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Brief Report

Hematology

Juvenile myelomonocytic leukemia (JMML) is a pediatric myeloproliferative neoplasm that bears distinct characteristics associated with abnormal fetal development. JMML has been extensively modeled in mice expressing the oncogenic $Kras^{G12D}$ mutation. However, these models have struggled to recapitulate the defining features of JMML due to in utero lethality, nonhematopoietic expression, and the pervasive emergence of T cell acute lymphoblastic leukemia. Here, we have developed a model of JMML using mice that express $Kras^{G12D}$ in multipotent progenitor cells ($Flt3Cre^+$ $Kras^{G12D}$ mice). These mice express $Kras^{G12D}$ in utero, are born at normal Mendelian ratios, develop hepatosplenomegaly, anemia, and thrombocytopenia, and succumb to a rapidly progressing and fully penetrant neonatal myeloid disease. Mutant mice have altered hematopoietic stem and progenitor cell populations in the BM and spleen that are hypersensitive to granulocyte macrophage-CSF due to hyperactive RAS/ERK signaling. Biased differentiation in these progenitors results in an expansion of neutrophils and DCs and a concomitant decrease in T lymphocytes. $Flt3Cre^+$ $Kras^{G12D}$ fetal liver hematopoietic progenitors give rise to a myeloid disease upon transplantation. In summary, we describe a $Kras^{G12D}$ mouse model that reproducibly develops JMML-like disease. This model will prove useful for preclinical drug studies and for elucidating the developmental origins of pediatric neoplasms.

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Mice expressing $Kras^{G12D}$ in hematopoietic multipotent progenitor cells develop neonatal myeloid leukemia

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Juvenile myelomonocytic leukemia (JMML) is a pediatric myeloproliferative neoplasm that bears distinct characteristics associated with abnormal fetal development. JMML has been extensively modeled in mice expressing the oncogenic $Kras^{G12D}$ mutation. However, these models have struggled to recapitulate the defining features of JMML due to in utero lethality, nonhematopoietic expression, and the pervasive emergence of T cell acute lymphoblastic leukemia. Here, we have developed a model of JMML using mice that express $Kras^{G12D}$ in multipotent progenitor cells (Flt3Cre⁺ $Kras^{G12D}$ mice). These mice express $Kras^{G12D}$ in utero, are born at normal Mendelian ratios, develop hepatosplenomegaly, anemia, and thrombocytopenia, and succumb to a rapidly progressing and fully penetrant neonatal myeloid disease. Mutant mice have altered hematopoietic stem and progenitor cell populations in the BM and spleen that are hypersensitive to granulocyte macrophage-CSF due to hyperactive RAS/ERK signaling. Biased differentiation in these progenitors results in an expansion of neutrophils and DCs and a concomitant decrease in T lymphocytes. Flt3Cre⁺ $Kras^{G12D}$ fetal liver hematopoietic progenitors give rise to a myeloid disease upon transplantation. In summary, we describe a $Kras^{G12D}$ mouse model that reproducibly develops JMML-like disease. This model will prove useful for preclinical drug studies and for elucidating the developmental origins of pediatric neoplasms.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a pediatric myeloproliferative neoplasm (MPN) caused by somatic mutations in the RAS/MEK/ERK pathway signaling genes, including *KRAS*, *NRAS*, *PTPN11*, *NFI*, and *c-CBL* (1). These mutations result in a hypersensitivity of hematopoietic progenitors to granulocyte macrophage-CSF (GM-CSF) and lead to monocytosis, anemia, thrombocytopenia, hepatosplenomegaly, and infiltration of peripheral tissues with histiocytes (2–4). Compared with other pediatric hematologic malignancies, the prognosis of patients with JMML is very poor. Allogeneic hematopoietic stem cell (HSC) transplantation is the only curative therapy, which nonetheless has a 5-year overall survival rate of only 52% (5).

