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Pdx1 expression in hematopoietic cells activates *Kras*-mutation to drive leukemia in KC (*Pdx1-Cre ; LSL-Kras$^{G12D/+}$*) mice

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Abstract

The highly utilized KC model has a reported lethality rate of about 30%, which has been attributed to pancreas cancer. However, a competing cause of lethality in KC mice is due to activation of mutant-*Kras* gene (*Kras$^{G12D/+}$*) in the multipotent progenitor cells (MPP), and subsequent development of *Kras*-mutant T-cell acute lymphoblastic leukemia (T-ALL). Overall, 20% (5/25) of KC mice developed T-ALL by 9 months of age. Transplantation of pooled bone marrow from KC mice into CD45 congenic mice caused T-ALL in 100% of recipient mice, confirming that mutant-*Kras* expression in the hematologic compartment is driving the development of T-ALL in the KC mouse model. These results are an essential consideration for investigators using this model. Further, the lower penetrance of T-ALL in KC mice (versus existing leukemia models) suggest this model could be considered as an alternative research model to evaluate onset and factors that exacerbate development of T-ALL.

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Author's contributions

MW contributed to the data acquisition (prepared Figure 1, Figure 3, Figure 5, supplemental table 1), data analysis and writing of the manuscript. MN contributed to the data acquisition (prepared Figure 2, Figure 3, Figure 4, Figure 6, supplemental figures 1 and 2), data analysis, interpretation of data, and editing of the manuscript. MW and MN contributed equally to this work as co-first authors. ER contributed to the data acquisition, data analysis and editing of the manuscript. KM contributed to the data acquisition, data analysis and editing of the manuscript. SRK designed the study, contributed to the data acquisition, data analysis, interpretation of data, and writing of manuscript.

Competing interest

The authors declare that they have no competing interests

DECLARATIONS

Ethics approval and consent to participate

The use of animals for this study was approved by the University of Wisconsin School of Medicine and Public Health Institutional Animal Care and Use Committee (M005959).

Consent for publication

Not applicable

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Keywords

T-cell acute lymphoblastic leukemia; T-ALL; *Kras* ; Pdx1; hematopoietic; KC mice

INTRODUCTION

The KC mouse model is generated by crossing the *LSL-Kras*$^{G12D/+}$ (K) mouse to pancreatic and duodenal homeobox 1 (*Pdx1*)-*Cre* (C) mice resulting in expression of the *Kras*-mutation (*Kras* ^{G12D}) in cells expressing *Cre*-recombinase from pancreatic and duodenal homeobox 1 (*Pdx1*) promoter.[1] *Pdx1* is most highly expressed in pancreas lineage cells, and all KC mice develop pancreatic intraepithelial neoplasia (PanIN)-1 by age 5 months. With increasing age, the PanIN lesions progress such that a small percentage of KC mice develop PanIN-2, PanIN-3 and pancreas cancer by roughly 9 months of age.[1]

Despite the indolent pancreas pathology in KC mice, there is an approximate 30% rate of lethality or moribund status requiring euthanasia out to age 9 months, which matches existing literature.[2] No mice became moribund due to pancreas cancer, and there was no evidence for metastatic pancreas cancer in any of the moribund mice. Rather, KC mice became distressed – manifest by respiratory distress or significant weight loss – due to advanced hematologic cancer (T-cell acute lymphoblastic leukemia or T-ALL) resulting in large thymic tumors precipitating respiratory suppression and subsequent death.

This study presents the novel finding that in KC mice, *Pdx1* expression results in *Cre*-recombinase mediated excision of lox-stop and activation of mutant-*Kras* gene (*Kras*$^{G12D/+}$) in the hematopoietic cells, and subsequent development of *Kras*-mutant T-ALL with concomitant thymic tumor in a subset of mice. The use of a reporter mouse line and bone marrow transplant study supported these results. These notable results are an important consideration for investigators utilizing this model.

MATERIALS AND METHODS

Animals

All animal studies were conducted according to an approved protocol (M005959) by the University of Wisconsin School of Medicine and Public Health (UW SMPH) Institutional Animal Care and Use Committee (IACUC). Mice were housed in an Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited selective pathogen-free facility (UW Medical Sciences Center) on corncob bedding with chow diet (Mouse diet 9F 5020; PMI Nutrition International), and water ad libitum. The Lox-Stop-Lox (*LSL*) *Kras*$^{G12D/+}$ (B6.129S4-*Kras* tm4Tyj/J #008179), and *Pdx1-Cre* (B6.FVB-Tg(*Pdx1*-cre)6Tuv/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were bred to develop *LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* (KC) mice. The B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J or Ai14 mice (#007914) were purchased from the Jackson Laboratory. The Ai14, *Pdx1-Cre* and *LSL-Kras*$^{G12D/+}$ were bred to develop *Rosa26*$^{LSL-tdTomato}$;*LSL-Kras*$^{G12D/+}$;*Pdx1-Cre* (AiKC) mice. CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

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Genotyping was performed according to Jackson Laboratory's protocols (*Cre*: Protocol #21298, *Kras*: Protocol #29388 and Ai14: Protocol #29436). Activated *Kras* genotyping was performed as previously published.[1, 3] All mice were housed under identical conditions and are congenic on a C57BL/6J background (backcrossing > 15 generations). Both male and female mice of all strains were used. A total of 77 KC and 30 control mice were evaluated. Six AiKC mice were evaluated (reporter) for mutant-*Kras* expression. Twenty CD45.1, 3 AiKC mice and 3 C57BL6/J (CD45.2) [negative controls] mice at age 8 weeks were used for the bone-marrow transplant studies. All mice from each study and control group were included in the analysis. The health and well-being of the mice were monitored closely by research and veterinary staff. Mice that showed signs of distress were immediately euthanized through CO^2 asphyxiation.

Histology

Thymus, pancreas, liver, spleen, and bone marrow from mice were collected and formalin-fixed and paraffin embedded (FFPE) by standard methods. Blocks were sectioned at 4 μm thickness and 50 μm intervals for a total of 6–10 slides. Sectioning and hematoxylin and eosin (H&E) staining was performed by the University of Wisconsin Experimental Animal Pathology Lab (EAPL) core facility. All histology was evaluated by a board certified gastrointestinal pathologist (KAM) or hematopathologist (EAR) as appropriate, and each pathologist was blinded to the genotype of the mouse.

