Regular Article

MYELOID NEOPLASIA

Timing of the loss of Pten protein determines disease severity in a mouse model of myeloid malignancy

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Key Points

- Early postnatal loss of Pten protein in mice with *Nf1* haploinsufficiency causes a fatal juvenile myeloproliferative neoplasm.
- Akt and MAPK activities are elevated in juvenile mice with *Nf1* haploinsufficiency and Pten protein loss.

Juvenile myelomonocytic leukemia (JMML) is an aggressive pediatric mixed myelodysplastic/myeloproliferative neoplasm (MDS/MPN). JMML leukemogenesis is linked to a hyperactivated RAS pathway, with driver mutations in the *KRAS*, *NRAS*, *NF1*, *PTPN11*, or *CBL* genes. Previous murine models demonstrated how those genes contributed to the selective hypersensitivity of JMML cells to granulocyte macrophage–colony-stimulating factor (GM-CSF), a unifying characteristic in the disease. However, it is unclear what causes the early death in children with JMML, because transformation to acute leukemia is rare. Here, we demonstrate that loss of Pten (phosphatase and tensin homolog) protein at postnatal day 8 in mice harboring *Nf1* haploinsufficiency results in an aggressive MPN with death at a murine prepubertal age of 20 to 35 days (equivalent to an early juvenile age in JMML patients). The death in the mice was due to organ infiltration with monocytes/macrophages. There were elevated activities of protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) in cells at physiological concentrations of GM-CSF. These were more pronounced in mice with *Nf1* haploinsufficiency than in littermates with wild-type *Nf1*, but this model is insufficient to cause cells to be GM-CSF hypersensitive. This new model represents a murine MPN model with features of a pediatric unclassifiable mixed MDS/MPN and mimics many clinical manifestations of JMML in terms of age of onset, aggressiveness, and organ infiltration with monocytes/macrophages. Our data suggest that the timing of the loss of PTEN protein plays a critical role in determining the disease severity in myeloid malignancies. This model may be useful for studying the pathogenesis of pediatric diseases with alterations in the Ras pathway. (*Blood*. 2016;127(15):1912-1922)

Introduction

Juvenile myelomonocytic leukemia (JMML) is an aggressive, mixed myelodysplastic/myeloproliferative neoplasm (MDS/MPN) with a median age at presentation of 2 years. Left untreated, the majority of patients die within a year after diagnosis, primarily due to organ failure and bleeding resulting from infiltration with monocytes/macrophages.[1] Hematopoietic stem cell (HSC) transplant is the only cure for JMML, but the relapse rate remains high at 30% to 40%.[2,3] Although many investigators have shown that JMML is caused by hyperactivity of the RAS pathway due to driver mutations in *KRAS*, *NRAS*, *NF1*, *PTPN11*, and *CBL* genes,[4-11] clinical outcomes are independent of these driver mutations.[12] Two recent publications of whole-exome sequencing in JMML arrived at a similar conclusion; that is, the absolute number of somatic alterations at diagnosis is a major determinant of survival.[13,14]

Although the majority of JMML patients experience an aggressive disease course, small subsets have more indolent disease and/or even spontaneous remission,[15] leading to difficult clinical decisions. JMML is also a very age-restricted disease. It rarely presents congenitally, but often develops within the first year after birth, with 96% of patients presenting by age 5 years. Children with clinical neurofibromatosis have a 200- to 500-fold increased risk of developing JMML. Other pediatric diseases with a deregulated RAS/mitogen-activated protein kinase (MAPK) pathway, such as neuro-cardio-facial-cutaneous syndromes and Noonan syndrome, also have a markedly increased risk for JMML.[3] Of interest, in nearly all of these inherited genetic syndromes, the children "outgrow" their risk for JMML by approximately age 6 years.[16] The mechanism underlying this dramatic shift in risk for JMML development remains unknown.

Previous murine models of JMML were developed based on single-gene disruptions and demonstrated how mutant driver genes contribute to selective granulocyte macrophage–colony-stimulating factor (GM-CSF) hypersensitivity, a unifying characteristic of JMML. However, none of them mimic the JMML clinical phenotype of a very age-restricted disease onset coupled with significant aggressiveness. Most of them were absent in demonstrating hyperactivities of protein kinase

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B (Akt) and MAPK,[4,8,17-21] which are found in 55% and 73% of JMML patients, respectively.[22] Nevertheless, they have tremendously advanced our understanding underlying JMML leukemogenesis. It is unfortunate that it remains unclear what causes the early death in JMML patients, given that very few transform to acute leukemia.

PTEN (phosphatase and tensin homolog) negatively regulates phosphatidylinositol-3-kinase (PI3K) and MAPK signaling, which are downstream of RAS. Loss of PTEN protein is frequently found in solid tumors, especially breast, lung, prostate, and ovarian cancers, as well as in acute lymphoid leukemia (ALL).[23-28] We reported that 67% of JMML patients were PTEN protein deficient.[22] Other groups reported that myeloid-specific somatic deletions of *Pten* in fetal or adult mice produced a transient MPN, with eventual death in adulthood from acute myeloid leukemia (AML) or ALL.[29-31] It was also reported that fetal and adult mouse HSCs display marked differences in their self-renewal, differentiated cell output, and gene expression properties, with persistence of a fetal phenotype until 3 weeks after birth.[32] The role of Pten in regulating mouse hematopoiesis has been reported to be age dependent.[33] We hypothesized that PTEN protein loss at a specific stage in early human childhood might contribute to the early death in JMML patients. In this study, we demonstrate that by inducing Pten protein deficiency at postnatal day (PND) 8 in mice (equivalent to the age of a full-term newborn in humans[34]), along with haploinsufficiency of *Nf1*, results in a lethal murine MPN that mimics human pediatric MPNs, including JMML, in terms of age of onset, aggressiveness, and organ infiltration with monocytes/macrophages. Our data suggest that the timing of PTEN protein loss plays a critical role in determining the disease severity in myeloid malignancies.

Materials and methods

For more information on materials and methods, see supplemental Methods, available on the *Blood* Web site.

Study design and mice

Because mechanisms of PTEN protein loss in human cancers are complex and not well classified, we chose to induce *Pten* gene deletion at a specific time point to simulate Pten protein loss in mice, even though *PTEN* mutations are not found in JMML. By crossbreeding the founder mice bearing $Pten^{floxP/floxP}$ ($Pten^{fl/fl}$; B6.129S4-$Pten^{tm1Hwu}$/J), $Nf1^{Fcr/+}$ (B6.129S6-$Nf1^{tm1Fcr}$/J), and *Mx1-Cre* (B6.Cg-Tg[Mx1-Cre]1Cgn/J) (The Jackson Laboratory), we generated experimental mice with $Pten^{fl/fl}$ with or without $Nf1^{+/Fcr}$ ($Nf1^{+/-}$) and *Mx1-Cre*. *Pten* deletion was induced by intraperitoneal injection of 30 μL of polyinosinic polycytidylic acid (poly[I:C]) at a concentration of 1 μg/μL in normal saline (InvivoGen) on days 8 and 10 after birth (hereafter referred collectively as PND8), or at the age of 6 weeks (180 μL of poly[I:C] twice; supplemental Figure 1). Mouse tails were clipped at age 2 weeks, and genotyping was performed according to the instructions provided by the vendor. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Competitive BMT

Competitive bone marrow transplant (BMT) was performed according to the method published by Zhang et al,[30] with modifications. Briefly, adult C57BL/6 recipient mice with $CD45.1^+$ background at age 6 to 8 weeks (The Jackson Laboratory) were lethally irradiated with a single dose of 9.5 Gy delivered by a Mark I ^{137}Cs irradiator with a rotating platform (JL Shepherd and Associates, San Fernando, CA) at a rate of 1.08 Gy/min. Unfractionated bone marrow nucleated cells (WBM cells) from 3-week-old wild-type (WT) controls (2×10^5) or *PtenΔ/Δ; Nf1*$^{+/-}$ mice (1×10^6) with $CD45.2^+$ background were mixed with 2×10^5 rescue cells from WT $CD45.1^+$ mice. Donor cells were suspended in 120 μL of phosphate-buffered saline/5% mouse serum (Fisher Scientific) and injected into the retro-orbital venous sinus of irradiated recipient mice at 6 hours postirradiation. Transplanted mice were maintained with regular mouse chow and sterile water without additives. Four weeks after BMT, peripheral blood (PB) was collected from recipient mice serially at 2-week intervals for complete blood cell (CBC) count or flow cytometric analysis (fluorescence-activated cell sorting [FACS]) until 18 weeks posttransplant, or when recipient mice were euthanized for overall evaluation.

