

Dual inhibition of JAK and MEK suppresses T-cell acute lymphoblastic leukemia induced by mutant *IL7R* and *RASGRP1* overexpression

by Steven Hsu, Giselle O. L. Rodrigues, Nathan Wong, Timothy Gower, Julie A. Hixon, Stephen K. Anderson, Wenqing Li and Scott K. Durum

Received: February 20, 2026.

Accepted: September 29, 2026.

Citation: Steven Hsu, Giselle O. L. Rodrigues, Nathan Wong, Timothy Gower, Julie A. Hixon, Stephen K. Anderson, Wenqing Li and Scott K. Durum. Dual inhibition of JAK and MEK suppresses T-cell acute lymphoblastic leukemia induced by mutant *IL7R* and *RASGRP1* overexpression. *Haematologica*. 2026 Oct 8. doi: 10.3324/haematol.2026.300752 [Epub ahead of print]

Publisher's Disclaimer.

E-publishing ahead of print is increasingly important for the rapid dissemination of science.

Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication.

E-publishing of this PDF file has been approved by the authors.

After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval, the final version of the manuscript will then appear in a regular issue of the journal.

All legal disclaimers that apply to the journal also pertain to this production process.

Dual inhibition of JAK and MEK suppresses T-cell acute lymphoblastic leukemia induced by
mutant *IL7R* and *RASGRP1* overexpression

Steven Hsu^{1, 2, 3*}, Gisele O. L. Rodrigues^{1*}, Nathan Wong^{4, 5}, Timothy Gower⁶, Julie A. Hixon¹,
Stephen K. Anderson¹, Wenqing Li^{1#} and Scott K. Durum^{1#}

¹Cancer Innovation Laboratory, National Cancer Institute, Frederick, MD, USA

²Comparative Biomedical Scientist Training Program, National Institutes of Health,
Bethesda, MD, USA

³Veterinary Diagnostic Laboratory, Department of Pathobiology and Diagnostic
Investigation, Michigan State University, Lansing, MI, USA

⁴CCR Collaborative Bioinformatics Resource, National Cancer Institute, National Institutes
of Health, Bethesda, Maryland.

⁵Advanced Biomedical Computational Science, Frederick National Laboratory for Cancer
Research, Frederick, Maryland.

⁶Laboratory Animal Science Program, Frederick National Laboratory for Cancer Research

*S.H. and G.O.L.R. contributed equally as co-first authors.

#WL and SKD contributed equally as senior authors.

Running title: Targeting JAK–MEK in IL7R/RAS T-ALL

Competing Interests: The authors declare there are no competing financial interests in relation to the work described.

Acknowledgments

These studies were performed in partial fulfillment of the doctorate degree (SH) at Michigan State U. This work utilized the computational resources of the NIH HPC Biowulf cluster (<https://hpc.nih.gov>).

Author Contributions

SH was responsible for the conceptualization of the study, contributing to the methodology used, investigation, visualization of the results, writing of the original draft, and reviewing and editing the final draft. GOL was responsible for the conceptualization of the study, reviewing and editing the final draft, contributing to the methodology used, and conducting additional experiments to address the reviewers' comments. NW was responsible for the softwares used, data curation, visualization of the results, investigation, and reviewing and editing the final draft. TG was responsible for conducting the animal experiments. JH contributed to the methodology used and was responsible for reviewing and editing the final draft. SKA contributed valuable suggestions. WL was responsible for the conceptualization of the study, contributing to the methodology used, and reviewing and editing the final draft. SKD was responsible for the writing of the original draft, reviewing and editing of the final draft, and supervision.

Disclosure

The contributions of the NIH authors were made as part of their official duties as NIH federal employees, are in compliance with agency policy requirements, and are considered Works of the United States Government. However, the findings and conclusions presented in this paper are those of the authors and do not necessarily reflect the views of the NIH or the U.S. Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

Funding statement

This research was supported by the Center for Cancer Research, National Cancer Institute, National Institutes of Health Intramural Research Program project numbers ZIA BC 009287 and ZIA BC 011150, and federal funds from the National Cancer Institute, National Institutes of Health, under contracts HHSN261200800001E and 75N91019D00024.