CD3 Immunohistochemistry

CD3 staining was performed using the automated immunohistochemistry machine, the Ventana Discovery Ultra BioMarker Platform. Deparaffinization and heat-induced epitope retrieval in the form of "cell conditioning" with CC1 buffer (Ventana #950–500), a Tris based buffer, for approximately 32 minutes at 95°C was performed. Primary antibody anti-CD3 (2GV6) (Ventana #790–4341) was added undiluted and incubated for 20 minutes at 36°C followed by rinse with Reaction Buffer (Ventana #950–300). The secondary antibody, Discovery Omni-Map anti-Rabbit HRP (Ventana #760–4311) was added and incubated for 16 minutes at 37°C followed by rinse with Reaction Buffer (Ventana #950–300). Finally, the Discovery ChromoMap DAB detection kit (Ventana #760–159) was applied according to manufacturer instructions. The samples were removed from the instrument and rinsed with dawn dish soap, warm tap water and dH_2O. Lastly, the harris hematoxylin counterstain 1:5 was applied for 45 seconds, followed by dH_2O rinse, dehydration to xylene and cover slip.

Genotyping for the activation of *Kras^{G12D}* mutation construct

We followed the genotyping method for activated-*Kras^{G12D}* mutation previously described.[3] Genomic DNA was isolated from formalin fixed paraffin embedded thymus slides using ReliaPrep FFPE gDNA MiniPrep System (Promega, Madison, WI). The DNA was then amplified using polymerase chain reaction (PCR) with the following probes: 5'-GGGTAGGTGTTGGGATAGCTG-3' (OL8403) and 5'-CCGAATTCAGTGACTACAGATGTACAGAG-3' (OL8404) with conditions previously published.[3] These primers amplified a 325 bp band corresponding to the activated *Kras^{G12D}* mutant allele and a 285 bp band corresponding to the WT allele.

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tdTomato immunohistochemistry (IHC)

FFPE samples of AiKC mouse liver, thymus and spleen were sectioned by the University of Wisconsin Experimental Animal Pathology Lab (EAPL) core facility. Immunohistochemical staining for red fluorescence protein (tdTomato) was also performed by the EAPL. Sections were deparaffinized in xylenes and hydrated through graded alcohols to distilled water. Antigens were retrieved using citrate buffer pH 6.0 (10 mM Citric Acid, 0.05% Tween 20). Endogenous peroxidase was blocked with 0.3% H_2O_2 in PBS for 10 minutes at room temperature and blocking of non-specific binding was performed using 10% goat serum. Sections were incubated with rabbit anti-RFP antibody (600-401-379, Rockland Inc, Pottstown, PA) (1:1600) overnight at 4°C. After rinsing, sections were incubated with ImmPRESS goat anti-rabbit IgG (MP-7451, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Detection was performed using DAB substrate kit (8059S, Cell Signaling Technology, Danvers, MA). Samples were counterstained using Mayer's hematoxylin (MHS32, Millipore-Sigma, St. Louis, MO) for one minute.

tdTomato Fluorescent Visualization

AiKC mouse bone marrow was formalin-fixed and frozen embedded. Sectioning was performed by the University of Wisconsin EAPL core facility. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Confocal images were acquired x10 objectives by a Zeiss Axio observer fluorescence microscope. Excitation was achieved using a Zeiss Colibri 7 (DAPI: 385 nm, tdTomato: 555 nm). The emitted fluorescence was collected with emission filters of DAPI (370–400 nm) and AF546 (540–570 nm).

RNA isolation

RNA isolation was performed as previously described.[3] Briefly, samples were stored in RNAlater (ThermoFisher Scientific, Waltham, MA) until RNA isolation (24 hours later). Following Qiazol lysis, samples were homogenized using a tissue homogenizer (Brinkmann Instruments, Model PT 10/35, 110 Volts, 6 Amps, 60 Hz) for spleen and thymus while RLT (Qiagen, Hilden, Germany) and hand homogenization was used to isolate BM. RNA was isolated using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany). The extracted RNA was quantified using a spectrophotometer (ClarioStar Plate Reader, BMG LABTECH, Ortenberg Germany) and diluted to 50 ng/uL.

Quantitative reverse transcription PCR

The qPCR was done as previously described.[3] Briefly, 500 ng of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, MA) per manufacturer protocol. The qPCR was performed on the ThermoFisher QuantStudio 7 (ThermoFisher, Waltham, MA). All reactions were run in triplicate. Results were analyzed using the delta-delta CT method. The following TaqMan® probes were used: *Cre* (Enterobacteria phage P1 cyclization recombinase, Mr00635245_cn), *Pdx1* (pancreatic and duodenal homeobox 1, Mm00565835_cn) and the house keeping gene Hprt (hypoxanthine guanine phosphoribosyl transferase, Mm03024075_m1) (ThermoFisher, Waltham, MA).

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Flow Cytometry

Bone marrow was collected from the femurs and tibias of AiKC mice and filtered through a 70 μm cell strainer. The single cell suspension was adjusted to 1 million cells per sample, suspended in 400ul of staining buffer (IMDM + 10% FBS) and stained with an antibody cocktail (supplemental table 1). Stained cells were run using the Attune Nxt (ThermoFisher) and analyzed using FlowJo. The following gating strategy was used to delineate live cells: FSC-A X SSC-A to FSC-A X FSC-H to SSC-A X SSC-H to FSC-A X DAPI. Live cells are DAPI negative. To identify bone marrow subsets from the live cells the following gating strategy was used (supplemental figure 1): FITC x FSC (FITC negative = lineage negative cells) to Kit x Sca1 (gated on c-Kit^+Sca1^+) to CD150 negative (multipoint progenitor cells) and $CD150^+$ (hematopoietic stem cells). All final subsets were analyzed as FSC-A x tdTomato to identify the tdTomato positive cells in each group.

To determine the donor chimerism, peripheral blood was collected in blood collection tube with K_2EDTA (BD Microtainer tubes), and the red blood cells were lysed with RBC lysis buffer (Santa Cruz Biochemicals, Dallas, TX). The leukocytes were labelled with CD45.1-PE (Biolegend, 12-0453-81) and CD45.2-FICT (Biolegend, 11-04540-81) antibodies. The cells were analyzed using the Attune Nxt (ThermoFisher) and analyzed using FlowJo.

Bone Marrow Transplant

B6 CD45.1 recipient mice were sub-lethally irradiated (5.5 Gy, twice) at 9–10 weeks of age. Bone marrow cells from 3 pooled donor mice (C57BL6/J mice [negative controls] or AiKC mice [study / test group]) were isolated and bone marrow transplantation performed via retro-orbital sinus injection. Following transplantation, the recipient mice were placed on a Uniprim diet containing 275 parts-per-million (ppm) trimethoprim and 1365 ppm sulfadiazine (Test Diet Company TD.06596) for 3 weeks post-irradiation in place of standard chow. Recipient mice were monitored closely for 3 weeks following transplantation to ensure no lethality related to the transplant procedure. Retro-orbital bleeds were performed to assess for donor chimerism. Mice were evaluated until time of onset of leukemia as evidenced by signs of duress or respiratory distress, or until the time of study endpoint at 5 months post-transplant.