Results

Myeloid-specific deletion of *Pten* at PND8 results in a lethal juvenile MPN

In attempting to test our hypothesis that PTEN protein loss is a second event that contributes to early death in JMML, we designed experimental mice to mimic the putative dynamics of molecular defects in JMML, which is loss of PTEN protein in the first few months after birth following a germ line *NF1* mutation. *NF1*, encoding neurofibromin, negatively regulates Ras activity,[35] and was the first well-documented gene that was linked to the selective GM-CSF hypersensitivity in JMML.[4,8,17] Mice with germ line homozygous *Nf1* inactivation, caused by interrupting the *Nf1* exon 31 ($Nf1^{Fcr/Fcr}$), die during embryonic development. Conversely, heterozygotes do not exhibit any overt disease symptoms until adulthood, with an increased rate of tumorigenesis and a spectrum of tumors similar to WT mice.[36,37] Somatic inactivation of *Nf1* ($Nf1^{floxP/floxP}$) or loss of heterozygosity (LOH) caused a JMML-like disease, but with a significant latency (mice did not become sick until age 3 months) (reviewed in Table 1; supplemental Table 4).[8,17] In the present study, we generated mice bearing $Pten^{fl/fl}$ with or without $Nf1^{+/-}$ and $Mx1\text{-}Cre^+$, which were born healthy. To simulate an age-specific Pten protein loss in a subject with genetic susceptibility, loss of the *Pten* gene was induced by intraperitoneal injection with poly(I:C) at PND8, which is equivalent to the age of a full-term newborn in humans.[34] Mouse genotypes were identified at an age of 2 weeks, 1 week post–poly(I:C) (supplemental Figure 2). Mice with biallelic *Pten* deletion and *Nf1* haploinsufficiency (*PtenΔ/Δ; Nf1*$^{+/-}$) started to display signs of disease (fuller abdomen) in the third week of life (2 weeks post–poly[I:C]). Without intervention, all mice with *PtenΔ/Δ; Nf1*$^{+/-}$ died at age 20 to 35 days (equivalent to the age of an early juvenile in humans). Mice with *PtenΔ/Δ; Nf1*$^{+/-}$ had significantly shorter median survival (26 days, n = 13) compared with *PtenΔ/Δ; Nf1*$^{+/+}$ mice (35 days, n = 11; $P < .001$), and with both *Pten*$^{+/+}$*; Nf1*$^{+/-}$ (n = 10) and WT mice (n = 9, 100% survived past the 120-day observation; $P < .001$) (Figure 1A). Necropsy showed that *PtenΔ/Δ* mice had substantial hepatosplenomegaly, regardless of *Nf1* background, in comparison with both *Pten*$^{+/+}$*; Nf1*$^{+/-}$ (n = 10) and WT littermates (n = 9). No lymphadenopathy or thymus enlargement was found, differing substantially from previous models in which *Pten* deletion occurred at either the prenatal or the adult stage and in which the mice always died of AML or ALL in adulthood (Table 1; supplemental Table 4).[29-31,33] We also noticed that littermates with hemizygous *Pten* deletion or WT *Pten* did not show any overt disease symptoms during the 120-day observation, regardless of *Nf1* background (Figure 1A). Because our focus was primarily on juvenile leukemia, we studied the mice with only *PtenΔ/Δ; Nf1*$^{+/-}$. We euthanized the diseased mice and littermates at age 3 to 4 weeks (2-3 weeks post–poly[I:C]). Tissue genotyping demonstrated that

Table 1. Comparison of JMML patients and the *Pten*$^{fl/fl}$*Nf1*$^{+/-}$ mouse model with previously reported mouse models

		Mx1-*Cre*								
				Pten$^{fl/fl}$				*Lox-Stop-Lox (LSL)*		
	JMML patients	*Pten*$^{fl/fl}$ *Nf1*$^{+/-}$		p55$^{-/-}$	p55$^{+/-}$	*Pten*$^{fl/fl}$	*Nf1*$^{fl/fl}$ or *Nf1*$^{-/fl}$	*Kras*^{G12D}	*Nras*^{G12D}	*Ptpn11*$^{D61Y/+}$
Age mutation introduced	NApp	PND8	6 wk	PND2	6 wk	3 or 6 wk	PND3-PND5	3 wk	3 wk	3-4 wk
Age MPN occurs (postinduction)	2 y, often <1 y	3 wk (2 wk)	9 wk (3 wk)	T-ALL (7 wk)	T-ALL (3 wk)	7 wk (1-4 wk)	>3 mo (3 mo)	6 wk (3 wk)	>7 mo (6 mo)	5 mo (4 mo)
Survival	<1 y	3.5 wk*	11 wk	6-8 wk		7-12 wk	7.5 mo	>3 mo	>12 mo	>10 mo
Penetrance, %	NApp	100	100	100		90	100	100	100	100
Leukocytosis	Yes	Yes	Yes	Yes		Yes	No	Yes	Yes	Yes
Monocytosis	Yes	Yes	Yes	N/A		Yes	Yes	Yes	N/A	Yes
Lymphocytes	N/A	Decreased	Yes	Yes		N/A	Increased	N/A	Increased	N/A
Hemoglobin (hematocrit)	Decreased	Decreased	No	N/A		N/A	Normal	Decreased	Decreased	Decreased
Platelets counts	Normal/decreased	Elevated	No	N/A		N/A	Normal	Normal	Normal	Normal
Hypersensitivity to GM-CSF	Yes	No	N/A	N/A		N/A	Selective	Independent†	Independent†	Independent†
Hepatosplenomegaly	Yes	Yes	Yes	N/A		Yes	Yes	Yes	Yes	Yes
Cell infiltration in Lung	Yes	Yes	Yes‡	N/A		N/A	No	N/A	Yes§	No
AML or ALL	No (blasts <20%)	No	T-ALL	T-ALL		Yes	No	No	No	No
Other organs	Skin, lymph nodes, colon	Not found	Thymus, lymph nodes	Thymus, lymph nodes		Lymphoma	No	N/A	Lymph nodes, histiocytoma	No
Elevated Akt activity	55% patients	Constitutive	N/A	No	Yes	N/A	No	Yes	N/A	Yes
Elevated MAPK activity	73% patients	Yes‖	N/A	N/A		N/A	Yes‡	Yes	No	Yes
Reference	1, 22	The present study		33		29, 30	17	20, 21	19	18

NApp, Not applicable; N/A, not available.
*Equivalent to 1 to 3 y old in humans.
†Colony growth in the absence of GM-CSF.
‡Only observed in Mac-1^{+} cells.
§Lymphocytes.
‖Preference to GM-CSF over IL-3.