Abstract

Gain-of-function mutant IL-7R α (mutIL7R) occurs in approximately 10% of patients with T-cell acute lymphoblastic leukemia (T-ALL), an aggressive pediatric cancer. Nearly half of patients with T-ALLs also overexpress RAS guanyl nucleotide exchange factor (RASGRP1), an activator of RAS. Since mutant RAS itself has been shown to cooperate with mutIL7R in leukemogenesis, we evaluated whether RASGRP1 overexpression could do so through activation of wild type RAS. Immature murine thymocytes were transduced with mutIL7R combined with overexpression of wild type RASGRP1. The combination of the two transgenes was sufficient to induce transformation into aggressive T-ALL, whereas neither transgene alone could do so. The resulting leukemia was polyclonal with heterogeneous T-cell markers, and its subclones showed different clinical behaviors as do genetic subtypes of patient T-ALLs. Like a leukemia model driven by *NRAS*^{G13D} and mutIL-7R, overexpression of RASGRP1 increased ERK1/2 phosphorylation and enriched RAS gene expression signature. The leukemia driven by mutIL7R and RASGRP1 was susceptible to ruxolitinib inhibition of Janus kinase, a key component of the IL-7R pathway, and trametinib inhibition of MEK, a downstream component of the RAS pathway. Combination therapy with ruxolitinib and trametinib resulted in the near elimination of the leukemia. This study demonstrates that these two oncogenes, which frequently occur in T-ALL, can drive leukemia and suggests targeting drugs to the pathways they activate.

Introduction

Acute lymphoblastic leukemia is the most common cancer in children, and approximately 15% of cases are of T-cell origin (T-ALL) ^{1,2}. The lymphoid progenitors that give rise to T-ALLs exhibit partially blocked ontogeny as a result of diverse genetic alterations, some of which define classified subtypes ³. While treatment outcomes differ between immunophenotypically or genetically defined subtypes, generally, the 5-year survival rate is 80-90% ^{4,5}; however, multi-agent, risk-adjusted, intensive chemotherapy is required. Treatment-associated complications aside, the prognosis for relapsed cases is poor ⁶. A recent T-ALL classification system combines extensive genetic characterization and immunophenotypic data to achieve prognostically significant risk strata ⁷. Understanding the molecular mechanisms of leukemogenesis and blocked ontogeny can more effectively target leukemia cells and minimize undesirable effects.

IL7R is a heterodimer, comprised of IL7R α and γ_c , and functions in normal T-cells to regulate development and peripheral homeostasis ^{8,9}. Approximately 10% of T-ALLs express a gain-of-function mutant IL7R (mutIL7R), frequently via insertion of a cysteine near the transmembrane domain. This generates a disulfide-linked homodimer of two IL7R α chains, creating a ligand-independent, constitutively active signaling complex ^{8,9}.

It has been shown that the mutIL7R alone is insufficient for leukemogenesis, but sufficiency can be achieved in combination with mutant NRAS^{G13D} ¹⁰ or wild-type MYC

overexpression ¹¹. The experimental demonstration of the leukemogenic cooperation of mutIL7R-mediated activation of JAK1-STAT5 and PI3K-AKT, together with RAS-ERK signaling pathways, indicates the oncogenic potential of these three axes. In patients, mutations in the cytokine receptor and RAS signaling pathways have been found in approximately 67% of early T-cell progenitor (ETP)-ALLs ¹², and mutations in the RAS pathway account for 30-50% of ALL relapses ¹³. An underappreciated regulator of RAS activation is RAS guanyl-releasing protein 1 (RASGRP1), a RAS guanine exchange factor that activates RAS via the facilitation of GDP-GTP exchange. Its overexpression has been found in approximately 50% of T-ALLs ¹⁴, and can cooperate with mutant NOTCH1 ¹⁵, a positive regulator of IL7R expression, to achieve sufficiency for leukemogenesis ¹⁶.

The present study evaluated whether mutIL7R combined with overexpression of RASGRP1 was sufficient to generate T-ALL from murine T-cell progenitors. We show that this led to an aggressive T-ALL and, through phenotypic and transcriptional characterization at the single-cell level, identified a pair of therapeutic agents that targeted the relevant pathways, yielding a marked suppressive effect on the most aggressive subclones of the leukemia.

Materials and Methods

Retroviral vector construction, transfection, and transduction

Human mutant IL7R (mutIL7R) and wild-type RASGRP1 cDNAs were cloned into pMIG and IRES-mCherry retroviral vectors, respectively. The mutIL7R construct contained the in-frame insertion c.731_732insTTGTCCCAC⁸. Retrovirus was produced in Phoenix cells using pCL-Eco helper plasmid and used to transduce CD4⁺/CD8⁺-depleted thymocytes isolated from 3–6-week-old C57BL/6J female mice. Transduced thymocytes were cultured on OP9-DL4 stromal cells prior to transplantation¹⁷. All animal experiments were conducted in accordance with NIH guidelines and approved by the NCI Animal Care and Use Committee.