Statistics

All data are presented as mean ± SD. Statistical significance was assessed using Fisher's Exact test (significance was assigned for P-values < 0.05). Graph generation and statistical analyses were performed using GraphPad Prism software8.0 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Development of T-ALL in the KC mouse model

The KC mice evaluated in this study developed the expected rates of pancreatic intraepithelial neoplasia (PanIN), with 100% (36/36) of KC mice developing PanIN-1, 5.6% (2/36) developing PanIN-2, and 8.3% (3/36) developing PanIN-3. Meanwhile, 13.9% (5/36) developed pancreas cancer and 36.1% (13/36) developed surface tumors called

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papillomas. Thirty-three survived to age 9 months for a 70.2% survival rate (33/47), with eleven mice unable to be evaluated due to being found deceased (age range 14.1 – 29.1 weeks). Three mice did not reach 9 months due to moribund status (>20% weight loss) and were euthanized (age 15.7–26.7 weeks). Two of the moribund mice had abdominal cavity evaluated at necropsy and no abnormalities were identified; the pancreas demonstrated only PanIN-1 with mostly preserved pancreatic architecture. Subsequently, the third moribund KC mouse (age 15.7 weeks) demonstrated chest bulging at necropsy and thus the thoracic cavity was evaluated. This revealed a large thymic tumor (pancreas demonstrated only PanIN-1) (Figure 1A, B). On histopathologic examination of the thymic tumor, there was a uniform infiltration of immature blast cells with frequent mitotic figures, consistent with the established murine models of mutated *Kras*-driven T-ALL (figure 1C).[4, 5] The diagnosis of T-ALL was confirmed by expression of cytoplasmic CD3 (figure 1D).[6] Subsequently, 30 KC mice were included in the study group and 5 KC mice were found to develop respiratory distress and/or weight loss prompting euthanasia which revealed large thymic tumors on necropsy. Of note, there were 5 KC mice that were found deceased (not able to be evaluated). Comprehensive analysis revealed no other pathology that could have precipitated duress in these mice (only PanIN-1). Only KC mice developed tumors, while no age-matched negative controls including LSL-$Kras^{G12D/+}$ (n = 10), *Pdx1-Cre* (n = 10) or C57BL/6J (n=10) mice developed leukemia, Pan-IN, pancreas cancer or papillomas; 100% of control mice lived to age 9 months.

T-ALL development in KC mouse model is driven by mutated-*Kras* gene

Given that the thymic tumors only developed in mice harboring a conditional mutant-$Kras^{G12D}$ gene, we hypothesized that the *LSL-*$Kras^{G12D/+}$ gene was being activated in the hematopoietic cells driving the development of T-ALL (and the concomitant thymic tumor). To detect the activated $Kras^{G12D}$-mutation (L-$Kras^{G12D}$), DNA was isolated from KC normal thymus and KC thymic tumor and was assessed for activated *Kras*-mutation. PCR analysis of the genomic DNA demonstrated L-$Kras^{G12D}$ in the thymic tumors of KC mice, but only wild type *Kras* (*Kras*$^{+}$) in normal thymus of KC mice without T-ALL (Figure 2). This finding indicates the T-ALL / thymic tumors are driven by the mutated $Kras^{G12D}$ gene.

Pdx1-Cre is expressed in the thymus, spleen and bone marrow

The $Kras^{G12D}$ mutation is activated by *Pdx1* driven *Cre* indicating *Pdx1* expression outside of the pancreas causes leukemia development in KC mice. Evidence clearly demonstrates *Pdx1* expression in other organ systems such as anal epithelium.[3] Further, evaluation of human and mouse hematologic cell databases reveals *Pdx1* expression in hematopoietic cells.[7, 8] To ascertain where *Pdx1-Cre* is being expressed within the KC mouse model, we crossed the KC mice to Ai14 mice to generate the AiKC ‘marker’ mouse model ($Rosa26^{LSL\text{-}tdTomato}$; *LSL-*$Kras^{G12D/+}$; *Pdx1-Cre*). AiKC mice harbor the *LSL-tdTomato* red fluorescent gene in the *Rosa26* locus, and in the presence of *Cre*-recombinase, the stop sequence is excised allowing for expression of tdTomato protein, localizing *Pdx1* (*Pdx1-Cre*) expression and functioning as a marker for expression of mutant-$Kras^{G12D}$ gene. Through IHC staining for tdTomato, we found *Pdx1-Cre* was expressed in pancreas as expected, and also in thymus and spleen (Figure 3 A,B,C,D,E,F). In conjunction, immunofluorescence (IF)

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demonstrated *Pdx1* expression in thymus, spleen, liver and high expression in bone marrow, which contains the hematopoietic cells (Figure 3 G,H,I). This was further confirmed through qPCR showing *Pdx1* expression in these tissues of WT and KC mice (Figure 3 J, K).

Pdx1 is expressed in multipotential progenitor cells (MMP)

Given the development of T-ALL, bone marrow was isolated from AiKC mice and C57BL/6J mice (control) and analyzed for *Pdx1* expression using flow cytometry including the lineage-committed cells, the lineage negative cells, the multipotential progenitor cells (MPP) and hemopoietic stem cells (HSC). We found that *Pdx1* was statistically significantly expressed in the bone marrow cells, lineage negative cells, and MPP cells in AiKC mice compared to controls, and not within the lineage positive cells or HSC population (Figure 4). This indicates that *Pdx1* allows for expression of *Cre* which activates the mutated-$Kras^{G12D}$ gene in these cells, thus likely driving the development of the T-ALL found in these mice.

Bone marrow transplant confirms hematopoietic etiology of T-ALL

Kras-mutant leukemia development in KC mice is driven by *Pdx1* expression and Cre mediated excision of lox-stop, and we hypothesized that the hematopoietic compartment likely contains the cell of origin for the T-cell malignancy seen in these mice. To confirm, we performed a bone marrow transplant (BMT) study where pooled bone marrow collected from three AiKC mice was transplanted into irradiated B6 CD45.1 mice (AiKC mice express CD45.2). The AiKC mice were otherwise healthy 8-week-old mice with no evidence of pathology on gross analysis. As a control, pooled bone marrow from three B6 CD45.2 mice was transplanted into irradiated B6 CD45.1 mice. No mice in either group died as a result of toxicity related to the irradiation or BMT. We found 100% (10/10) of the CD45.1 mice transplanted with AiKC bone marrow died of their disease by 4 months post-transplant. All deceased mice demonstrated large thymic tumors with uniform cellular infiltration and presence of *Kras* mutation confirmed by PCR analysis (Figure 5). In contrast, none (0/8) of the age matched CD45.1 mice transplanted with C57BL6/J (CD45.2) bone marrow died. Flow cytometry confirmed successful transplant of the CD45.2 bone marrow into CD45.1 mice (Supplemental Figure 2), and thymus from these wild-type BMT controls demonstrated normal histopathology. These results confirm that *Pdx1* expression in the hematopoietic compartment promotes Cre expression (*Pdx1-Cre* transgene) in KC mice, resulting in activation of mutant $Kras^{G12D}$ in hematopoietic cells and development of T-ALL, with subsequent death in the afflicted KC mice.

