-GEMMs (Figure 3). We also observed increased numbers of day 8 CFU-S colonies in the bone marrows of recipients of transplants with *Nf1*$^{-/-}$, *beta c*$^{-/-}$ versus *Nf1*$^{+/-}$, *beta c*$^{-/-}$ cells (Figure 3).

Loss of β_c attenuates MPD in *Mx1-Cre, Nf1*$^{flox/flox}$ mice

While our adoptive transfer experiments were in progress, Zhu and colleagues reported a conditional mutant *Nf1* allele, which they generated by flanking exons 31 and 32 with loxP sites.[16] Somatic inactivation of this *Nf1*^{flox} allele, which is functionally wild-type in the basal state, can be achieved by expressing Cre recombinase in specific tissues. *Mx1-Cre, Nf1*$^{flox/flox}$ mice in a mixed C57BL/6 × 129/Sv genetic background consistently develop a JMML-like MPD that closely resembles the disorder seen in mice given transplants with *Nf1*$^{-/-}$ fetal liver cells.[11] We generated *Mx1-Cre, Nf1*$^{flox/flox}$ mice of all 3 possible β_c genotypes to examine the effects of ablating GM-CSF signaling on the MPD phenotype in this model. Cre recombinase was induced in offspring of *Mx1-Cre, Nf1*$^{flox/flox}$, *beta c*$^{+/-}$ intercrosses at 3 to 5 days of age by injection of pI-pC.[11] PCR analysis of DNA extracted from blood leukocytes at 6 weeks of age demonstrated efficient *Mx1-Cre* transgene-dependent inactivation of *Nf1* (data not shown).

Blood counts were compared over time in *Mx1-Cre*$^{+}$ and *Mx1-Cre*$^{-}$ *Nf1*$^{flox/flox}$ mice of different *beta c* genotypes. Because mice with heterozygous inactivation of *beta c* were similar to *beta c*$^{+/+}$ animals (data not shown), we focused on 4 cohorts: (1) *Mx1-Cre, Nf1*$^{flox/flox}$, *beta c*$^{+/+}$; (2) *Mx1-Cre, Nf1*$^{flox/flox}$, *beta c*$^{-/-}$; (3) *Nf1*$^{flox/flox}$, *beta c*$^{+/+}$; and (4) *Nf1*$^{flox/flox}$, *beta c*$^{-/-}$. Leukocyte counts were significantly elevated in the *Mx1-Cre, Nf1*$^{flox/flox}$ animals by 3 months of age irrespective of *beta c* genotype, which