Founder and passaged leukemia generation

Transduced thymocytes (5×10^5 cells/mouse) were injected intravenously into sub-lethally irradiated (3 Gy) 6–12-week-old female Rag1^{-/-} mice. Engraftment was monitored biweekly by flow cytometry for GFP⁺mCherry⁺ cells in peripheral blood. Mice were monitored daily for leukemia-associated morbidity and euthanized when humane endpoints were reached. Hematopoietic and lymphoid tissues were collected for downstream analyses. For serial transplantation, spleen-derived leukemic GFP⁺mCherry⁺ cells were injected into irradiated Rag1^{-/-} recipients ($1\text{--}5 \times 10^6$ cells/mouse), and disease progression was assessed as above.

Drug treatment study

Spleen-derived leukemic cells were transplanted into Rag1^{-/-} mice (5 x 10⁶ cells/mouse). Forty-eight hours post-transplantation, mice received vehicle, ruxolitinib, trametinib, or combination therapy for 10 days. Ruxolitinib was administered orally and trametinib intraperitoneally. Leukemic burden and clinical response were evaluated by flow cytometry and survival monitoring.

Flow cytometric analysis

Single-cell suspensions from blood and tissues were stained with fluorochrome-conjugated antibodies against T-cell markers and analyzed to quantify GFP⁺mCherry⁺ leukemic cells. Intracellular phospho-flow cytometry was performed to assess STAT5, AKT, and ERK1/2 activation. Formalin-fixed, paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin for histopathologic evaluation.

scRNA- and scTCR-sequencing data processing

Single-cell 5' mRNA and TCR libraries were generated using the 10x Genomics platform and sequenced on Illumina instruments. Data were processed using Cell Ranger and analyzed in Seurat¹⁸. Differential expression, gene set enrichment analysis, and clonotype identification were performed using standard computational pipelines.

Statistical analysis

Statistical analysis was performed on Prism version 7 (GraphPad, San Diego, CA, USA).

Unpaired, two-tailed Student's *t*-test was used to compare two groups, and one-way

ANOVA was used for multiple comparisons. $p < 0.05$ was considered statistically

significant. Error bars represented standard deviation.

A detailed description of other methods used is provided in the Supplementary Methods.

Results

MutIL7R and RASGRP1 induced leukemia in a mouse model

Gain-of-function, oncogenic, mutant *IL7R*, wild-type *IL7R*, and wild-type *RASGRP1*

overexpression can be found in approximately 10%^{8,9,12}, 54%¹⁹, and 50% of T-ALL patients

^{14,15}, respectively. Multiple groups have shown that while the mutIL7R alone cannot induce

T-ALL, sufficiency for leukemogenesis can be achieved in cooperation with a secondary

oncogene¹¹, often in the RAS signaling pathway^{10,20,21}. Retroviral co-transduction of

thymocytes with mutant *IL7R* and *RASGRP1* was performed to determine whether this is a

novel pair of oncogenes sufficient to induce T-ALL (Fig. 1A), and to recapitulate a pair of co-

occurrent genetic alterations in T-ALL patients (Fig. 1B)²²⁻²⁴. Mice that received

mutIL7R+RASGRP1-transduced thymocytes reached the humane endpoint at 3 to 4 weeks

post-injection, and GFP+ mCherry+ cells were identified by flow cytometry in the blood,

bone marrow, thymus, spleen, and lymph nodes (Fig. 1C, Supplemental Fig. 1A). Passage

studies were performed to distinguish non-transformed leukocytosis from leukemia.

Recipient mice that received mutIL7R+RASGRP1-transduced cells, but not mutIL7R-alone, developed severe clinical signs and met humane endpoint parameters (Fig. 1D). Recipient mice that received RASGRP1-transduced cells did not develop clinical signs (data not shown). A dose response was observed showing decreased survival with increasing numbers of transferred cells (Fig. 1E). The ability of mutIL7R+RASGRP1-transduced cells to pass from a founder to secondary recipients demonstrates a neoplastic process, and this disease process will be referred to as the mutIL7R+RASGRP1 leukemia.

Clinicopathologic features of the mutIL7R+RASGRP1 leukemia

To further describe and support the transformed state of the mutIL7R+RASGRP1 leukemia, clinicopathologic features were evaluated and compared with those of other established mutIL7R-driven T-ALL models. As expected, both splenomegaly and hepatomegaly were observed in passaged leukemia mice compared with founder leukemia and control mice (Fig. 1F)^{10, 20}. The histopathologic features in the lymph nodes, spleen (Fig. 2A), and liver (photomicrographs not shown) of founder leukemia mice were consistent with leukemia; furthermore, the severity of the findings was greatly intensified in the passaged leukemia mice. The clinicopathologic changes observed in both the founder and passaged mutIL7R+RASGRP1 leukemia mice were similar to those observed in other mutIL7R-driven T-ALL models^{10, 20}. The only notable discordant finding was that the livers and kidneys of passaged leukemia mice (Fig. 2B) were more severely affected than previously reported. Taken together, the mutIL7R+RASGRP1 leukemia falls within the spectrum of disease compared to other mutIL7R-driven T-ALL models.