DISCUSSION

The KC pancreas-cancer pre-cursor mouse model (i.e. PanINs) was first developed in 2003[1] and has since continued to be highly utilized in pancreas cancer studies. Often, the *Pdx1-Cre* driven $Kras^{G12D}$-mutant (KC) mouse is typically crossed to mice harboring additional genetic mutations (e.g., Trp53, Ink4a/Arf) or mice are subjected to carcinogenic environmental changes (e.g., high-fat diet) to explore advanced pancreas cancer.[9–11] Notably, while we did not identify evidence for metastatic pancreas cancer in moribund mice or at necropsy, we did find mice have evidence of advanced hematologic cancer (T-ALL) resulting in a large thymic tumor which caused respiratory suppression and death.

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To confirm the origin of the leukemia, we used pooled bone marrow from 3 AiKC mice to transplant into 10 mice because roughly 30% of mice were deceased of leukemia by 9 months in the KC mice, so we suspected pooling of three separate samples would heighten the likelihood of hematopoietic cells containing mutant-*Kras*. Thus, this pooling of bone marrow resulted in a 'solitary sample' with activated $Kras^{G12D}$ and explains the difference between the BMT mice (where 100% developed T-ALL) and the KC mice (where 30% die by age 9 months). Similar to other *Kras*-mutant leukemia models, [5, 12] BMT mice receiving AiKC bone marrow were deceased within 3–4 months of transplantation. This reflects the aggressiveness of *Kras*-mutant leukemia, where onset of *Kras*-mutation in the hematopoietic cells causes rapid demise. In fact, in the KC mice that were found to harbor thymic tumors with T-ALL, the age of onset was roughly 14–29 weeks, with a median age of 18 weeks. Moreover, in KC mice that were spontaneously deceased in this study (prior to identification of the leukemia phenotype), all were found deceased near the median age of 18 weeks. When evaluating the pancreas of KC mice at age 4.5 months,[1] the majority of the pancreas is normal with few foci of the earliest pancreatic pre-cancerous lesions (PanIN-1), thus further refuting an underlying pancreas pathology as the cause of early death in KC mice. These results are highly indicative of the bone marrow containing the cell of origin, but an important limitation in this study is that we did not perform irradiation followed by transplantation with splenic or thymic cells to definitively confirm that spleen or thymus was not the cell of origin. In follow up work, to definitively confirm the bone marrow compartment (and begin to home in on the cell of origin), we aim to address this limitation by performing splenic/thymic cell isolation from KC mice and transplant into irradiated CD45.1 mice.

The aggressive nature of the *Kras*-mutant leukemia manifest in the KC mice (deceased within 3–4 months of BMT) is also reflected in human disease. T-cell acute lymphoblastic leukemia is a heterogeneous and lethal malignancy of immune cells that accounts for 10–15% of pediatric ALL, and 25% of adult ALL. Although high intensity combination chemotherapeutic regimens have success in pediatric and adult cases, ~25% of pediatric and ~50% of adult patients fail initial therapy or relapse.[6, 13] Prognosis is poor in these patients, with 80% mortality in spite of aggressive and toxic salvage protocols (e.g. intensive chemotherapy followed by allogeneic stem cell transplant).[14] Notably, *Kras*-mutation is disproportionately present in recurrent disease (40%) in comparison to newly diagnosed T-ALL (5–10%),[15] providing insight into refractory T-ALL. Thus a *Kras*-driven mouse model of T-ALL provides an excellent platform to understand high-risk T-ALL (i.e. refractory disease).[16] Moreover, because of the onset of T-ALL and death at a median age of 18 weeks, and the fact that there is not full penetrance with the KC model (not all KC mice developed overt hematologic malignancy), this model could be considered to evaluate factors (e.g. transcription factors) that exacerbate development of *Kras*-mutant T-ALL.

However, a more complete evaluation as compared to established models of *Kras* driven T-ALL would be necessary to properly assess changes in the development of T-ALL in the KC model. A more comprehensive characterization would include further immunophenotyping for CD4 and CD8 expression as done by Zhang et al and Junco et al in *Kras*-driven leukemia.[5, 12] This is because T-ALL is typically described by an aberrant CD4+CD8+ double-positive immunophenotype, and additional characterization could include markers

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such as CD44 and CD25.[4, 17] In addition to immunophenotyping, further characterization of the genetic heterogeneity in the T-ALL from KC mice would be beneficial. For instance, mutations in other genes, such as *Pten* or *Notch1*, are commonly found in established mouse models of T-ALL.[18] While concomitant *PTEN* and *KRAS* mutations in human T-ALL are highly uncommon, mutations in both have been identified in patients.[19] More often, *KRAS* mutation coincides with *NOTCH1* mutation. For instance, in murine models of $Kras^{G12D}$ T-ALL, mice typically acquire the *Notch1* mutation with progression of disease.[4] Future studies will further characterize the genetic characteristics of the T-ALL in the KC mouse, to identify the concomitant mutations that develop in addition to $Kras^{G12D}$. Finally, there are several classifications of T-ALL that can be defined based on the T-cell receptor (TCR) phenotype and the expression of different v genes within different T-cell subsets including malignant T-cell development.[20] Further characterization of the specific subtype in future studies will be done in conjunction with immunophenotyping and sequencing.

It is important to note that not all mice were able to be evaluated either at age 9 months, or when captured at moribund status. Some mice spontaneously died without obvious signs that precipitated the need for euthanasia, and these mice had undergone autodigestion of organs prior to effective evaluation. We identified 5 KC mice with thymic tumors from 25 KC mice that were evaluated, but there were five additional mice during this time period that were spontaneously deceased (i.e. ten out of thirty mice died prior to age 9 months). It is plausible that other hematologic malignancies (e.g. acute myelogenous leukemia) may arise in the KC mice that does not result in respiratory distress / thymic tumors, but that may cause spontaneous death. Evidence to support this is the fact that Pdx1 is expressed in the MPP cells and lineage negative cells in KC mice, which may result in other types of leukemia. However, additional studies are required to specifically address this current limitation.