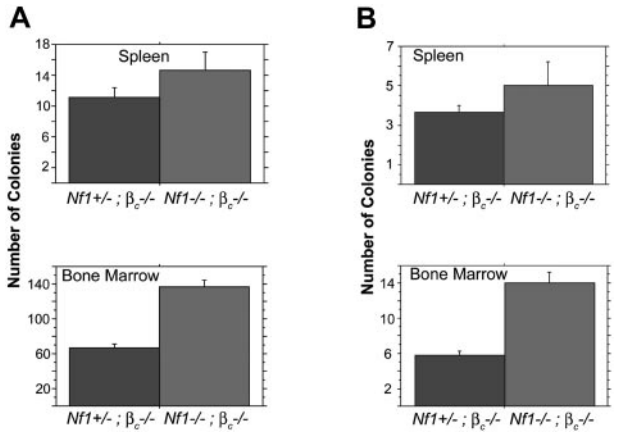

Figure 3. Recipients of *Nf1*$^{-/-}\beta_c^{-/-}$ bone marrow have increased CFCs and CFU-Ss. (A) Spleen and bone marrow cells from 5 secondary mice given transplants with *Nf1*$^{-/-}$, $\beta_c^{-/-}$ or 4 secondary mice given transplants with *Nf1*$^{+/-}$, $\beta_c^{-/-}$ fetal stem cells were assayed for CFU-GMs, -Gs, -Ms, and -GEMMs and erythroid burst-forming units. Spleen cells from animals given transplants do not show a significant difference from sample to sample, $P = .164$. Bone marrow plated from 5 mice given transplants with *Nf1*$^{-/-}$, $\beta_c^{-/-}$ or *Nf1*$^{+/-}$, $\beta_c^{-/-}$ fetal stem cells have a significantly higher number of cells capable of colony formation than the *Nf1*$^{+/-}$, $\beta_c^{-/-}$ transplants, $P < .001$. (B) Spleen and bone marrow cells from 6 mice given transplants with *Nf1*$^{-/-}$, $\beta_c^{-/-}$ or 9 mice given transplants with *Nf1*$^{+/-}$, $\beta_c^{-/-}$ fetal stem cells were injected intravenously into mice irradiated with 750 rads to assay for CFU-Ss. Injected spleen cells do not show a significant difference from sample to sample, $P = .228$. Bone marrow cells in animals given *Nf1*$^{-/-}$, $\beta_c^{-/-}$ transplants have a significantly higher number of CFU-S progenitor cells than the *Nf1*$^{+/-}$, $\beta_c^{-/-}$ transplants, $P < .001$. Error bars indicate SE.

was due to increased numbers of lymphoid and myeloid cells (Figure 4A-B). The leukocyte counts of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* mice were persistently elevated at 6 months of age, whereas the counts of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice were similar to those of *beta c$^{+/+}$* and *beta c$^{-/-}$* mice that did not inherit the *Mx1-Cre* transgene and therefore retained wild-type *Nf1* function (Figure 4A). *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* mice developed MPD between 5 and 7 months of age, characterized by hunching, abnormal gait, a disheveled appearance, and rising white blood cell (WBC) counts (Figure 4A). These sick mice were humanely killed and analyzed with littermates of the other 3 groups. Importantly, none of the *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* animals were killed due to signs of systemic illness. The bone marrows of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* mice were highly cellular with myeloid cells at various stages of differentiation and a paucity of erythroid elements, whereas *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* marrows showed less myeloid proliferation and residual erythropoiesis (data not shown). Pathologic analysis of sick *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* mice also uncovered extensive splenic infiltration by myeloid and erythroid cells with effacement of germinal centers (Figure 5A). Splenic infiltration was also present in some age-matched *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice, but was typically less prominent than in *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* littermates (Figure 5A). Fluorescence-activated cell sorting (FACS) analysis demonstrated substantial numbers of erythroid (Ter119^{+} or CD71^{+} or both) and myeloid (Gr-1^{+} or Mac-1^{+} or both) cells in the spleens of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* and *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice that had morphologic evidence of infiltration (Figure S1, available on the *Blood* website; see the Supplemental Figure link at the top of the online article). We also observed extensive periportal invasion within the livers of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* animals that was markedly reduced in *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mutant mice (Figure 5A). The spleens of *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* and *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice were enlarged (Figure 5B) and contained granulocyte-macrophage progenitors (Figure 5C).

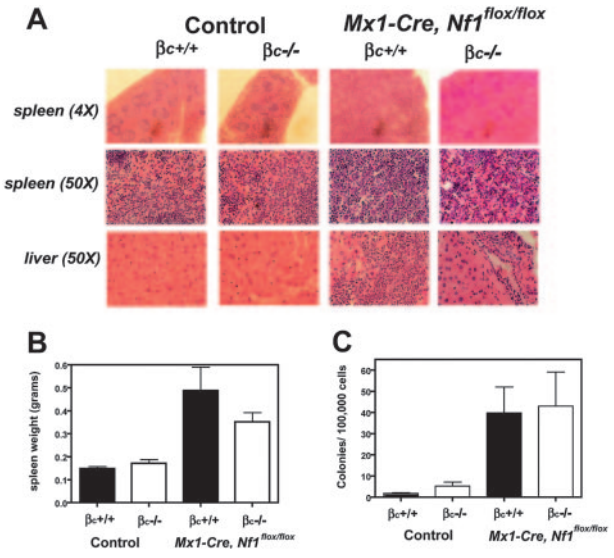

Figure 5. Splenic infiltration in *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* and *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* mice. (A) Inactivation of *Nf1* leads to increased numbers of myeloid lineage cells in spleen and liver, which is most pronounced in 6 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* animals and absent in 7 wild-type and 5 *beta c$^{-/-}$* spleens. (B) Spleen weights in 7 control and 6 *Mx1-Cre, Nf1$^{flox/flox}$* mice. Loss of *Nf1* results in splenomegaly and is attenuated 6 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice. (C) CFU-GM colony numbers from 4 individual experiments of mice of all 4 genotypes demonstrate infiltration in the *Nf1* mutant animals.