T-cell lineage classification of the polyclonal mutIL7R+RASGRP1 leukemia

Lineage switch has been described in a mutIL7R-driven ALL model ²⁵. Flow cytometry and scRNA-sequencing, and scTCR-sequencing, were used to determine the phenotype and clonality of the mutIL7R+RASGRP1 leukemia, respectively. GFP+ mCherry+ cells were predominantly CD8+ and TCRβ+ (Fig. 2C; Supplemental Fig. 1B, Supplemental Fig. 2B, C), similar to other T-ALL models driven by mutIL7R and RAS pathway dysregulation ^{10,20}.

Transcriptionally, the mutIL7R+RASGRP1-transduced cells were predominantly T-cell-like, of which they were subclassified as naïve thymus-derived, memory, and effector CD8+ αβ T-cells (Supplemental Fig. 3A). Similar to other mutIL7R-driven T-ALL models ^{10,21}, the mutIL7R+RASGRP1 leukemia was polyclonal (Fig. 2D; based on TCR CDR3 sequence).

However, over 50% of the founder population was dominated by four clones, which suggests a weaker oncogenic potential of RASGRP1 compared to mutNRAS^{G13D} and MYC and the transformation of a smaller pool of initiating cells ²¹. The low frequency of leukemia initiating cells is also suggested by the five-fold increase (5×10^6 vs 1×10^6) in the total number cells needed to transfer leukemia (Fig. 1E). Interestingly, in a subset of passaged leukemia mice (6 out of 13), the leukemia shifted towards an immature immunophenotype, characterized by the loss of TCR and CD3 expression (Supplemental Fig. 3B) and increased proportion of CD8+ and CD4+ CD8+ cells (Supplemental Fig. 3C), consistent with immature single positive and double positive stages of normal thymocyte maturation, respectively ²⁶. Specific to the bone marrow, a minor subpopulation of TCR- CD3- CD4- CD8- leukemia cells was also identified in two passaged leukemia mice

(Supplemental Fig. 3C). The transcriptional phenotype corroborated the immunophenotype of the founder mutIL7R+RASGRP1 leukemia. Analogous to the mutIL7R+mutNRAS^{G13D} and mutIL7R+MYC T-ALL models ^{10, 20}, and unlike the mutIL7R+TLX3 model ²⁵, the mutIL7R+RASGRP1 leukemia retained T-cell lineage features. The observed shift towards an immature phenotype was accompanied by increased aggressiveness of the passaged leukemia, as observed in patients with early T cell progenitor (ETP) compared to non-ETP ALLs ²⁷⁻²⁹.

Oligoclonal origin of an aggressive thymocyte-like subpopulation of the mutIL7R+RASGRP1 leukemia

T-ALL classification moved beyond immunophenotypic or cytogenetic subtypes to include next-generation sequencing ^{7, 30-33}, which can have prognostic significance ⁷. To determine whether these observations in T-ALL patients are recapitulated in this mouse T-ALL model, the clinical parameters of immature thymocyte-like versus mature T-cell-like passaged leukemias were compared. Mice with immature thymocyte-like passaged leukemia had significantly shorter survival than the mature T-cell-like counterpart (Fig. 2E) and worsened pathologic parameters such as splenomegaly and hepatomegaly (Fig. 2F). To interrogate the marked clinical differences between these two passaged leukemia subtypes, the origin of the immature thymocyte-like subpopulation within the founder leukemia was explored. TdT is a component of the TCR gene rearrangement process that is normally expressed in immature thymocytes. Consistent with this, TdT was widely expressed (48%) by passaged leukemia cells with the immature thymocyte-like phenotype (Fig. 3A, Supplemental Fig.

3D), but less frequently (11%) by the founder leukemia cells (Fig. 3A). A few dominant clones already seen in the founder were mostly annotated as naïve, non-effectors T-cells, whereas the more numerous clones were mostly annotated as various effectors (Fig. 3B). These data suggest that the passaged leukemia with more aggressive immature thymocyte-like characteristics likely originated from the oligoclonal minority of cells in the founder that were less mature and expressed TdT. On the other hand, the polyclonal leukemia cells with an effector T-cell-like phenotype, which were more frequent in the founder, generated less aggressive disease upon transfer. The differences in the severity of pathologies and pace of clinical progression between these two subtypes recapitulate similar observations in T-ALL patients comparing immature versus mature immunophenotypic subtypes ⁷.