This study identified the spontaneous development of T-ALL in the KC mouse model, a model highly utilized in pancreatic cancer research. The development of T-ALL in the KC model stems from the expression of Pdx1 within the BM which is likely driving the expression of the mutated $Kras^{G12D}$ gene in this cell population resulting in the development of T-cell leukemia. T-ALL, particularly refractory T-ALL, is a highly lethal cancer with 80% of those who relapse or fail first line therapies succumbing to their disease. Notably, *Kras*-mutation is disproportionately present in recurrent disease (40%) in comparison to newly diagnosed T-ALL (5–10%) presenting a unique etiology of the recurrent disease.[14] Given the KC mice develop $Kras^{G12D}$ driven T-ALL that is not 100% penetrant and is transplantable, this model has the potential to be utilized to investigate the onset and exacerbation of the T-ALL. Finally, the results of this study are highly important to those utilizing the KC model and should be of particular relevance for those investigating immunologic changes within this model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Availability of data and materials

The data generated or analyzed during the study are included in the published article (and the supplementary information).

LIST OF ABBREVIATIONS

Ai14	B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J
AiKC	B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J ; *Pdx1-Cre ; LSL-Kras*$^{G12D/+}$
BMT	Bone marrow transplant
FFPE	Formalin-fixed, paraffin embedded
H&E	Hematoxylin and eosin
HSC	Hematopoietic stem cells
IHC	Immunohistochemistry
KC	Pdx1-Cre ; LSL-Kras$^{G12D/+}$
LSL	Lox-stop-lox
MPP	Multipotent progenitor cells
PanIN	Pancreatic intraepithelial neoplasia
Pdx1	Pancreatic and duodenal homeobox 1
T-ALL	T-cell acute lymphoblastic leukemia

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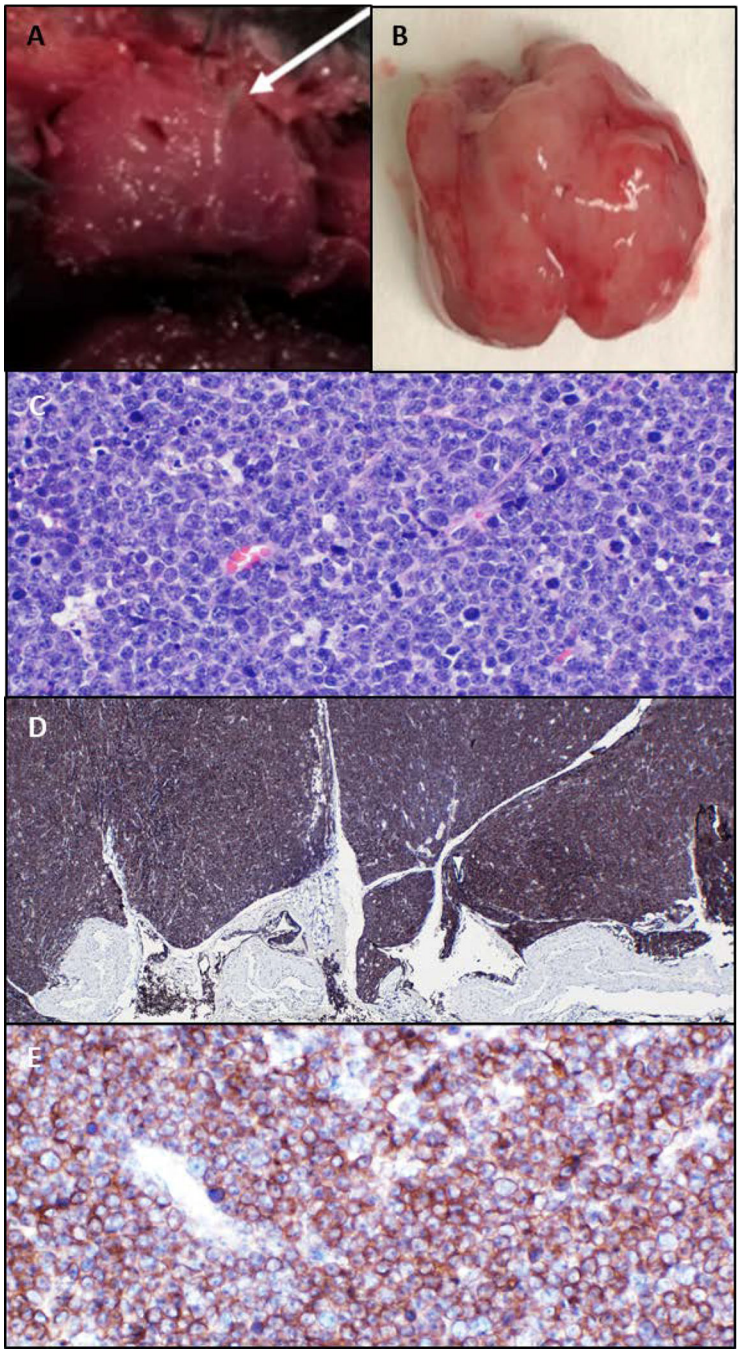

Figure 1: T-ALL driven by activated *Kras*-mutation.

KC mice develop thymic masses, demonstrated *in situ* (A), and *ex-vivo* (B). On histopathologic analysis (H&E), there is diffuse infiltration of immature blast cells with convoluted nuclear membrane, indistinct nucleoli and frequent mitotic figures (C, 400x magnification). These abnormal appearing cells obliterate the normal cortex and medulla and are positive for cytoplasmic CD3, confirming T-ALL diagnosis (D, 40x magnification; E, 400x magnification).

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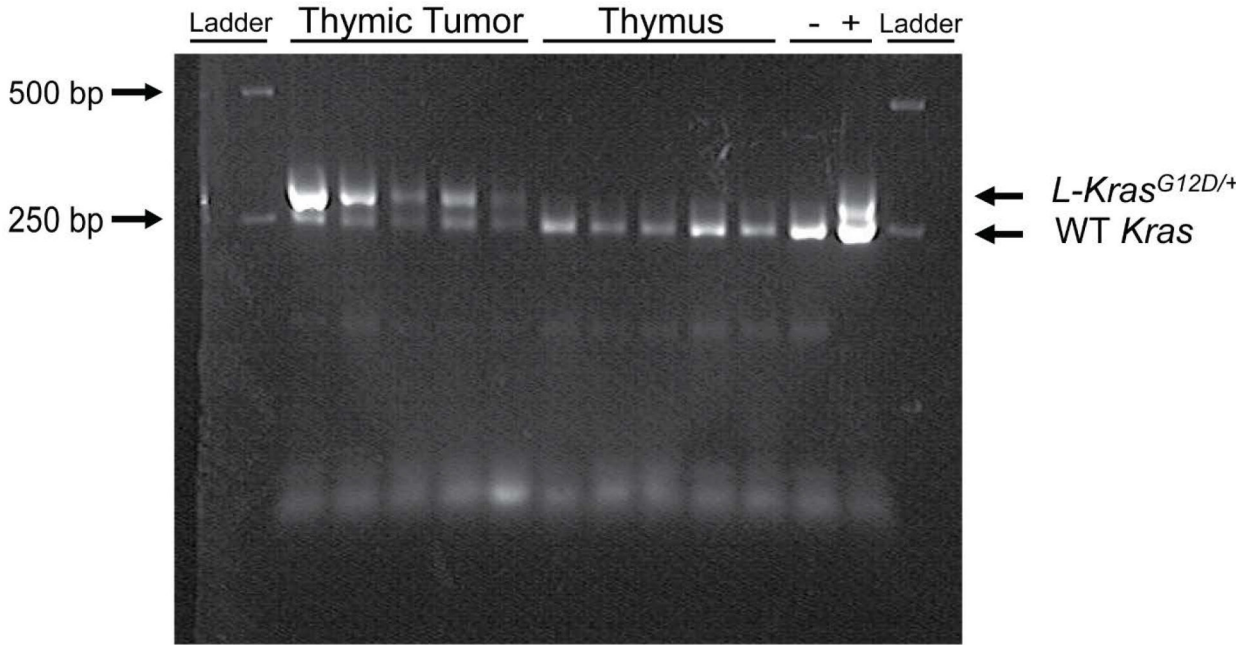