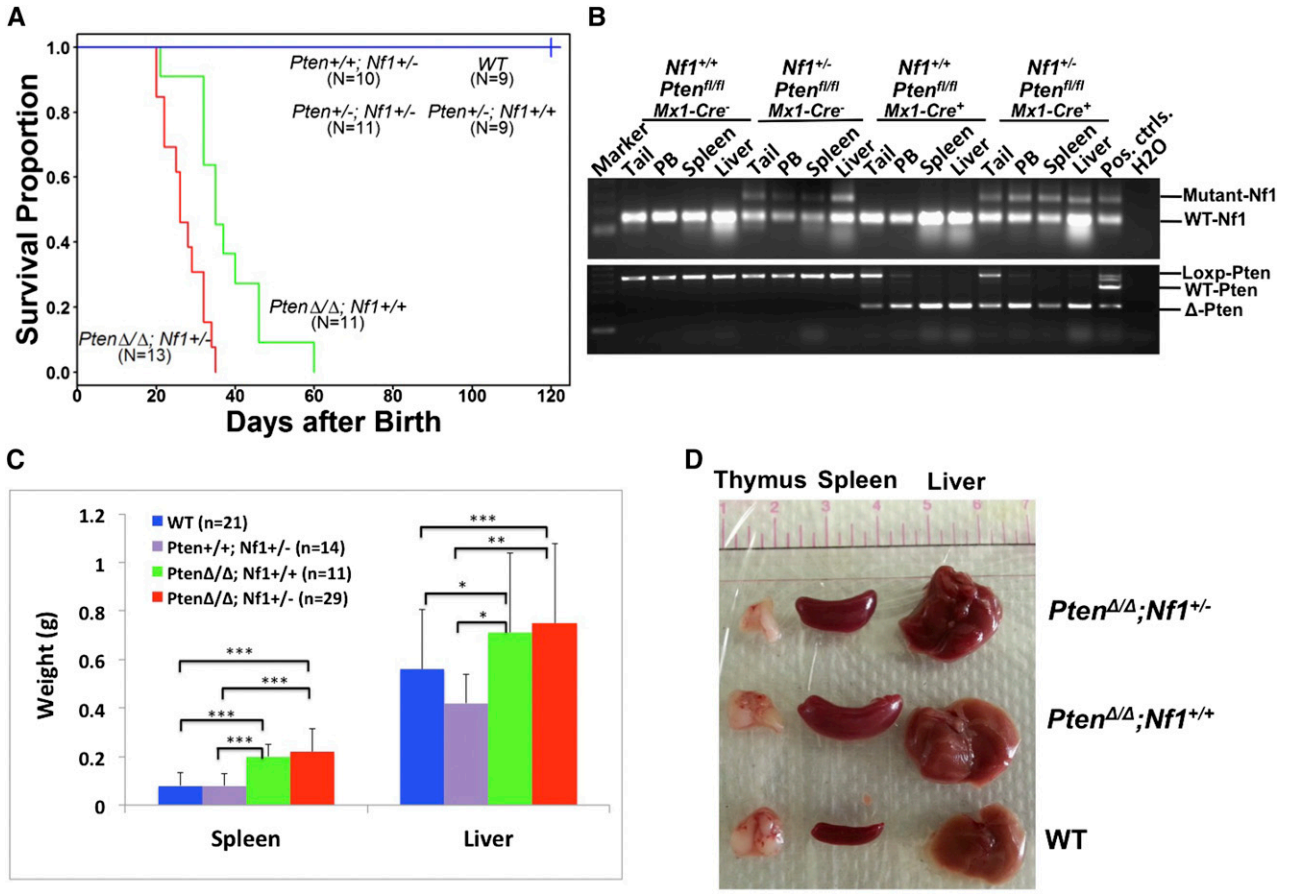

Figure 1. Tissue genotype, phenotype, and survival of mice with or without *Pten* deletion and *Nf1* mutation. (A) Kaplan-Meier analysis demonstrated the high penetration (100%) of disease and early lethal effect of *Pten* deletion that was induced at PND8 in mice with or without *Nf1* mutation. WT mice were euthanized after age 120 days. Survival in mice with *PtenΔ/Δ; Nf1*$^{+/-}$ was significantly shorter, with a median survival of 26 days (95% confidence interval [CI], 22-32) (n = 13), and differed from *PtenΔ/Δ; Nf1*$^{+/+}$ (35 days [95% CI, 32-46]; $P = .001$), *Pten*$^{+/-}$*; Nf1*$^{+/-}$, *Pten*$^{+/+}$*; Nf1*$^{+/-}$, and WT mice ($P < .001$). Survival in mice with *PtenΔ/Δ; Nf1*$^{+/+}$ significantly differed from littermates with *Pten*$^{+/-}$*; Nf1*$^{+/-}$ or *Pten*$^{+/+}$*; Nf1*$^{+/-}$ and with WT ($P < .001$). (B) Genotype analysis by polymerase chain reaction on tissues that were collected at age 3 to 4 weeks from the littermate mice received poly(I:C) at PND8, demonstrating that *Pten* was deleted in the PB, spleen, and liver of mice with *Pten*$^{fl/fl}$ and *Mx1-Cre*$^{+}$, whereas the WT *Nf1* allele was detectable in mice with all genotypes, suggesting that the *Nf1* LOH had not occurred in diseased mice. In agreement with prior studies regarding *Nf1*, the intensity of signals of the mutant and WT *Nf1* alleles combine to roughly equal totals. Thus, when a mutant *Nf1* allele is present, the density of the WT allele is proportionately decreased (haploinsufficient). (C) Average spleen and liver weights demonstrate hepatosplenomegaly in *PtenΔ/Δ* mice. Data are presented as mean ± SD, *$P < .05$; **$P < .01$; ***$P < .001$. (D) Photographs of organs from representative littermate mice at age 4 weeks demonstrating hepatosplenomegaly; however, the thymus was spared in *PtenΔ/Δ* mice regardless of *Nf1* background, in comparison with WT littermates (additional relevant data are presented in supplemental Figures 1 and 2). SD, standard deviation.

Pten was deleted in PB, spleens, and livers of mice with *Pten*$^{fl/fl}$ and *Mx1-Cre*$^{+}$, whereas the WT allele of *Nf1* remained detectable in mice with all genotypes (Figure 1B), suggesting that *Nf1* LOH had not occurred in diseased mice. The size and the weight of spleens and livers were significantly increased in mice with *PtenΔ/Δ*, regardless of *Nf1* background, in comparison with WT and *Pten*$^{+/+}$*; Nf1*$^{+/-}$ mice, but there was no difference between *Pten*$^{+/+}$*; Nf1*$^{+/-}$ and WT mice (Figure 1C-D). CBC counts showed modestly elevated white blood cells only in *PtenΔ/Δ; Nf1*$^{+/-}$ mice, which was significantly different from littermates with WT *Pten* with or without *Nf1* haploinsufficiency (Figure 2A). Significantly increased platelets and anemia were also found in *PtenΔ/Δ* mice, regardless of *Nf1* background, but mice with *PtenΔ/Δ; Nf1*$^{+/-}$ had significantly lower platelets than those with *PtenΔ/Δ; Nf1*$^{+/+}$ (Figure 2B-D). Blood smear evaluation showed markedly elevated monocytes and granulocytes with normal morphology, but decreased lymphocytes in all *PtenΔ/Δ* mice (Figure 2E-F; supplemental Table 2). Tissue sections of formalin-fixed organs showed substantial monocyte/macrophage infiltration in the spleens, livers, and lungs from all *PtenΔ/Δ* mice (Figure 3). Of interest, total cell numbers in bone marrow (BM) were significantly reduced in *PtenΔ/Δ; Nf1*$^{+/-}$ mice in comparison with WT mice ($48.88 \times 10^6 \pm 6.49 \times 10^6$ vs $82.80 \times 10^6 \pm 24.38 \times 10^6$; $P < .05$). BM cytospins demonstrated increases in monocytes and granulocytes with normal morphology in *PtenΔ/Δ; Nf1*$^{+/-}$ mice (Figure 3). FACS analysis confirmed that *PtenΔ/Δ* mice with or without *Nf1* haploinsufficiency had significant increases of monocytes/macrophages (Mac-1^{+}Gr-1^{int}) and granulocytes (Mac-1^{+}Gr-1^{high}) in BM, blood, and spleen (Figure 4A-B; supplemental Figure 3). Importantly there was a significant difference in monocytes/macrophages and granulocytes in spleens from mice with *PtenΔ/Δ; Nf1*$^{+/-}$ and those with *PtenΔ/Δ; Nf1*$^{+/+}$ (Figure 4A-B). We also found significantly decreased B-cell (CD19^{+}) and T-cell (CD3e^{+}) populations in PB and spleen in *PtenΔ/Δ* mice, regardless of *Nf1* background (Figure 4D-E; supplemental Figure 3). Mature monocytes/macrophages (Mac-1^{+}CD115^{+}) were elevated significantly in BM and PB only in *PtenΔ/Δ; Nf1*$^{+/-}$ mice, but not in *PtenΔ/Δ; Nf1*$^{+/+}$ mice, compared with WT mice (Figure 4C; supplemental Figure 3A). Macrophage (F4/80^{+}) infiltration was confirmed in spleens from *PtenΔ/Δ* mice regardless of *Nf1* background (Figures 3 and 4F; supplemental Figure 3B). FACS profiles showed no difference