MutIL7R+RASGRP1 leukemia hyperactivated the RAS-ERK pathway

The mechanism of cooperation of these two oncogenic pathways was further investigated. The mutIL7R has been shown to activate the JAK1-STAT5, PI3K-AKT, and RAS-ERK signaling pathways ^{8, 9, 20}, and RASGRP1 overexpression has been shown to preferentially activate RAS-PI3K-AKT over RAS-RAF-MEK-ERK signaling pathways and to potentiate IL2-, IL7-, and IL9-mediated proliferation in T-ALL cells ^{16, 26}. To determine whether the cooperation of mutIL7R and RASGRP1 was mediated through the hyperactivation of the JAK-STAT, RAS-PI3K-AKT, and/or RAS-RAF-MEK-ERK signaling pathways, GSEA and phospho-flow cytometry were performed to compare the differential activation of the aforementioned pathways compared to the mutIL7R-driven leukocytosis. Enrichment of IL2-STAT5 and RAS

signaling signatures and underrepresentation of the PI3K-AKT signaling signature were observed (Fig. 3C); only increased phosphorylation of ERK1/2 (Fig. 3D) was functionally validated. Although the JAK-STAT pathway was implicated, we did not observe increased STAT5 phosphorylation (Fig. 3E); the GSEA result suggests that other mechanisms may amplify this pathway downstream. Consistent with the underrepresentation of the PI3K-AKT signaling signature, a functional decrease in the phosphorylation of AKT was observed (Supplemental Fig. 4A). The intensified clinicopathologies exhibited by the immature thymocyte-like passaged leukemia were not due to further hyperactivation of the RAS-ERK signaling pathway (Supplemental Fig. 3B). These results suggest that RASGRP1¹⁶, despite being an activator of RAS, cooperates with the IL-7R pathway differently from mutNRAS^{G13D}²⁰ in that the mutIL7R+RASGRP1 leukemia did not preferentially activate the RAS-PI3K-AKT signaling pathway (Supplemental Fig. 4A), increase *Myc* (data not shown), or enrich the MYC target signature (Fig. 3C).

Ruxolitinib and trametinib suppressed leukemia progression and prolonged survival

The GSEA result implicated the JAK-STAT pathway in cooperation between the two oncogenes (Fig. 3C). JAK inhibitors are effective in ALLs with JAK-STAT pathway dysregulation^{10, 34}, and their clinical applications in the treatment of ALLs are currently being explored at various phases of clinical trials (NCT04996160, NCT06128070, NCT03571321, NCT05592015). However, the effectiveness of MEK inhibitors in RAS dysregulated ALLs is unclear. Specifically, the MEK inhibitor trametinib was effective in the mutIL7R+mutNRAS^{G13D} T-ALL model¹⁰ and numerous T-ALL PDXs in vitro³⁵, but ineffective

in a *Rasgrp1*-overexpressing acute myeloid leukemia (AML) model ³⁶. The mutIL7R+RASGRP1 leukemia showed enriched JAK-STAT5 and RAS signaling signatures (Fig. 3C) and increased p-ERK1/2 (Fig. 3D). Therefore, ruxolitinib (JAK1/2 inhibitor) and trametinib (MEK inhibitor) were selected to test their efficacy as single agents and in combination (Supplemental Fig. 4C) in an early engraftment treatment model (Supplemental Fig. 4D, E). Two dosages, high and low, were selected for both inhibitors in recognition that their combination is toxic in chronic studies. While both ruxolitinib and trametinib at low and high doses resulted in variable suppression of the leukemia, only the combination of low-dose ruxolitinib and trametinib and high-dose ruxolitinib and high-dose trametinib rescue resulted in consistent suppression across all 3 tissue compartments (Fig. 4A, B, C). This leukemia suppression translates to prolonged survival with treatment compared to vehicle control (Fig. 4D). The therapeutic responses of the mutIL7R+RASGRP1 leukemia to ruxolitinib and trametinib as single agents or in combination were similar to the mutIL7R+mutNRAS^{G13D} model ¹⁰ and T-ALL PDXs ³⁵.