Figure 2: The activated *Kras*-mutation is present in thymic tumors but not normal thymus.
PCR of genomic DNA from thymus of KC mice without T-ALL (Thymus) reveals wild type *Kras* (WT *Kras*). In KC mice with T-ALL, the active form of *Kras*-mutant allele (L-$Kras^{G12D/+}$) is present in the thymic tumors (tumor), indicating *Cre*-mediated excision of Lox-Stop sequence. Positive control (+) consisted of splenocytes from *Vav-Cre ; LSL-*$Kras^{G12D/+}$ mice (as previously described)[3] and negative control (−) was tail from C57BL/6J mice. These are shown along with a ladder demonstrating the 250 and 500 bp markers.

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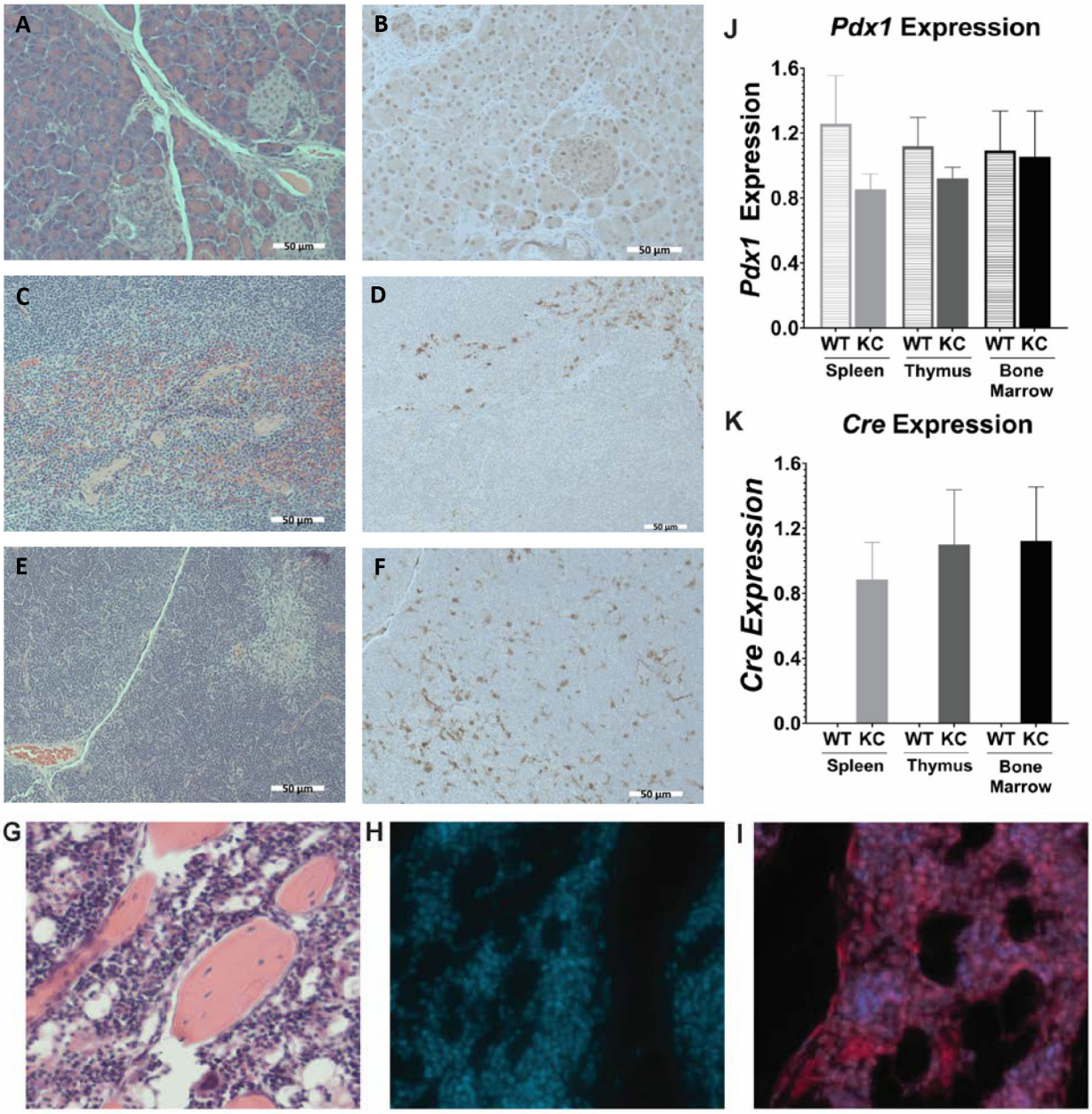

Figure 3: *Pdx1*-cre expression in pancreas, spleen, thymus and bone marrow.

AiKC mice (*Rosa26*$^{LSL\text{-}Tdtomato}$;KC) express tdTomato in cells expressing *Pdx1*. Pancreas (A,B), spleen (C,D), thymus (E,F), and femur (G,H,I) H&E (A,C,E,G) with anti-tdTomato antibody (B,D,F brown dots) demonstrate cells with *Pdx1* expression. Immunofluorescence shows DAPI stained cells (H) and DAPI with tdTomato (I), confirming *Pdx1* expression in the bone marrow. *Pdx1* (J) and *Cre* (K) expression was confirmed using qPCR. Scale bars equal 50 µm.