The majority of JMML cases result from a mutation in a single gene (6–8). As such, disease models using the most common JMML-initiating mutations have been readily generated (9–11). The Mx1Cre $Kras^{G12D}$ mouse was the first conditional animal model of JMML and continues to be studied extensively (12, 13). However, these mice succumb with MPN that can be exacerbated by T cell leukemia/lymphoma (T-ALL) and that is confounded by nonhematopoietic $Kras^{G12D}$ expression (14–18). While the use of inducible Mx1Cre serves to limit oncogene expression until after birth, in utero $Kras^{G12D}$ expression owing to spontaneous Mx1Cre activity was not assessed in this model. Moreover, in utero $Kras^{G12D}$ expression induced by LysMCre or VavCre led to lung adenocarcinoma

or prenatal lethality, respectively (19, 20). Thus, existing $Kras^{G12D}$ models do not directly address the fetal origin of JMML and do not reliably recapitulate the myeloid-restricted nature of the disease.

Converging clinical evidence suggests that the origin of JMML is closely associated with fetal development. Patients present very young with a median age of less than 2 years, and retrospective analyses indicate that the somatic disease-initiating mutation is frequently present at birth (6, 21, 22). Furthermore, BM progenitors of most patients exhibit a fetal-like gene expression signature, which correlates with an inferior prognosis (23). These findings strongly implicate a developmental origin of JMML and imply that disease-initiating mutations occur within a specific spatial and temporal context.

Fetal hematopoietic progenitors are functionally distinct from adult progenitors. Murine fetal progenitors have greater engraftment efficiency, biased lineage differentiation, and altered susceptibility to transformation compared with adult counterparts (24–27). Analogous studies in humans showed that fetal and cord blood CD34⁺ cells are more proliferative and have a greater propensity to form myeloid colonies in methylcellulose culture than do adult cells (28, 29). These characteristics of fetal progenitors suggest a mechanism through which they may evoke clinical features of JMML when challenged with a somatic oncogenic mutation.

We hypothesized that temporal expression of $Kras^{G12D}$ during fetal hematopoiesis that was limited functionally to the hematopoietic progenitor population would produce a JMML-like disease. Recently, the expression pattern of Flt3Cre has been extensively studied using lineage-tracing methods (30–32). Robust Flt3Cre activity begins in multipotent progenitors (MPPs) at E10.5 and is subsequently observed in more than 90% of mature leukocytes. We now demonstrate that $Kras^{G12D}$ is sufficient to produce a peri-

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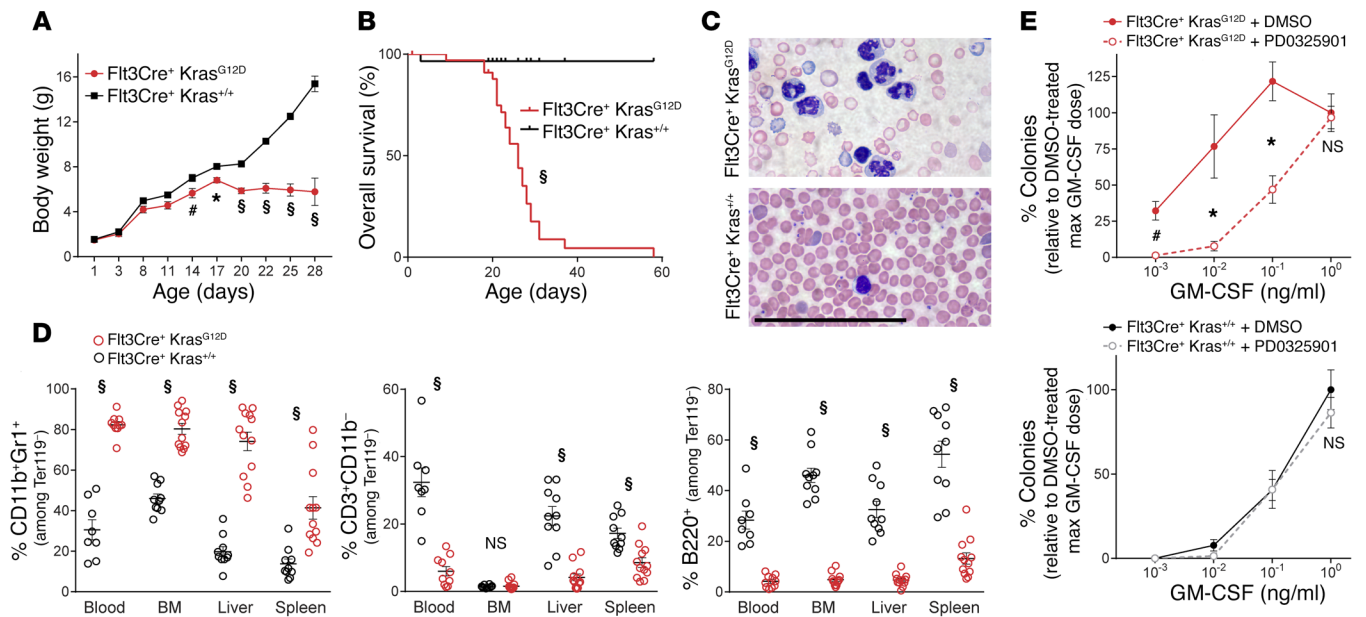