Discussion

Birnbaum et al intercrossed *Gmcsf* and *Nf1* mutant mice to investigate whether GM-CSF was essential for MPD.[18] Whereas GM-CSF production by either donor hematopoietic cells or by the irradiated recipients was sufficient to induce MPD, inactivating *Gmcsf* in both donor cells and recipients attenuated the disease phenotype. However, only 6 primary *Gmcsf$^{-/-}$* recipients of *Nf1, Gmcsf* doubly mutant fetal liver cells survived due to an unexpected requirement for GM-CSF signaling for efficient engraftment.[21] Moreover, 4 of 6 *Gmcsf* mutant mice that were repopulated with doubly mutant fetal liver cells developed MPD with prolonged latency.[18] The α and β subunits of the GM-CSF receptor form ligand-independent complexes, and it has been suggested that these preformed receptors normally provide a weak constitutive survival signal in hematopoietic cells.[22] This raised the possibility that cytokine-independent signaling accounts for the observation that most of the recipients of *Nf1, Gmcsf* doubly mutant fetal liver cells ultimately develop MPD.[18] Resolving this question has therapeutic implications because inhibitors of GM-CSF signaling have been proposed as a potential strategy for treating JMML.[23,24]

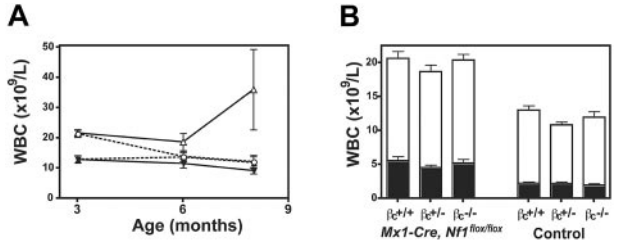

Figure 4. Effects of *beta c* genotype on leukocyte counts in *Mx1-Cre, Nf1$^{flox/flox}$* mice. (A) Data from 6 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$*(○) and 6 *Mx1-Cre, Nf1$^{flox/flox}$, beta βc$^{+/+}$* mice (△) show a modest elevation in total leukocyte counts relative to 5 *beta c$^{-/-}$*(●) or 7 wild-type (▲) animals. Leukocyte counts are significantly higher in the *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$* group by 6 months of age. (B) Lymphoid (□) and myeloid (■) cell counts are shown at 3 months of age for mice of each genotype: 12 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/+}$*; 12 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{+/-}$*; 8 *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$*; 8 *beta c$^{+/+}$*; 12 *beta c$^{+/-}$*; 9 *beta c$^{-/-}$*. Error bars indicate SD from the mean.

In contrast to recipients of doubly mutant fetal liver cells, somatic inactivation of *Nf1* in *Mx1-Cre, Nf1$^{flox/flox}$, beta c$^{-/-}$* mice attenuated, but did not eliminate, the MPD phenotype. These data indicate that *beta c* function is not essential for myeloid disease. Deregulated signaling from other cytokine receptors might contribute to MPD that arises in some *Nf1$^{-/-}$, beta c$^{-/-}$* fetal liver cells. Interestingly, *Nf1$^{-/-}$* progenitors are hypersensitive to stem-cell factor (SCF), which signals through the c-kit receptor.[20] Although *Nf1$^{-/-}$* myeloid progenitors demonstrate a normal pattern of colony growth in response to IL-3 alone, these cells are hypersensitive to the combination of IL-3 plus SCF.[20] Importantly, despite phenotypic differences between recipients of

We first used a fetal liver cell adoptive transfer model to demonstrate that loss of *Nf1* results in expansion of early hematopoietic progenitors (CFU-GEMMs and day 8 CFU-Ss) that is independent of beta c function. Consistent with these data, competitive repopulation experiments showed *Nf1*-deficient donor cells have a proliferative advantage in all hematopoietic lineages, which is greatest in the myeloid compartment.[20] Our data extend these studies by demonstrating that *Nf1* perturbs myelopoiesis independent of signaling through *beta c*.