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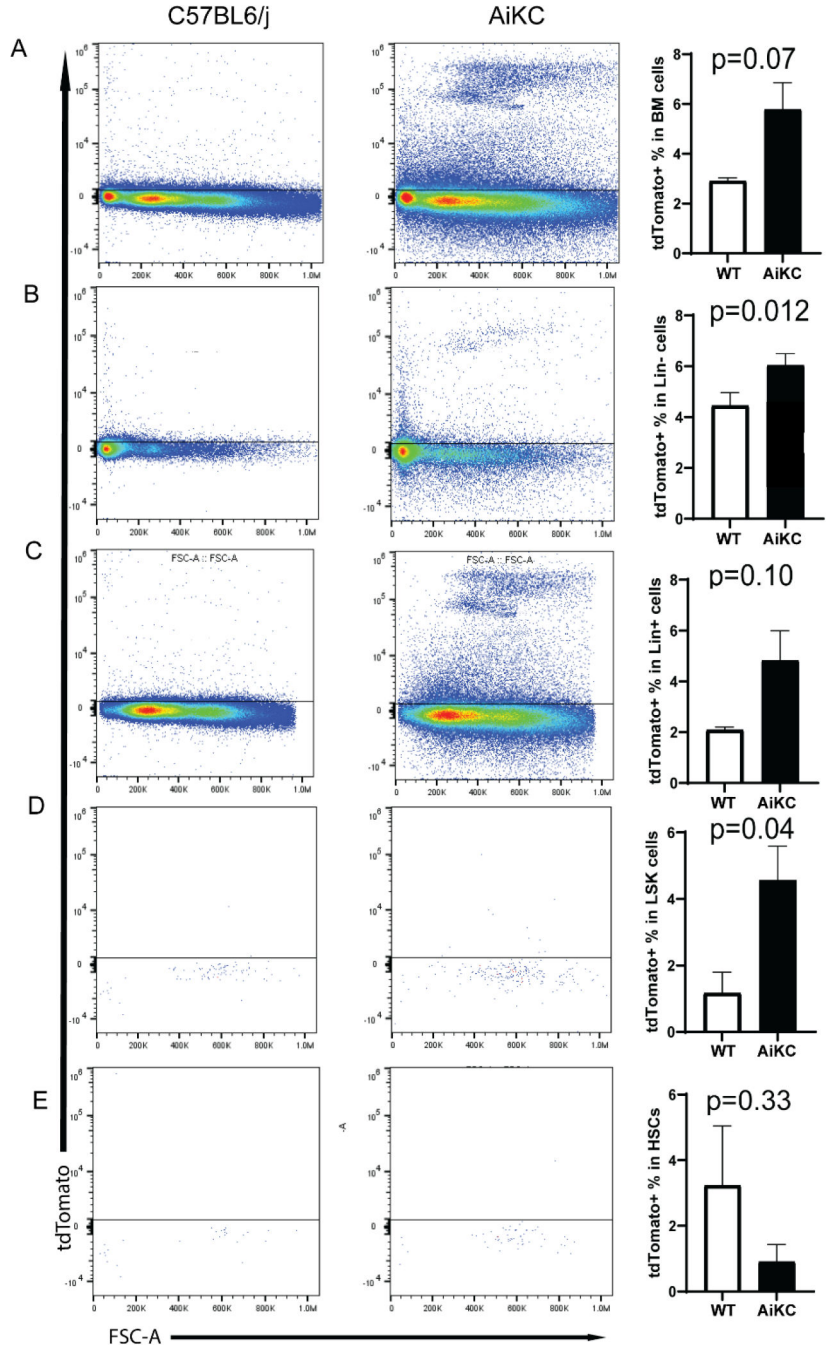

Figure 4: *Pdx1* expression within the multipotential progenitor cells in the bone marrow.

Bone morrow was isolated from the AiKC mice and the cells expressing *Pdx1* were analyzed. (A) The expression level of tdTomato cells in the bone marrow (BM) (P=0.07). (B)There is significant expression of tdTomato cells in lineage negative BM cells (P=0.012), (C) but not lineage positive BM cells (P=0.10). (D)There is significant expression in the multipotential progenitor cells (P=0.04). (E) There is not significant expression within the hematopoietic stem cells (P=0.33).

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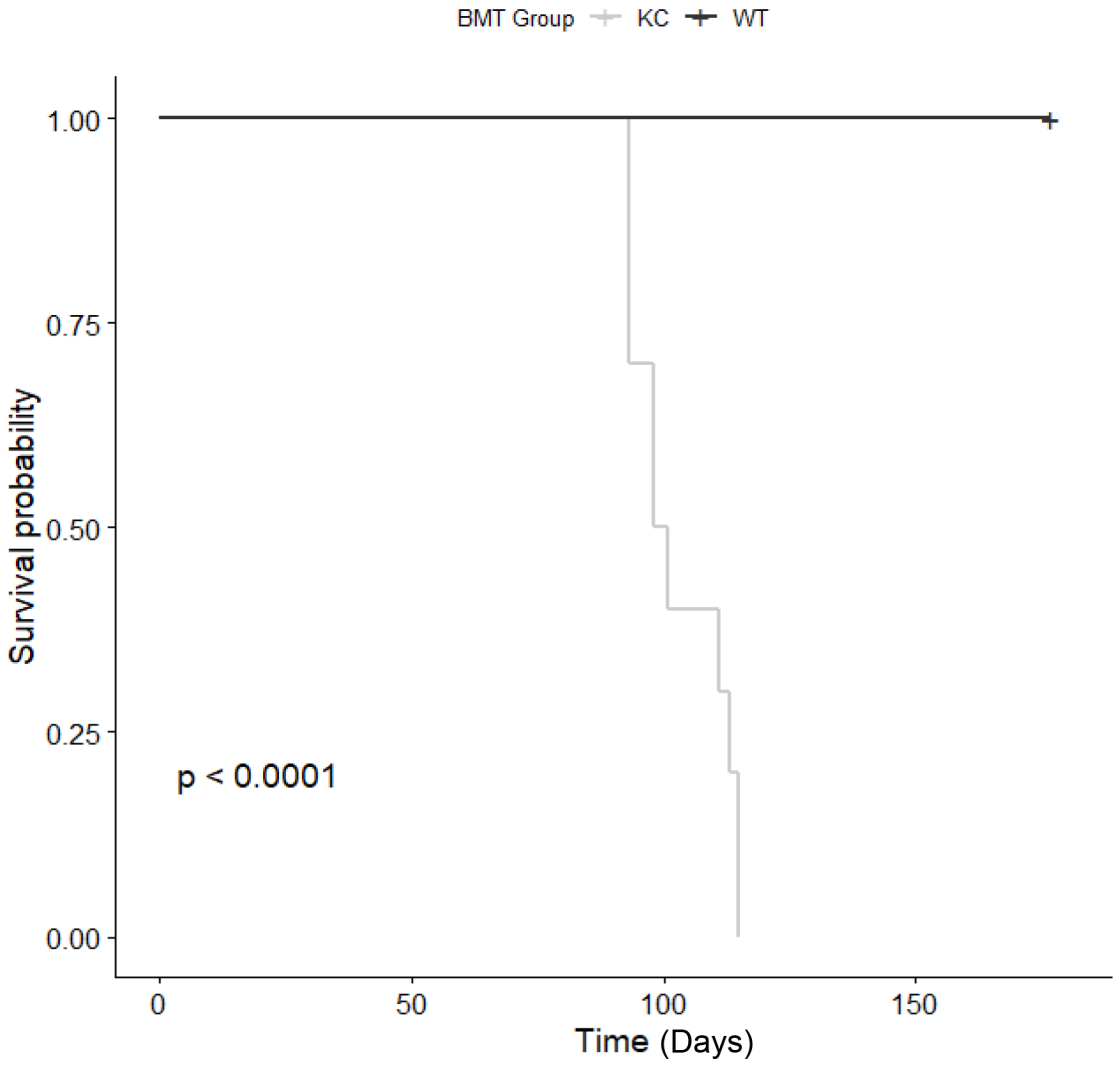

Figure 5: Kaplan-Meier survival curve in bone marrow transplant mice.