Figure 1. *Flt3Cre⁺ Kras^{G12D}* mice develop a JMML-like disease. (A) Weight gain from birth ($n = 12$ mutants and 19 controls). (B) Overall survival (statistical analysis by Mantel-Cox test). (C) Peripheral blood smear ($n = 5$). Scale bar: 100 μm . (D) Flow cytometric quantification of tissue leukocytes. (E) Seven-day BM colony formation with 100 nM PD0325901 or 0.1% DMSO ($n = 3$ biological replicates/group). All analyses were performed on 3- to 4-week-old moribund *Flt3Cre⁺ Kras^{G12D}* mice and age-matched littermates. * $P < 0.05$, * $P < 0.01$, and § $P < 0.001$, by unpaired, 2-tailed Student's t test (A, D, and E).

natal MPN when expressed in fetal hematopoietic progenitors using *Flt3Cre* and have thereby defined the crucial cellular and developmental environment needed to produce authentic features of *Kras^{G12D}*-induced JMML.

Results and Discussion

We mated *Flt3Cre⁺ ROSA^{mTmG/mTmG}* studs with dams bearing a conditional *Lox-STOP-Lox Kras^{G12D/+}* allele (*LSL-Kras^{G12D/+}*) to generate *Flt3Cre⁺ ROSA^{mTmG/+} LSL-Kras^{G12D/+}* mice (hereafter referred to as *Flt3Cre⁺ Kras^{G12D}* mice), in which oncogene expression could be monitored by a switch from Tomato to GFP expression. *Flt3Cre⁺ Kras^{G12D}* mutants were born at expected Mendelian ratios and had weight gain comparable to that of their littermates until 2 weeks of age (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI94031DS1>). Mutants and littermates had equivalent activity of *Flt3Cre*, as measured by the percentage of GFP⁺ cells, and *LSL-Kras^{G12D}* recombination in mutant mice was confirmed by PCR (Supplemental Figure 1, B and C). After 2 weeks, *Flt3Cre⁺ Kras^{G12D}* mice showed progressive weight loss, leukocytosis, anemia, thrombocytopenia, and hepatosplenomegaly and died at a median age of 26 days (Figure 1, A–C, Supplemental Figure 2, and Supplemental Figure 3). Histological organ examination revealed a histiocytic infiltrate in the spleen, liver, lung, and intestines (Supplemental Figure 4), and a markedly increased frequency of CD11b⁺Gr1⁺ cells in the blood, BM, liver, and spleen was confirmed by flow cytometry (Figure 1D and Supplemental Figure 5). Notably, the frequency of CD3⁺ T lymphocytes and B220⁺ B lymphocytes was decreased, and *Flt3Cre⁺ Kras^{G12D}* mice had an atrophied thymus compared with that seen in the littermates (Figure 1D, Supplemental Figure 3, and Supplemental Figure 4C). Consistent with a faithful model of hyperactive RAS-induced JMML, BM

progenitors from *Flt3Cre⁺ Kras^{G12D}* animals demonstrated hypersensitivity to GM-CSF in colony-forming assays, which was corrected by MEK inhibition (Figure 1E and Supplemental Figure 6).