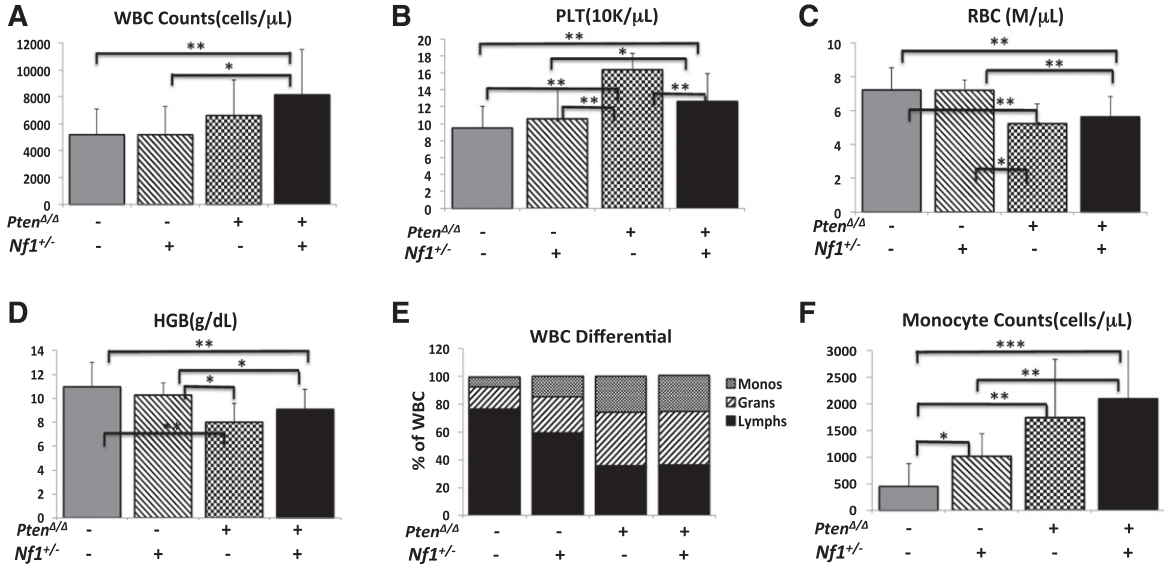

Figure 2. CBC counts and white blood cell (WBC) differential analysis in mice at age 3 to 4 weeks (2-3 weeks post–poly[I:C]). CBC counts were performed by a Vet ABC Hematology Analyzer. WBCs (A), platelets (PLT) (B), red blood cells (RBC) (C), hemoglobin (HGB) (D), and differentials (E) were manually counted from blood smears stained with May-Grünwald-Giemsa (MGG). (F) Absolute numbers of monocytes from 4 groups of mice were compared. Data are presented as mean ± SD (n = 10-14 per group). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. Additional details are presented in supplemental Table 2. Gran, granulocytes; Lymphs, lymphocytes; Monos, monocytes.

in mice with WT *Pten* with or without *Nf1* haploinsufficiency. Chromosome stability analysis of BM cells showed no significant difference between mice with *PtenΔ/Δ; Nf1*$^{+/-}$ (n = 5) and WT littermates (n = 5) ($P = .107$) (data not shown).

In summary, mice with *PtenΔ/Δ; Nf1*$^{+/-}$ induced at PND8 had decreased hemoglobin; markedly elevated normal-appearing monocytes/macrophages infiltrating BM, spleen, liver, and lungs; and the mice died rapidly at an age equivalent to that of an early juvenile in humans. On the basis of the Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice,[38] we believe that *PtenΔ/Δ; Nf1*$^{+/-}$ mice represent a juvenile mouse MPN model that has features of an unclassifiable juvenile mixed MDS/MPN and mimics many JMML clinical manifestations in terms of age of onset, aggressiveness, and organ infiltration with monocytes/macrophages. Our data also suggest that *Nf1* haploinsufficiency worsens the accumulation of monocytes/macrophages in the BM and blood (Figure 4C) and the infiltration of monocytes/macrophages and granulocytes in the spleens of mice with *PtenΔ/Δ*; *Nf1*$^{+/-}$ compared with littermates with *PtenΔ/Δ*; *Nf1*$^{+/+}$ (Figure 4A-B), which results in a substantially shorter survival (Figure 1A).

Loss of Pten protein promotes HSC migration but does not alter cell sensitivities to GM-CSF and IL-3

We previously reported that selective GM-CSF hypersensitivity is a unifying characteristic in JMML.[1,39] Other groups reported that circulating CD34^{+} cells were significantly increased, with a lower apoptosis rate in JMML.[40] WBM and spleen cells from *PtenΔ/Δ; Nf1*$^{+/-}$ or WT mice were serum starved for 4 hours after overnight depletion of adherent cells and seeded in semisolid medium with varying concentrations of recombinant mouse GM-CSF or interleukin-3 (IL-3). We found a slight leftward shift in GM-CSF and IL-3 growth curves in spleen cells, but not in WBM cells (Figure 5A-B,D-E). We performed FACS analysis on BM and