Bone morrow was isolated from three separate AiKC mice and pooled for transplant via retro-orbital injection into irradiated CD45.1 B6 mice. Additionally, bone marrow was isolated from three separate C57BL/6J (CD45.2) mice and pooled for transplant into irradiated CD45.1 B6 mice. Following transplant, mice were monitored for signs of duress. There were no deaths in the immediate post-transplant period. Time of survival following BMT was measured and recorded. One-hundred percent of the CD45.1 mice receiving AiKC bone marrow (KC: light grey) died due to T-ALL and thymic tumor development within 4 months of transplant, compared to none of the CD45.1 mice receiving wild-type bone marrow (WT: dark grey) [$p<0.0001$].

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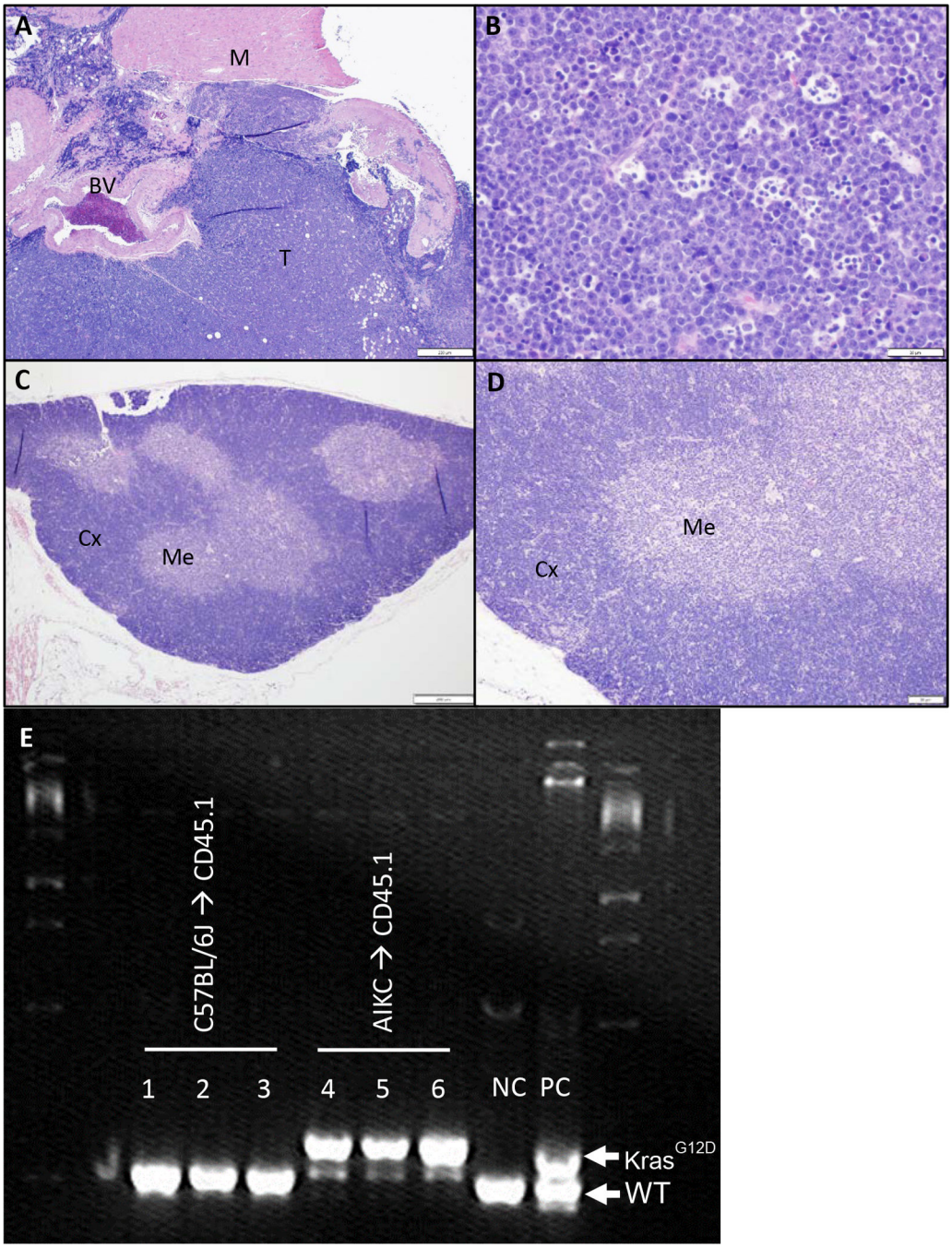

Figure 6: Transplant of KC bone marrow into wild-type results in *Kras*-mutant T-ALL.

Pooled bone marrow from three KC mice was transplanted into sub-lethally irradiated B6 CD45.1 mice. Thymic tumors in recipient mice were removed for histopathologic analysis. Demonstrated is the thymic tumor (T) adjacent to major blood vessels (BV) and myocardium (M) [40x; scale bar = 200 μm]. On closer magnification (B), there is diffuse infiltration of immature T-cells with convoluted nuclear membrane, indistinct nucleoli and frequent mitotic figures, consistent with T-ALL seen primarily in the KC mice (200x; scale bar = 20 μm). Histopathology from CD45.1 mice transplanted with C57BL6/J bone marrow

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revealed normal thymus containing the cortex (Cx) and medulla (Me) [40x; scale bar = 200 μm] (C). These epithelial compartments are also evident on higher magnification (D) [100x; scale bar = 50 μm]. DNA isolated from thymus of CD45.1 recipients of C57BL6/J bone marrow (1, 2, 3), and KC bone marrow (4, 5, 6) and demonstrated wild-type *Kras* and activated *Kras*-mutation, respectively (E). Positive control (PC) consisted of splenocytes from *Vav-Cre ; LSL-Kras*$^{G12D/+}$ mice (as previously described)[3] and negative control (NC) was tail from C57BL/6J mice.