Discussion

Type 1a gain-of-function mutant IL-7R ^{8,9,37} has been shown to cooperate with a small, and likely incomplete, repertoire of oncogenes ^{11,20,21,25} to achieve sufficiency for leukemogenesis; several of which are in the RAS signaling pathway ^{10,20,21}. RASGRP1 ¹⁶ has been suggested to cooperate with the IL-7 signaling pathway in T-ALL ^{14,38} due to the observation that *RASGRP1* is overexpressed in approximately 50% of T-ALLs ^{14,26} and activates RAS ¹⁶. Herein, it was demonstrated that RASGRP1 can indeed cooperate with

mutIL7R to achieve sufficiency for T-cell leukemogenesis, and the mutIL7R+RASGRP1 leukemia represents a novel model to study RAS-dysregulated, mutIL7R-driven T-ALLs.

The mechanism of RASGRP1 overexpression in T-ALLs remains elusive despite an extensive genomic and transcriptional characterization study of patient T-ALLs ⁷, which suggests a non-mutational basis of dysregulation. Regardless, the intersections between mutIL7R, MYC ^{10, 20}, mutant NOTCH1 ^{8, 21, 32, 39}, and mutant RAS ¹⁰ in the T-ALL context have been the subject of interest ^{11, 21, 38}, and RASGRP1 should be included therein ^{15, 40}. Since a direct regulatory link between NOTCH1 and RASGRP1 is not known to exist, indirect links should be considered ⁴¹⁻⁴⁴. The existence of convergent regulatory axes on RASGRP1 may have clinical implications, as evidenced by the marked therapeutic response of the mutIL7R+RASGRP1 leukemia to dual targeted therapy with ruxolitinib and trametinib, which will be further discussed.

The ability of the mutIL7R to cooperate with multiple RAS pathway effectors suggests that mutIL7R-expressing T-cell progenitors are especially prone to RAS pathway dysregulation and subsequent transformation. Functionally equivalent mutations have been found in B-ALLs ^{9, 45}, and likewise, mutIL7R-driven B-ALLs are also susceptible to RAS pathway dysregulation ⁴⁶. Whether RASGRP1 overexpression has similar cooperative effects on B-cell progenitors remains to be seen. Further work in this area will inform how prone particular lymphoid progenitors are to mutIL7R- and RAS-mediated transformation and potentially respond to targeted therapeutic agents. Interestingly, our GSEA analysis

revealed negative enrichment of PI3K/AKT/mTOR- and MYC-associated gene sets in mutIL7R+RASGRP1 leukemia relative to mutIL7R-driven leukocytosis. IL7R signaling is not restricted to PI3K/AKT but engages multiple interconnected downstream pathways, including JAK/STAT and RAS/MEK/ERK. Thus, rather than uniformly enhancing all pathways associated with mutant IL7R signaling, RASGRP1 overexpression may reshape their relative downstream outputs. Consistent with the GSEA results, AKT phosphorylation was also reduced in mutIL7R+RASGRP1 cells relative to mutIL7R cells, whereas ERK1/2 phosphorylation was increased. Although these findings support differential utilization of downstream signaling pathways in the presence of RASGRP1 overexpression, the underlying mechanism cannot be established from the current experiments and will require further investigation.

IL-7R-mediated signaling is known to enrich for leukemia-initiating cells (LICs) in T-ALL^{47, 48}. A subsequent study that examined the frequency of LICs between MYC and mutIL7R+MYC leukemias²¹ reaffirmed the earlier studies and posed the question of whether this effect of IL-7R signaling on the frequency of LICs is restricted to the context of MYC. The observations herein suggest that the frequency of LICs is also affected by the secondary oncogene in cooperation, as the number of cells needed to initiate a passaged leukemia in the mutIL7R+RASGRP1 model was 5-fold that of the mutIL7R+mutNRAS^{G13D} model^{10, 20}. As such, the mechanism of RAS signaling pathway activation affects the frequency of LICs between these two T-ALL models. Factors such as survival and proliferative potential of the initiating cell confound all indirect methods of LIC identification^{10, 20, 21}. In search of and to

more precisely characterize the mutIL7R+RASGRP1 leukemia LICs, the expression of TdT was identified as a potential marker, as these rare, oligoclonal, TdT-expressing founder leukemic cells were disproportionately expanded in a subset of passaged leukemia mice. While these TdT-expressing founder leukemia cells were not solely responsible for reconstituting the passaged leukemia, they exhibited the greatest initiation potential. As such, the expression of TdT may be used in future studies as a direct comparison of LIC frequency in TdT-expressing T-ALL models.