Despite increased numbers of immature hematopoietic progenitors in recipients of *Nf1$^{-/-}$, beta c$^{-/-}$* fetal liver cells, loss of *beta c* suppressed any evidence of MPD for over 13 months. Although the lack of a positive control is a potential concern, we did not transplant *Nf1$^{-/-}$, beta c$^{+/+}$* cells in parallel because previous studies found that *Nf1$^{-/-}$* fetal livers consistently cause MPD in primary and secondary recipients.[17-19]

Nf1$^{-/-}$, *beta c*$^{-/-}$ fetal liver cells and *Mx1-Cre, Nf1*$^{flox/flox}$, *beta c*$^{-/-}$ mice, both models infer an important role of GM-CSF signaling in modulating myeloproliferation in vivo.

The absence of MPD in the adoptive transfer model may have been due to decreased replicative potential of the donor cells after 2 rounds of transplantation, particularly given the interaction between GM-CSF signaling and fetal liver cell engraftment.[21] It is also possible that transplant recipients would have ultimately developed MPD had we observed them beyond 13 months. On the other hand, because the *Mx1-Cre* promoter is active in multiple cell types, it is possible that nonhematopoietic cells contribute to MPD pathogenesis in *Mx1-Cre, Nf1*$^{flox/flox}$, *beta c*$^{-/-}$ mice by secreting abnormal amounts of cytokines. This idea is consistent with studies showing that heterozygous inactivation of the *Nf1* gene in mice can induce a "field effect" that promotes neurofibroma formation due to interactions between Schwann cells that have inactivated both *Nf1* alleles and heterozygous mutant mast cells and fibroblasts.[25]

Based on our data and the existing literature, we speculate that immature and lineage-specific hematopoietic progenitors require neurofibromin to negatively regulate multiple extracellular stimuli in the bone marrow microenvironment that orchestrate the differentiation of stem cells into progenitors and then mature myeloid cells. Loss of *Nf1* results in increased numbers of cells at all stages of myeloid differentiation. Although we have shown that immature hematopoietic progenitors accumulate in the absence of *beta c*, our data also demonstrate that GM-CSF signaling is required for a fully penetrant MPD phenotype. Previous in vitro and in vivo data support the idea that interfering with GM-CSF signaling may inhibit the growth of JMML cells.[18,23,24] However, the GM-CSF–independent proliferation of immature *Nf1* mutant progenitors also infers that curing this aggressive MPD will require inhibiting beta c in combination with other strategies.

Acknowledgments

This work was supported by grants from the National Cancer Institute (R29 CA78269-01; D.A.L.), American Cancer Society (RPG LIB-106632; D.A.L.), Leukemia and Lymphoma Society of America Specialized Center of Research (LLS 7019-04; D.A.L. and K.S.), National Cancer Institute (R01 CA72614; K.S.), and the US Army Neurofibromatosis Research Program (Project DAMD17-02-1-0638; K.S.). K.M. was supported by a predoctoral training grant (Young Investigator Award) from the National Neurofibromatosis Foundation, now called the Children's Tumor Foundation, and J.O.L. was supported by a training grant from the National Institutes of Health.

We would like to thank the members of the Largaespada and Shannon laboratories for technical advice and input on experimental design.

Authorship

Contribution: K.M., D.A.L., and K.S. designed research; A.K., K.M., D.E.H., S.M.W., and D.T.L. performed research; A.K., K.M., J.O.L., and S.C.K. analyzed data; D.E.H., J.L.G., and M.D.D. maintained the mouse colony; S.C.K. and L.F.P. contributed reagents; and A.K., K.M., J.O.L., K.S., and D.A.L. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A.K. and K.M. authors contributed equally to the study.

Correspondence: David A. Largaespada, 6-160 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455; e-mail: larga002@tc.umn.edu.