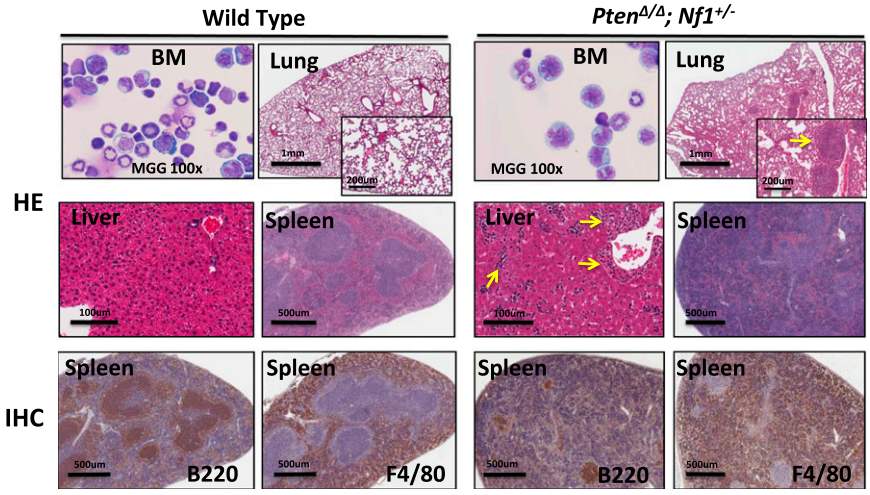

Figure 3. Morphologic analysis of organ tissues from *PtenΔ/Δ; Nf1*$^{+/-}$ mice induced at PND8 and from control littermates at age 3 weeks (2 weeks post–poly[I:C]). Cytospins from BM show increased granulocytes and monocytes and substantial monocytic infiltration was found in the lungs, liver, and spleen of mice with *PtenΔ/Δ; Nf1*$^{+/-}$. The lungs show nodular monocytic infiltration (indicated by an arrow). Monocytic infiltration was observed in the sinuses of the liver (indicated by arrows). Monocytic infiltration involved both red and white pulps of the spleen, which is accompanied by decreased lymphoid tissues. Images were acquired using a Nikon Eclipse NV with a 100× lens (BM cytospins) and an Aperio CS2 scanner (HE and IHC). BM, bone marrow; HE, hematoxylin and eosin staining, IHC, immunohistochemistry staining with indicated specific antibodies.

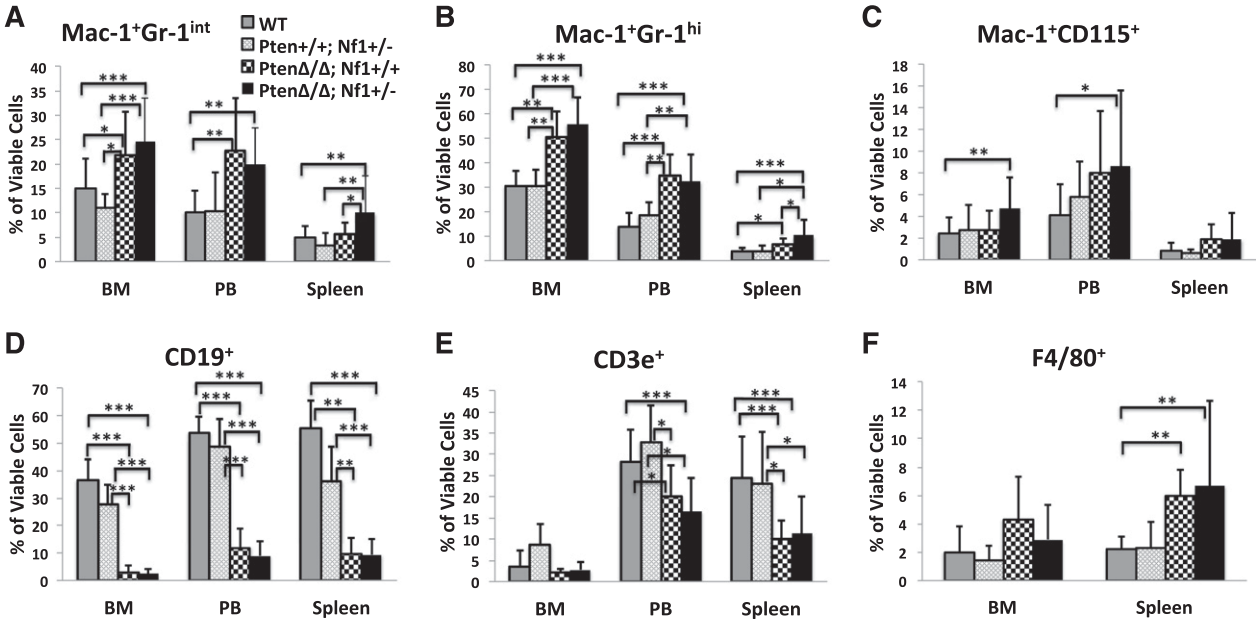

Figure 4. FACS analysis of cell subpopulations from the BM, PB, and spleens of *PtenΔ/Δ; Nf1*$^{+/-}$ mice induced at PND8 and of control littermates at age 3 to 4 weeks (2-3 weeks post–poly[I:C]). Mice with *PtenΔ/Δ*, regardless of *Nf1* background, had significantly increased monocytes/macrophages and granulocytes (A-C) in BM, PB, and spleen, but elevated mature monocytes were seen in BM and PB from mice with *PtenΔ/Δ; Nf1*$^{+/-}$ but not *PtenΔ/Δ; Nf1*$^{+/+}$ (C). B cells (D) and T cells (E) were both markedly decreased in PB and spleen from mice with *PtenΔ/Δ*, regardless of *Nf1* background; macrophage infiltration was confirmed in spleens from mice with *PtenΔ/Δ*, regardless of *Nf1* background (F). Data are presented as mean ± SD (n = 7-21 per group). **P* < .05; ***P* < .01; ****P* < .001. Additional supportive data are presented in supplemental Table 3 and supplemental Figure 4.

spleen cells, and found that BM hematopoietic progenitor cells (HPCs), including LIN$^-$, LIN$^-$Scal-1$^-$cKit$^+$, and LIN$^-$Scal-1$^+$ cKit$^+$, were significantly decreased, with less apoptosis, whereas HPCs were increased in the spleen (Figure 5C,F; supplemental Figure 4). These findings, along with markedly decreased total cell numbers in the BM of *PtenΔ/Δ; Nf1*$^{+/-}$ mice, suggest an increased migration of HPCs to the spleen, which was also observed in mice with *Pten* deletion induced at the other ages.[29,30]