To confirm that the disease in *Flt3Cre⁺ Kras^{G12D}* animals was initiated in utero and could be propagated autonomously in vivo, we transplanted E14.5 fetal liver (FL) cells into adult BoyJ animals (Figure 2A). Progenitors from mutant donors showed robust engraftment and rapidly contributed to monocytosis, anemia, and thrombocytopenia (Figure 2, B and C, and Supplemental Figure 7). Mutant progenitors gave rise to expanded myeloid cell populations in the BM and spleen, leading to hepatosplenomegaly and a median survival of 9 weeks (Figure 2, D–H). In stark contrast to other *Kras^{G12D}* models, primary recipients of *Flt3Cre⁺ Kras^{G12D}* progenitors showed no signs of T-ALL (Figure 2I). Upon secondary transplantation with 4×10^6 primary BM cells, *Flt3Cre⁺ Kras^{G12D}* mutant cells engrafted, and 5 of 6 recipients rapidly succumbed with monocytosis, splenomegaly, and thymic atrophy (Supplemental Figure 8). These findings indicate that temporal expression of *Kras^{G12D}* in utero transforms fetal hematopoietic progenitors into transplantable JMML-initiating cells.

We proceeded to analyze the effect of fetal *Kras^{G12D}* expression on the frequency and distribution of HSCs and progenitors. In contrast to *Mx1Cre⁺ Kras^{G12D}* animals (16), we observed a reduction of HSCs (Tomato⁺ LSK CD150⁺CD48⁻) and MPPs (GFP⁺ LSK CD150⁻CD48⁺) in both the BM and spleen of moribund *Flt3Cre⁺ Kras^{G12D}* mice (Figure 3A). This reduction corresponded with increased quiescence among HSCs in *Flt3Cre⁺ Kras^{G12D}* mice (Figure 3B and Supplemental Figure 9). These effects were mediated non-cell autonomously, since *LSL-Kras^{G12D}* was not recombined in HSCs (Supplemental Figure 10). We proceeded to analyze the progeny of *Flt3Cre⁺ Kras^{G12D}* progenitors and found that BM cells cultured in cytokine-free medium gave rise to histiocytes that expressed CD11c