References

1. Hope DG, Mulvihill JJ. Malignancy in neurofibromatosis. Adv Neurol. 1981;29:33-56.
2. Emanuel PD, Shannon KM, Castleberry RP. Juvenile myelomonocytic leukemia: molecular understanding and prospects for therapy. Mol Med Today. 1996;2:468-475.
3. Arceci RJ, Longley BJ, Emanuel PD. Atypical cellular disorders. Hematology (Am Soc Hematol Educ Program). 2002:297-314.
4. Xu G, O'Connell P, Viskochil D, et al. The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell. 1990;62:599-608.
5. DeClue JE, Papageorge AG, Fletcher JA, et al. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell. 1992;69:265-273.
6. Parada LF. Neurofibromatosis type 1. Biochim Biophys Acta. 2000;1471:M13-M19.
7. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. Tumor predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet. 1994;7:353-361.
8. Brannan CI, Perkins AS, Vogel KS, et al. Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Devel. 1994;8:1019-1029.
9. Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. Nat Genet. 1996;12:137-143.
10. Bollag G, Clapp DW, Shih S, et al. Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet. 1996;12:144-148.
11. Le DT, Kong N, Zhu Y, et al. Somatic inactivation of Nf1 in hematopoietic cells results in a progressive myeloproliferative disorder. Blood. 2004;103:4243-4250.
12. Nicola NA, Smith A, Robb L, Metcalf D, Begley CG. The structural basis of the biological actions of the GM-CSF receptor. Ciba Found Symp. 1997;204:19-27; discussion 27-32.
13. Okuda K, Foster R, Griffin JD. Signaling domains of the beta c chain of the GM-CSF/IL-3/IL-5 receptor. Ann N Y Acad Sci. 1999;872:305-312; discussion 312-313.
14. Nishinakamura R, Nakayama N, Hirabayashi Y, et al. Mice deficient for the IL-3/GM-CSF/IL-5 beta c receptor exhibit lung pathology and impaired immune response, while beta IL3 receptor-deficient mice are normal. Immunity. 1995;2:211-222.
15. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 1997;90:1073-1083.
16. Zhu Y, Romero MI, Ghosh P, et al. Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. Genes Dev. 2001;15:859-876.
17. Zhang Y, Taylor BR, Shannon K, Clapp DW. Quantitative effects of Nf1 inactivation on in vivo hematopoiesis. J Clin Invest. 2001;108:709-715.
18. Birnbaum RA, O'Marcaigh A, Wardak Z, et al. Nf1 and Gmcsf interact in myeloid leukemogenesis. Mol Cell. 2000;5:189-195.
19. Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. Nat Genet. 1996;12:137-143.
20. Zhang YY, Vik TA, Ryder JW, et al. Nf1 regulates hematopoietic progenitor cell growth and ras signaling in response to multiple cytokines. J Exp Med. 1998;187:1893-1902.
21. Katsumoto TR, Duda J, Kim A, et al. Granulocyte/macrophage colony-stimulating factor and accessory cells modulate radioprotection by purified hematopoietic cells. J Exp Med. 2005;201:853-858.
22. Woodcock JM, McClure BJ, Stomski FC, Elliott MJ, Bagley CJ, Lopez AF. The human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor exists as a preformed receptor complex that can be activated by GM-CSF, interleukin-3, or interleukin-5. Blood. 1997;90:3005-3017.
23. Iversen PO, Lewis ID, Turczynowicz S, et al. Inhibition of granulocyte-macrophage colony-stimulating factor prevents dissemination and induces remission of juvenile myelomonocytic leukemia in engrafted immunodeficient mice. Blood. 1997;90:4910-4917.
24. Iversen PO, Rodwell RL, Pitcher L, Taylor KM, Lopez AF. Inhibition of proliferation and induction of apoptosis in juvenile myelomonocytic leukemic cells by the granulocyte-macrophage colony-stimulating factor analogue E21R. Blood. 1996;88:2634-2639.
25. Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science. 2002;296:920-922.

2007 109: 1687-1691
doi:10.1182/blood-2006-05-025395 originally published online November 7, 2006

β common receptor inactivation attenuates myeloproliferative disease in *Nf1* mutant mice

Andrew Kim, Kelly Morgan, Diane E. Hasz, Stephen M. Wiesner, Jennifer O. Lauchle, Jennifer L. Geurts, Miechaleen D. Diers, Doan T. Le, Scott C. Kogan, Luis F. Parada, Kevin Shannon and David A. Largaespada

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.