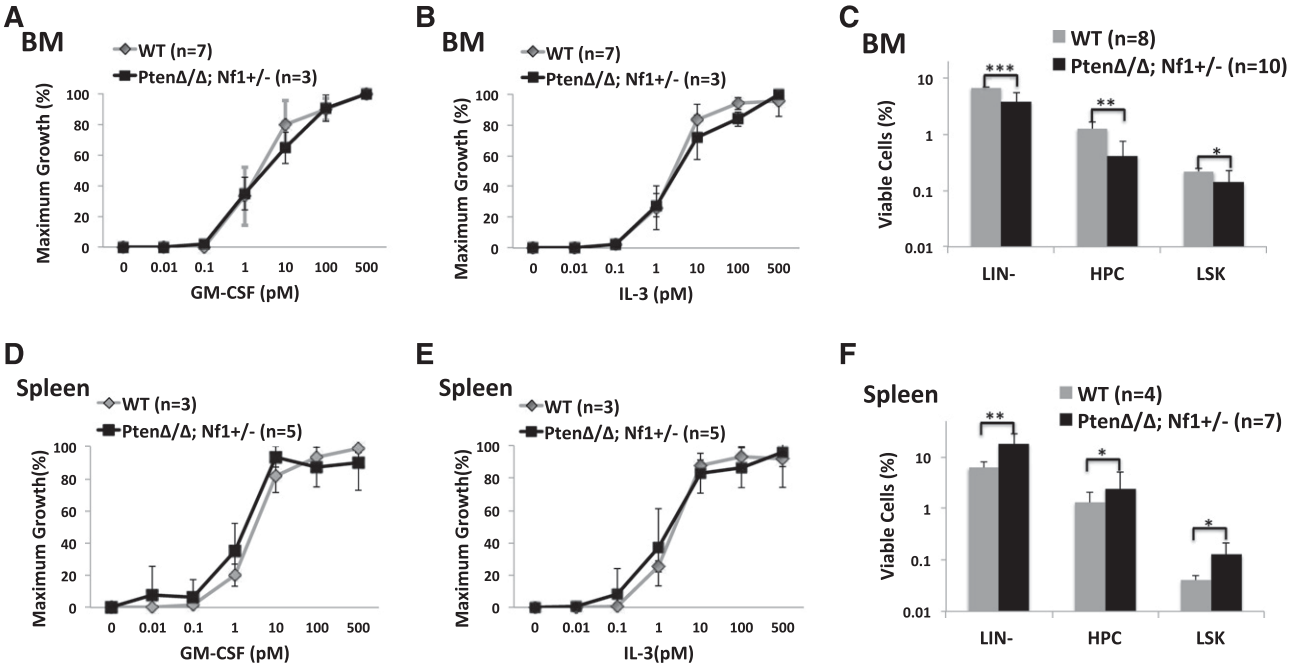

Figure 5. Analysis of progenitor cells from the BM and spleens of *PtenΔ/Δ; Nf1*$^{+/-}$ mice induced at PND8 and of control littermates at age 3 weeks (2 weeks post–poly[I:C]). Colony-forming unit–granulocyte-macrophage (CFU-GM) assays: after serum starvation for 4 hours, 5 × 10^4 BM cells (A-C) or 2 × 10^5 spleen cells (D-F) were seeded in triplicate on 1-mL semisolid cultures in 35-mm plates with 0.3% agar and McCoy's 5A Medium supplemented with nutrients and 15% fetal calf serum containing various concentrations of recombinant mouse GM-CSF or IL-3. (A-B,D-E) Data show there was no significant aberrant curve shift for GM-CSF and IL-3 sensitivities. (C,F) FACS analysis suggests hematopoietic migration from the BM to the spleen in mice with *PtenΔ/Δ; Nf1*$^{+/-}$. LIN$^-$, lineage markers negative; HPC, LIN$^-$Scal-1$^-$c-Kit$^+$; LSK, LIN$^-$Scal-1$^+$c-Kit$^+$. Data are presented as mean ± SD. **P* < .05; ***P* < .01; ****P* < .001. Additional supportive data are presented in supplemental Figure 3.

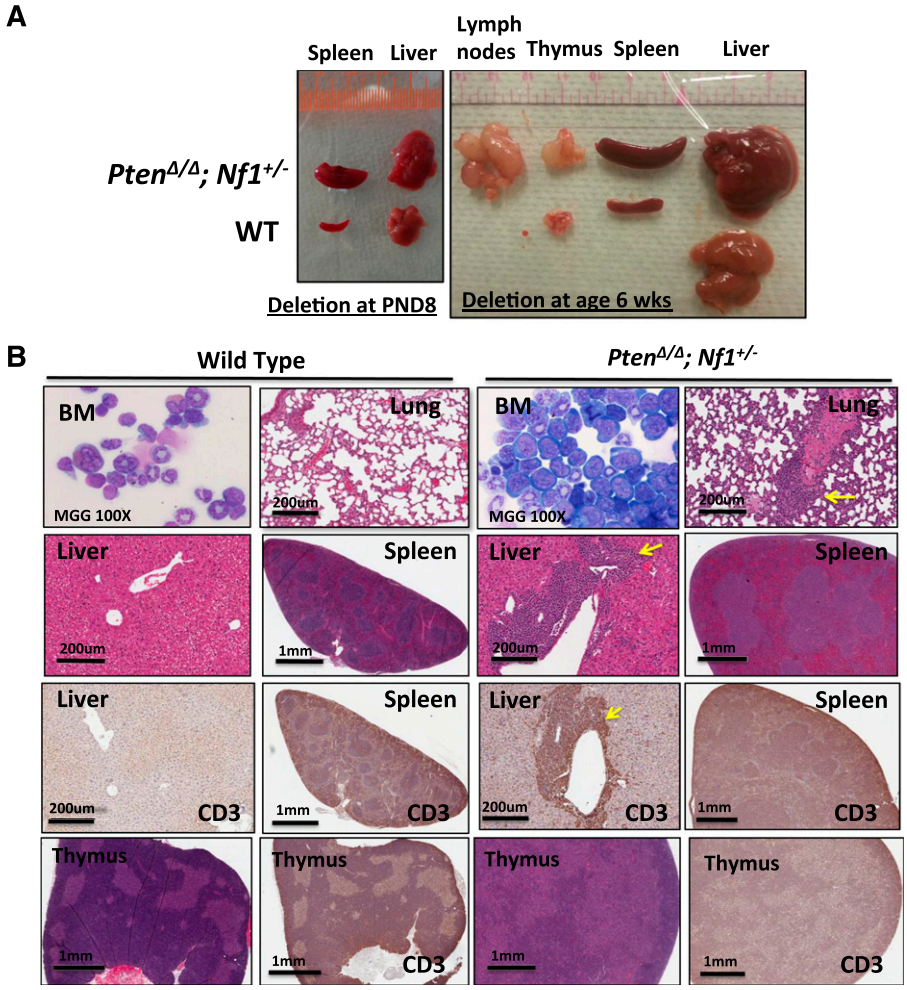

Figure 6. Significantly different phenotype in mice with *Pten* deletion induced at 6 weeks after birth in comparison with mice induced at PND8. (A) Photographs of organs from representative littermate mice with *Pten* deletion induced at PND8 (euthanized at age 3 weeks) or at age 6 weeks (euthanized at age 9 weeks). Hepatosplenomegaly and significant enlargement of lymph nodes and thymus are demonstrated in mice with *PtenΔ/Δ; Nf1*$^{+/-}$ induced at age 6 weeks. (B) Micrographs demonstrate BM with increased lymphoblasts and associated lymphocyte infiltration in the lung and liver (indicated by arrows), spleen, and thymus in mice with *PtenΔ/Δ; Nf1*$^{+/-}$. BM cytospins were stained by MGG; lung with HE; liver, spleen, and thymus with HE and IHC with antibody against CD3. Images were acquired by an Aperio CS2 scanner (additional relevant FACS data are presented in supplemental Figure 6).

MPN with induced Pten protein loss is transplantable

It was reported that *Pten* deletion in adult mice caused either a transient or lethal MPN, but mice in those models lived much longer (51-61 days) than that in this reported model, with a significant potential to transform into acute leukemia (reviewed in Table 1).[29,30] We questioned whether the MPN phenotype in mice with induced Pten protein loss at PND8 and *Nf1* haploinsufficiency could be transplantable without transforming into acute leukemia. WBM cells from *PtenΔ/Δ; Nf1*$^{+/-}$ or WT littermates with CD45.2^{+} background mixed with WBM cells from WT mice with CD45.1^{+} were transplanted into lethally irradiated WT mice (CD45.1^{+}). Recipient mice transplanted with cells from *PtenΔ/Δ; Nf1*$^{+/-}$ mice developed an indolent myelodysplastic syndrome (MDS) (1/8) or MPN (7/8) by 8 weeks posttransplantation (supplemental Figure 5A), whereas all mice transplanted with control WT BM (n = 5) lived without signs of disease through the 20-week post-BMT observation period. By 12 weeks posttransplantation, *PtenΔ/Δ; Nf1*$^{+/-}$ mice (3/8) started to die, so we euthanized 5 diseased recipients at 13 to 14 weeks posttransplantation and analyzed grafted tissues. We found that >50% of cells in recipient mice were positive for CD45.2. One recipient mouse was identified with donor-derived T-cell ALL (T-ALL) (CD45.2^{+}); the other 4 mice had signs of MPN after 13 weeks posttransplantation without any signs of acute leukemia (supplemental Figure 5). This finding suggests that the MPN phenotype in *Nf1*$^{+/-}$ mice with Pten protein loss induced at PND8 is transplantable and that *PtenΔ/Δ; Nf1*$^{+/-}$ cells possess a proliferative advantage over their WT counterparts. However, the disease latency and severity is significantly different from mice with *PtenΔ/Δ* induced after 3 weeks of age (adulthood), in which recipients always died of acute leukemia after 12 weeks posttransplantation.[29-31,33]