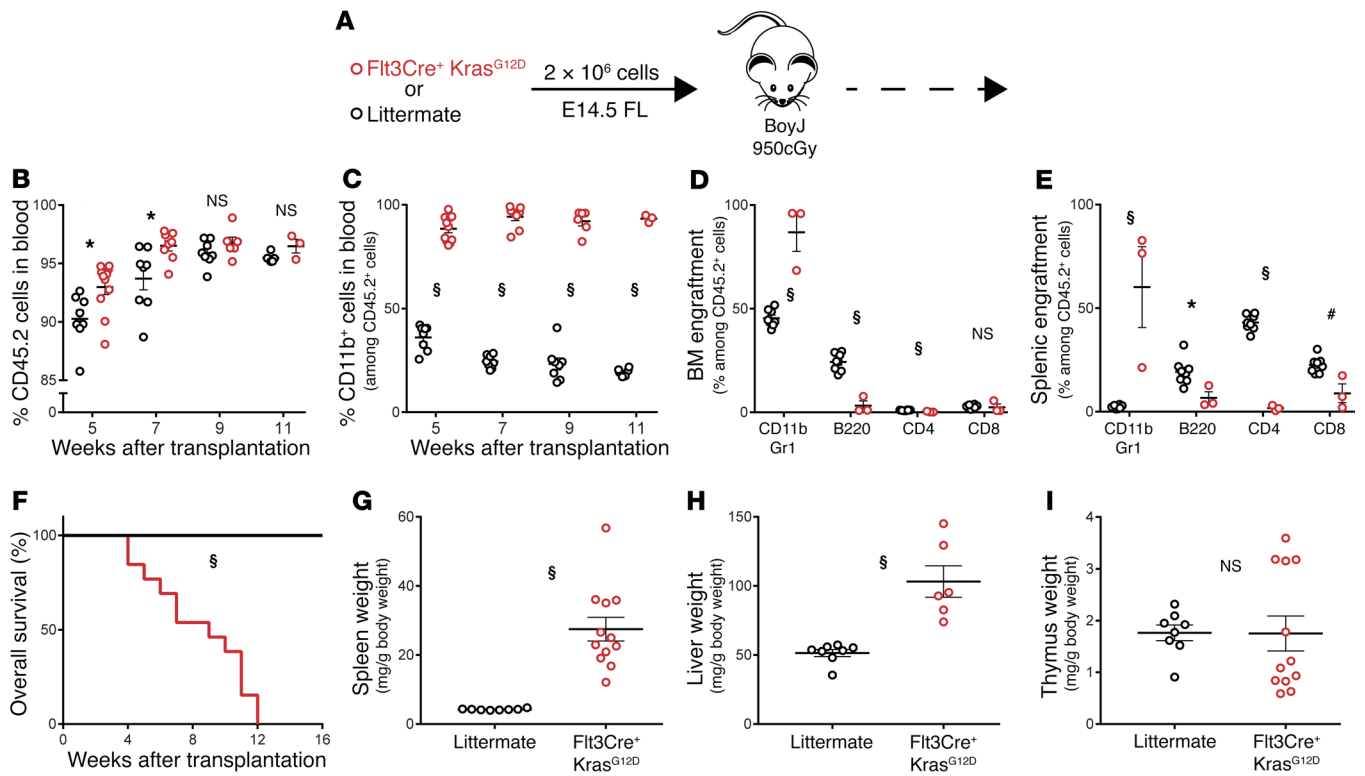


Figure 2. Fetal Flt3Cre⁺ Kras^{G12D} progenitors initiate a JMML-like disease upon transplantation. (A) Schematic of FL transplants ($n = 12$ mutant and 8 control recipients). Analysis of donor cell (B) engraftment and (C) myeloid contribution in peripheral blood. (D and E) Flow cytometric quantification of donor leukocytes in BM and spleens of moribund mutant recipients and control recipients 16 weeks after transplantation. max, maximum. (F) Overall survival following transplantation (statistical analysis by Mantel-Cox test). (G–I) Normalized tissue weights of analyzed animals. * $P < 0.05$, # $P < 0.01$, and § $P < 0.001$, by unpaired, 2-tailed Student's t test (B–E and G–I).

and CD135 (Supplemental Figure 11). This finding is reminiscent of reports describing DC-like tumor cells in patients with JMML (2, 33, 34) and prompted us to analyze DC populations in Flt3Cre⁺ Kras^{G12D} animals. We observed a marked increase in the frequency of CD11c⁺ cells in the BM and spleen (Figure 3C). Strikingly, the CD11c⁺ cell expansion was particularly prominent in the atrophied thymus, where we saw a concomitant deficit of CD4⁺CD8⁺CD25⁺ committed T cell progenitors and CD4⁺CD8⁺ double-positive cells (Figure 3, C–F, and Supplemental Figure 12). We also observed this propensity for preferential DC differentiation in recipients of Flt3Cre⁺ Kras^{G12D} FL progenitors (Supplemental Figure 13).

We present the first Kras^{G12D} model to our knowledge to unify JMML disease-defining features: an in utero origin; viability at birth followed by a failure to thrive; anemia; thrombocytopenia; monocytosis; hepatosplenomegaly; and infiltration of tissues with histiocytes. All Flt3Cre⁺ Kras^{G12D} mice succumbed to a myeloid disease that could be recapitulated following FL transplantation. This is in contrast to existing Kras^{G12D} models, whose MPN is exacerbated by nonhematopoietic oncogene expression and by the unpredictable coemergence of T-ALL (15–20).

Flt3Cre has an expression pattern markedly different from that of Mx1Cre, which may explain the observed respective myeloid versus lymphoid disease outcomes. Mx1Cre is used to target adult progenitors, whereas Flt3Cre becomes active in fetal MPPs. As such, Flt3Cre initiates Kras^{G12D} expression within an in utero progenitor that is more proliferative, has greater repopulating ability, and has

enhanced myeloid cell production compared with adult progenitors targeted by Mx1Cre (24, 27). This context emulates studies of JMML patients that highlighted the fetal origins of this disease: the causative somatic mutation commonly occurs before birth, and BM cells have a gene expression signature that is characteristic of fetal progenitors (22, 23). Therefore, in contrast to Mx1Cre, Flt3Cre targets Kras^{G12D} expression to hematopoietic progenitors at the appropriate developmental stage to recapitulate the origin of JMML.