Widely accepted T-ALL classification systems have focused on either immunophenotype, as with ETP-ALLs ^{12, 27-29, 49}, or genomic alterations ^{30, 32, 50}. A recent large-scale comprehensive (immunophenotypic, genomic, and transcriptomic characterizations) T-ALL classification study ⁷ found that for certain genomic subtypes, such as KMT2A and MLLT10, the integration of immunophenotypic classification, ETP-like vs non-ETP-like, has prognostic significance. Analogous to this difference in clinical behavior, the survival of mice with immunophenotypically defined immature thymocyte-like passaged mutIL7R+RASGRP1 leukemia was significantly shortened compared to the mature T-cell-like counterpart. The implication for future in vivo models is evident, in which an ideal model should recapitulate the genetic alterations of interest, the immunophenotype of the intended patient population, and the relative clinical behavior in patients based on both genomic and immunophenotypic characterization. Currently, only the ETP status has been combined with genomic classification to show differences in prognosis ⁷. While neither the founder nor the passaged mutIL7R+RASGRP1 leukemia was consistent with the ETP-like

classification, likely, the integration of other immunophenotypic subtypes into the proposed comprehensive T-ALL classification system ⁷ will expand the prognostic significance of certain genomic subtypes. This is evidenced by the observation that transcriptionally defined DP-like, $\alpha\beta$ -like, and $\gamma\delta$ -like subclassifications had prognostic implications for certain genomic subtypes ⁷.

T-ALL patients with mutant IL-7R are associated with very poor prognosis upon relapse ⁶ and may benefit from targeted therapeutics against the JAK-STAT, PI3K-AKT-mTOR, and RAS-ERK pathways ^{10, 34, 35, 51-53}. The suppressive effects of ruxolitinib and trametinib as single agents and in combination on the immature thymocyte-like passaged mutIL7R+RASGRP1 leukemia were expected and consistent with similar models ^{10, 35}. However, these results do not support 2 proposed mechanisms of RASGRP1-mediated MEK inhibitor resistance ^{16, 36}. Specifically, the overexpression of RASGRP1 does not necessarily confer MEK inhibitor resistance, nor does it necessarily hyperactivate the PI3K pathway, functionally or transcriptionally. As such, the perspective herein is that trametinib resistance, perhaps MEK inhibitors in general, is model-specific and cannot be generalized to RASGRP1 overexpression alone. The near elimination of leukemia cells by the combined treatment of ruxolitinib and trametinib, at both high and low doses, was a surprise, especially in the bone marrow, which is a difficult site to target and a niche that supports LIC survival ^{54, 55}. A limitation of this study is that, despite the marked reduction in leukemia burden following combined Ruxolitinib and Trametinib treatment, the improvement in overall survival was modest. The cause of this discrepancy could not be

definitively determined, as comprehensive pathological analyses were not performed. Further optimization of drug doses and/or treatment schedules may improve the balance between antileukemic efficacy and tolerability and potentially result in a greater survival benefit. Future studies incorporating dose optimization and pathological assessment will be important to address this question. Despite these limitations, the antileukemic activity observed with dual inhibition may have important clinical implications. This approach may offer an opportunity to achieve complete remission (less than 5% blasts in the bone marrow) and pave the way for hematopoietic stem cell transplantation, which remains one of the only paths for long-term cure.

Overall, the studies herein demonstrated that the combination of mutIL7R and RASGRP1 overexpression was sufficient to induce T-ALL development in mice, and that the oligoclonal, TdT-expressing founder leukemia cells were the most aggressive subpopulation and possessed the greatest leukemia initiation potential. This model also reaffirmed the observation in patient T-ALLs that the immunophenotype of a given genetic subtype can significantly affect clinicopathologic parameters, such as survival. Lastly, RASGRP1 overexpression drives functional activation and transcriptional enrichment of the RAS pathway and, as in the mutIL7R+mutNRAS^{G13D} model, renders the mutIL7R+RASGRP1 leukemia highly susceptible to the combined treatment of ruxolitinib and trametinib.