Timing of Pten protein loss plays a critical role in determining whether the disease transforms to acute leukemia

Other groups previously reported that mice with *Pten* deletion at age 6 weeks (equivalent to human adulthood) developed a transient MPN and eventually died of acute leukemia,[29,30,33] whereas mice with *PtenΔ/Δ* and *p53*$^{-/-}$ died of T-ALL at a similar time, irrespective of whether *PtenΔ/Δ* was induced at PND2 (equivalent to the third trimester of pregnancy in humans[34]) or 6 weeks after birth[33] (Table 1; supplemental Table 4). To investigate whether the timing of *Pten* deletion plays a role in determining phenotype and disease aggressiveness, we induced *PtenΔ/Δ* in mice with or without *Nf1*$^{+/-}$ at age 6 weeks. As expected, all 14 mice with *PtenΔ/Δ* developed a transient MPN after 3 weeks post–poly(I:C), and 6 of them subsequently transformed into T-ALL (Figure 6; supplemental Figure 6). Of interest, T-ALL occurred more frequently in mice with *PtenΔ/Δ; Nf1*$^{+/+}$ (5/7) than with *PtenΔ/Δ; Nf1*$^{+/-}$ (1/7), further supporting that Pten protein loss induced at PND8 determines disease severity and that the timing causes a more aggressive MPN in mice than when it occurs in adulthood.[29,30,33] This finding also suggests that *Nf1* haploinsufficiency contributes to the hematopoietic bias favoring the myeloid lineage.

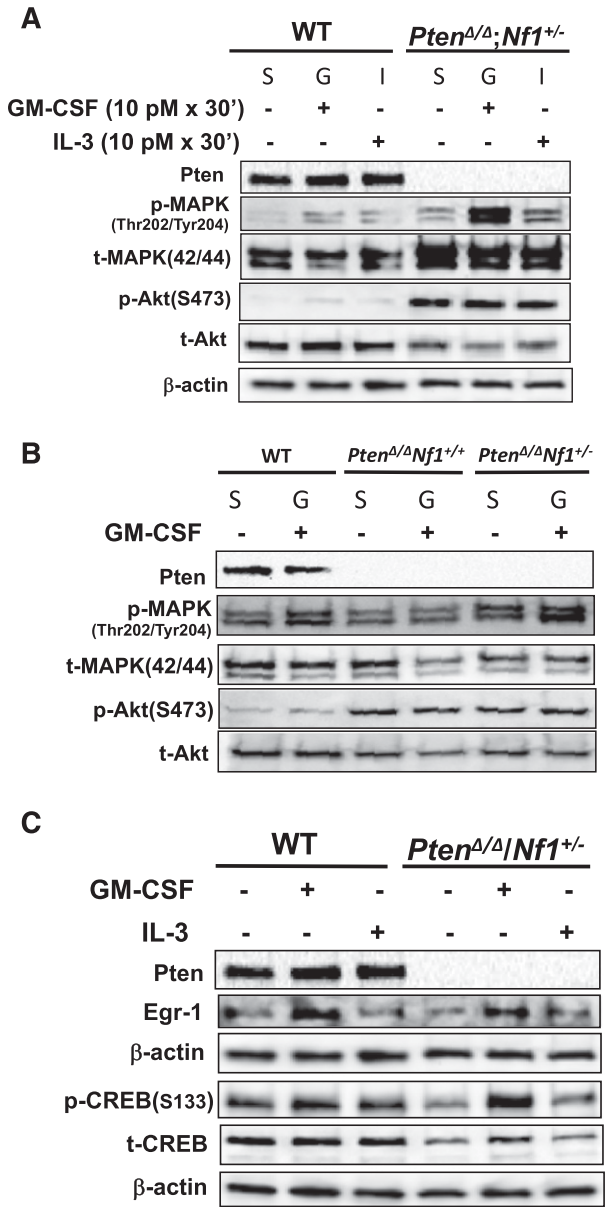

Figure 7. Western blot analysis of the elements in GM-CSF signal transduction pathway in *PtenΔ/Δ; Nf1*$^{+/-}$ mice induced at PND8 and in control littermates at age 3 weeks (2 weeks post–poly[I:C]). After serum starvation (S) and stimulation with GM-CSF (G) or IL-3 (I), mouse BM cells were lysed in a density of 2×10^7/mL in 2× Laemmli Sample Buffer (Bio-Rad Laboratories). One aliquot with 20 μL per well was loaded onto a 4% to 20% Mini-PROTEAN TGX gel (Bio-Rad). (A) Comparison of the activities of Akt and MAPK pathways in mice with WT and *PtenΔ/Δ; Nf1*$^{+/-}$ induced at PND8 in response to stimulation by GM-CSF and IL-3 at a physical concentration. Representative data from 8 experiments in littermate mice (age 3-4 weeks) are shown. (B) Comparison the activities of Akt and MAPK pathways in mice with *PtenΔ/Δ* with or without *Nf1*$^{+/-}$ in response to stimulation by GM-CSF. Representative data from 3 experiments are shown. (C) Comparison the activation of CREB and expression of Egr-1 in mice with WT and *PtenΔ/Δ; Nf1*$^{+/-}$. Representative data from 4 experiments are shown. CREB and Egr-1 were detected on 2 separate blots. p, phosphorylated protein; t, total protein. Additional relevant semiquantitative data are presented in supplemental Figure 7.

Pten loss at PND8 causes preferential MAPK activation in response to GM-CSF

Magee et al reported that *Pten* deletion induced in adult mice (6 weeks old) increased phosphorylated Akt levels in HSCs and multipotential progenitor cells, whereas *Pten* deletion at PND2 (equivalent to the third trimester of pregnancy in humans[34]) did not manifest until 3 to 4 weeks after birth.[33] However, MAPK was not impaired in either group.[33] To investigate the impact of Pten loss at PND8 on the Akt and MAPK pathways in response to stimulation by GM-CSF or IL-3, WBM cells were harvested from littermates who were WT or *PtenΔ/Δ* with or without *Nf1*$^{+/-}$ at age 3 to 4 weeks and stimulated with 10 pM GM-CSF after serum starvation for 4 hours, which is in the physiological concentration range that promotes a "survival-only" response in blood cells[41] and in the serum concentration range in AML and CML patients.[42,43] We found that Akt was constitutively active in WBM cells with *PtenΔ/Δ*, regardless of *Nf1* background (Figure 7A-B). Importantly, the MAPK pathway was hyperactivated, in a cytokine-dependent manner, with a preferential response to stimulation by GM-CSF over IL-3 in cells with *PtenΔ/Δ; Nf1*$^{+/-}$ (Figure 7A; supplemental Figure 7A). This finding suggests that disruption of the Akt signaling pathway was undifferentiated in GM-CSF and IL-3 pathways, whereas the MAPK pathway was preferentially upregulated in response to GM-CSF stimulation at physiological concentrations in mice with Pten loss. This observation has not been reported in other murine models (Table 1; supplemental Table 4). We also found that mice with *PtenΔ/Δ; Nf1*$^{+/-}$ had significantly higher MAPK activities compared with littermates with *PtenΔ/Δ; Nf1*$^{+/+}$ (Figure 7B; supplemental Figure 7B), suggesting that elevated MAPK activity may play a critical role in the disease severity in *Nf1*-deficient mice with *PtenΔ/Δ*.