The identity of the JMML-initiating cell has been controversial. On the one hand, case reports have shown that CD34⁺CD38[−] phenotypic HSCs express disease-initiating mutations (6, 35) and that xenotransplantation of patients' progenitors gives rise to mutated myeloid, B, and T cells with a common clonal origin (36). On the other hand, circulating T lymphocytes from most patients do not express the disease-initiating mutation (7, 37), suggesting that JMML is initiated within a MPP that undergoes a differentiation block during T lymphocyte commitment. Consistent with this hypothesis, case reports suggest that patients with JMML have decreased T cell frequencies in the BM and spleen (38, 39). These findings parallel our Flt3Cre⁺ Kras^{G12D} model, which has a paucity of T cells, an atrophied thymus, and abnormal T cell differentiation.

An earlier study found similarly skewed T lymphocyte development when Kras^{G12D} expression was restricted to DCs in p53^{−/−} mice (40). Our results advance these findings to show that Kras^{G12D} expression in multipotent progenitors results in widespread tissue infiltration with DCs that are distinct from the concomitantly

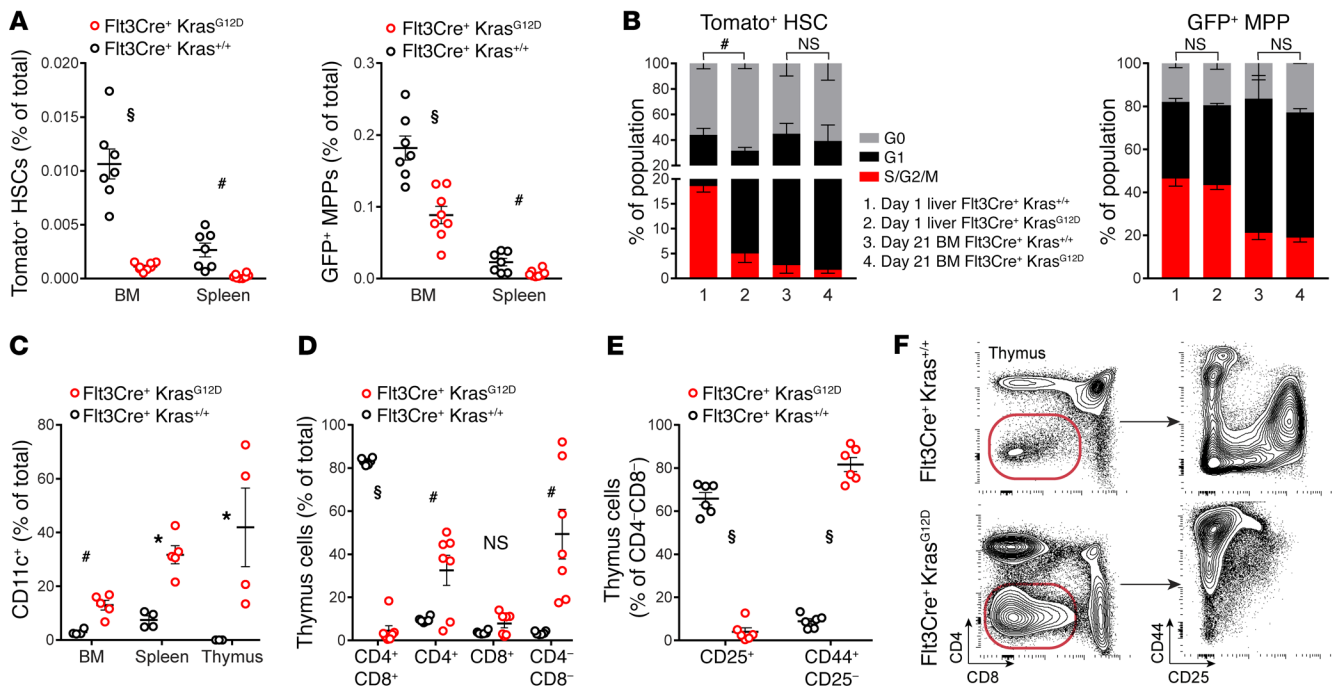


Figure 3. Analysis of Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ progenitor frequency and differentiation. (A) Frequency of HSCs and MPPs in the BM and spleens of mutants and littermates. (B) Cell-cycle analysis of HSCs and MPPs from 1-day-old liver ($n = 3$ WT and 4 mutants) and 21-day-old BM ($n = 2$ /group); representative gating is shown in Supplemental Figure 9. (C) Flow cytometric quantification of tissue DCs. (D–F) Flow cytometric quantification and representative gating of thymic cells. Error bars represent the SEM. Cell-cycle statistical analyses were performed using a χ^2 test, and other analyses were performed using an unpaired, 2-tailed Student's t test. * $P < 0.05$, # $P < 0.01$, and § $P < 0.001$.

expanded neutrophils (Supplemental Figure 13B). Importantly, case reports have equally noted that JMML patients' tissues are infiltrated by atypical histiocytes (2, 41) and that children with the DC disorder juvenile xanthogranuloma are at increased risk of JMML (42). Additionally, Langerhans cell histiocytosis and Erdheim-Chester disease, two aggressive DC disorders, are also characterized by hyperactive RAS signaling (43). These reports, along with our findings from Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ mice, suggest that a more formal investigation of DC involvement in JMML is warranted.

A unique feature of Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ mice is that their HSCs do not express the oncogene (Supplemental Figure 10). The full penetrance of a MPN in our model is therefore consistent with the hypothesis that the HSC is the cell of origin for $\text{Kras}^{\text{G12D}}$ -evoked T-ALL (16, 17). Notably, the quiescence of nononcogene-expressing HSCs in Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ mice demonstrates a profound non-cell-autonomous effect of this mutation. Our finding supports the conclusions of Sabnis et al., who noted that residual nonrecombined LSK Flt3^+ cells in Mx1Cre^+ $\text{Kras}^{\text{G12D}}$ mice did not expand to compensate for diminishing oncogene-expressing HSCs (16). Our results suggest that $\text{Kras}^{\text{G12D}}$ -expressing hematopoietic cells induce an aberrant BM microenvironment that stifles the expansion of normal neighboring HSCs. This yields the provocative hypothesis that patients with JMML relapse following allogeneic transplantation as a result of an adverse niche that impedes the proliferation of donor HSCs.

In summary, we describe what to our knowledge is the first $\text{Kras}^{\text{G12D}}$ mouse model that recapitulates defining features of JMML. Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ mice are viable, develop monocytosis, anemia, thrombocytopenia, and hepatosplenomegaly and die from a fully penetrant myeloid disease. This model further emu-

lates underappreciated features of JMML such as a paucity of mature T lymphocytes and an expansion of DCs and thereby hints at potential new therapeutic strategies. Flt3Cre^+ $\text{Kras}^{\text{G12D}}$ mice will prove useful for preclinical drug studies targeting the RAS/MEK/ERK signaling pathway and will help elucidate the developmental origins of JMML and pediatric leukemias.

Methods

Detailed methods, including all flow cytometry antibodies (Supplemental Table 1), are described in the Supplemental Methods.

Study approval. Animal studies were approved by the IACUC of the Indiana University School of Medicine. Animals were genotyped using primers outlined in Supplemental Table 2 of Supplemental Methods.

Statistics. P values comparing mutant and littermate groups were calculated using 2-tailed Student's t tests, Mantel-Cox log-rank tests, or χ^2 tests, as indicated in the figure legends. P values of less than 0.05 were considered significant. All error bars represent the SEM.

Author contributions

SPT conceived the study, designed, performed, and analyzed experiments, and wrote the manuscript. MK designed and performed experiments. RJC and MCY conceived the study, designed and analyzed experiments, and wrote the manuscript.

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