Pten protein loss at PND8 significantly decreases total CREB protein but does not alter Egr-1 expression

We reported that JMML patients were deficient in cyclic adenosine monophosphate response element–binding protein (CREB) and early growth response protein 1 (EGR1) levels (85% and 87%, respectively).[44] CREB has a dual function in regulating c-JUN, being a repressor in the absence of phosphorylation and an activator when phosphorylated.[45] c-JUN is elevated in JMML.[46] CREB phosphorylated at Ser133 differentially responds to stimulations by GM-CSF and IL-3[47] and upregulates Egr-1 transcription. Egr-1 plays a deterministic role in governing hematopoietic development along the macrophage lineage.[48] We hypothesized that deficiency of both CREB and Egr-1 was necessary for JMML cells to be selectively GM-CSF hypersensitive. To understand why the selective GM-CSF hypersensitivity was absent in *PtenΔ/Δ; Nf1*$^{+/-}$ mice, we investigated protein expression of CREB and Egr-1. We stimulated WBM cells with 10 pM GM-CSF or IL-3.[41] We found that although total CREB protein was consistently decreased in *PtenΔ/Δ; Nf1*$^{+/-}$ mice, the pattern of CREB phosphorylation was not altered in response to GM-CSF or IL-3 stimulation, nor was Egr-1 expression (Figure 7C). This finding supports the notion that the regulation of CREB activation and Egr-1 expression is intact in *PtenΔ/Δ; Nf1*$^{+/-}$ mice. It further suggests that without deregulated CREB activation or altered Egr-1 expression, Pten protein loss along with *Nf1* haploinsufficiency is insufficient to alter the cell sensitivity to GM-CSF (proliferation related), although it can cause the lethal monocyte/macrophage accumulation in organs by promoting the mechanism for "survival only."[41]

Discussion

Although JMML does not readily transform to acute leukemia, these very young patients still face an early death. We were therefore motivated to identify molecular defects responsible for the aggressiveness and early demise. Prior murine models of JMML have not reproduced the clinical phenotype typified by a very age-restricted

and aggressive disease that rarely transforms to acute leukemia (reviewed in Table 1; supplemental Table 4). The discoveries of differences in properties and transcriptional programs of HSCs during development and aging[32,49] prompted us to focus on the unique physical and pathological conditions of hematopoiesis in early development. The new murine model we report here simulates PTEN protein loss, a condition observed in many human malignancies. Importantly, we designed our model to occur in the setting of germ line *Nf1* haploinsufficiency. Although we recognize that *PTEN* mutations are not found in JMML, we chose to introduce *Pten* gene loss merely as a means to simulate the consequence of Pten protein loss. More important, the protein loss was induced at a specific time in juvenile mice at an age equivalent to that of human newborns. This timing resulted in a deadly murine MPN at a juvenile age equivalent to the age of JMML patients. This murine MPN is very aggressive, features monocyte/macrophage accumulation, and does not readily transform to acute leukemia. Our data support our hypothesis that PTEN protein loss in the first few months of life is a significant contributor to early death in JMML.

We previously reported that JMML PTEN protein deficiency was not due to *PTEN* mutation. If one considers that (1) although somatic deletion of *Nf1* or mutant *Ras* in mice can mimic aberrant GM-CSF hypersensitivity, these mice lack juvenile lethality; and (2) somatic loss of Pten without detectable mutation occurs in T-ALL murine cell lines with K-Ras$^{D12/G6450}$; then taken together, it suggests that PTEN protein loss is an event that follows driver mutations that cause GM-CSF hypersensitivity in JMML and results in lethal disease progression.

Our data demonstrated that MAPK pathway disruption was more pronounced in GM-CSF signaling in mice with *Nf1* haploinsufficiency than in WT *Nf1* littermates when Pten protein loss was induced at PND8. Others reported that the MAPK pathway was not impaired in mice with *Pten* deletion induced on either PND2 or at age 6 weeks, and the phosphorylated Akt did not manifest until 3 to 4 weeks after birth in HSCs/multipotential progenitor cells in mice with *PtenΔ/Δ* induced on PND2.[33] Taken together, these results suggest that the upregulated MAPK pathway is a critical player responsible for the early lethal monocyte/macrophage infiltration in vital organs, in addition to the deregulated Akt pathway in mice with *Nf1* haploinsufficiency and Pten protein loss induced at PND8. This reasoning may explain why *Nf1*-deficient mice with Pten loss developed a more severe MPN at a juvenile age, whereas other mice with *PtenΔ/Δ* induced in the prenatal or adulthood stages developed a latent and transient MPN but eventually transformed into acute leukemia in adulthood.[31,33] It is possible that the cells were able to accumulate more molecular defects during expansion, given a less aggressive course. Furthermore, it was reported that in vitro, somatic loss of *Pten* expression in cells with *K-Ras$^{D12/G64}$* is strongly correlated with resistance to MEK inhibition.[50] We also found that cells with *Pten* deletion are resistant to a MAPK inhibitor, PD0325901 (Y.L.L., unpublished observations). Therefore, using MAPK inhibitors as monotherapy should be approached cautiously in JMML patients with PTEN deficiency. Alternatively, because rapamycin, a mTOR inhibitor, can selectively inhibit colony formation of CFU-GM of JMML cells[22] and deplete leukemia-initiating cells while restoring normal HSC function,[29] mTOR inhibitors could be potential therapeutics for JMML and other pediatric MPNs with PTEN deficiency.

Our finding that the phenotypes in *Nf1* haploinsufficient mice with Pten protein loss induced at PND8 were dramatically different from mice induced at age 6 weeks provides a proof-of-concept that the timing of Pten loss determines the disease severity in myeloid malignancy. Furthermore, the notion that Pten disruption caused the differential MAPK activities in juvenile mice with or without *Nf1$^{+/-}$* (Figure 7B; supplemental Figure 7B) suggests that the role of Pten in the HSC switch during development and aging may have a different impact on hematopoiesis in hosts with *Nf1* haploinsufficiency compared with WT. This idea enriches the observation made by Magee et al,[33] wherein they demonstrated that Pten was not required for neonatal hematopoiesis in WT mice. Therefore, this murine model may provide insight for studying other molecules, such as *LIN28B*, *HMGA2*, *SOX17*, *BMI1*, and *CEBPA*, which are key players in regulating the HSC switch,[49] in patients with *Nf1* haploinsufficiency or other diseases with genetic defects.

Given the knowledge that mice with somatic inactivation of *Nf1* (*Nf1$^{floxP/floxP}$*) or LOH, which causes a JMML-like disease but with significant latency (reviewed in Table 1; supplemental Table 4),[8,17] we propose that driver mutations found in *NF1* and other genes in the Ras pathway in JMML initialize the cells to be GM-CSF hypersensitive but are insufficient to cause the full juvenile clinical phenotype with lethal results. This premise echoes 2 recent reports that demonstrate that the absolute number of mutations, in addition to the drivers, are critical in predicting the prognosis of JMML.[13,14]

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Authorship

Contribution: Y.L.L. conceived the project, designed and performed the experiments, analyzed the data, prepared the figures, and wrote the manuscript; Y.Y. designed and performed the experiments and analyzed the data; C.W. performed the experiments; L.S. designed and performed the FACS analyses and BMTs; S.Y.L. designed the experiments, performed the statistical analyses, prepared the figures, and wrote the manuscript; H.N. analyzed the morphologic data from blood, BM, and organ tissues and edited the manuscript; W.F. assisted with the FACS analyses; N.C. performed the blood profiling experiments and analyzed the data; R.P. performed the chromosome stability analysis; Z.X. designed the experiments, analyzed the data, prepared the figures, and edited the manuscript; M.H.-J. supervised an experiment and edited the manuscript; S.L. designed the experiments and edited the manuscript; D.Z. designed and supervised the experiments, analyzed the data, and wrote the manuscript; P.D.E. helped conceive the project, designed and supervised the experiments, analyzed the data, and wrote the manuscript. All authors reviewed the final version of the manuscript.

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Timing of the loss of Pten protein determines disease severity in a mouse model of myeloid malignancy

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