Reference

1. Bhojwani D, Yang JJ, Pui CH. Biology of childhood acute lymphoblastic leukemia. *Pediatr Clin North Am*. 2015;62(1):47-60.
2. Carroll WL, Raetz EA. Clinical and laboratory biology of childhood acute lymphoblastic leukemia. *J Pediatr*. 2012;160(1):10-18.
3. Ferrando AA, Neuberg DS, Staunton J, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
4. Pui CH, Evans WE. A 50-year journey to cure childhood acute lymphoblastic leukemia. *Semin Hematol*. 2013;50(3):185-196.
5. Hunger SP, Lu X, Devidas M, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *J Clin Oncol*. 2012;30(14):1663-1669.
6. Richter-Pechanska P, Kunz JB, Hof J, et al. Identification of a genetically defined ultra-high-risk group in relapsed pediatric T-lymphoblastic leukemia. *Blood Cancer J*. 2017;7(2):e523.
7. Polonen P, Di Giacomo D, Seffernick AE, et al. The genomic basis of childhood T-lineage acute lymphoblastic leukaemia. *Nature*. 2024;632(8027):1082-1091.
8. Zenatti PP, Ribeiro D, Li W, et al. Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. *Nat Genet*. 2011;43(10):932-939.
9. Shochat C, Tal N, Bandapalli OR, et al. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. *J Exp Med*. 2011;208(5):901-908.
10. Cramer SD, Hixon JA, Andrews C, et al. Mutant IL-7Ralpha and mutant NRas are sufficient to induce murine T cell acute lymphoblastic leukemia. *Leukemia*. 2018;32(8):1795-1882.
11. Rodrigues GOL, Cramer SD, Winer HY, et al. Mutations that collaborate with IL-7Ra signaling pathways to drive ALL. *Adv Biol Regul*. 2021;80:100788.

12. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. 2012;481(7380):157-163.
13. Tasian SK, Loh ML, Hunger SP. Childhood acute lymphoblastic leukemia: Integrating genomics into therapy. *Cancer*. 2015;121(20):3577-3590.
14. Hartzell C, Ksionda O, Lemmens E, et al. Dysregulated RasGRP1 responds to cytokine receptor input in T cell leukemogenesis. *Sci Signal*. 2013;6(268):ra21.
15. Oki T, Kitauro J, Watanabe-Okochi N, et al. Aberrant expression of RasGRP1 cooperates with gain-of-function NOTCH1 mutations in T-cell leukemogenesis. *Leukemia*. 2012;26(5):1038-1045.
16. Ksionda O, Melton AA, Bache J, et al. RasGRP1 overexpression in T-ALL increases basal nucleotide exchange on Ras rendering the Ras/PI3K/Akt pathway responsive to protumorigenic cytokines. *Oncogene*. 2016;35(28):3658-3668.
17. Rodrigues GOL, Li W, Cramer SD, et al. Co-Culture and Transduction of Murine Thymocytes on Delta-Like 4-Expressing Stromal Cells to Study Oncogenes in T-Cell Leukemia. *J Vis Exp*. 2023;196:e64271.
18. Lee M, Lee E, Han SK, et al. Single-cell RNA sequencing identifies shared differentiation paths of mouse thymic innate T cells. *Nat Commun*. 2020;11(1):4367.
19. Lenk L, Baccelli I, Laqua A, et al. The IL-7R antagonist lusvertikimab reduces leukemic burden in xenograft ALL via antibody-dependent cellular phagocytosis. *Blood*. 2024;143(26):2735-2748.
20. Winer H, Li W, Rodrigues G, et al. Mechanism of co-operation of mutant IL-7Ralpha and mutant NRAS in acute lymphoblastic leukemia: role of MYC. *Haematologica*. 2024;109(6):1726-1740.
21. Oliveira ML, Veloso A, Garcia EG, et al. Mutant IL7R collaborates with MYC to induce T-cell acute lymphoblastic leukemia. *Leukemia*. 2022;36(6):1533-1540.

22. de Bruijn I, Kundra R, Mastrogiacomo B, et al. Analysis and visualization of longitudinal genomic and clinical data from the AACR Project GENIE Biopharma Collaborative in cBioPortal. *Cancer Res.* 2023;83(23):3861-3867.
23. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012;2(5):401-404.
24. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* 2013;6(269):pl1.
25. Rodrigues GOL, Li WQ, Hixon J, et al. Cooperation between mutant IL7R and TLX3 generates mixed myeloid/lymphoid leukemia from thymocyte progenitors. *Blood.* 2022;140(Supplement 1):3032-3033.
26. Hsu TC, Rodrigues GOL, Winer H, et al. A focused review of ras guanine nucleotide-releasing protein 1 in immune cells and cancer. *Int J Mol Sci.* 2023;24(2):1652.
27. Ma M, Wang X, Tang J, et al. Early T-cell precursor leukemia: a subtype of high risk childhood acute lymphoblastic leukemia. *Front Med.* 2012;6(4):416-420.
28. Inukai T, Kiyokawa N, Campana D, et al. Clinical significance of early T-cell precursor acute lymphoblastic leukaemia: results of the Tokyo Children's Cancer Study Group Study L99-15. *Br J Haematol.* 2012;156(3):358-365.
29. Coustan-Smith E, Mullighan CG, Onciu M, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol.* 2009;10(2):147-156.
30. Buckley M, Yeung DT, White DL, Eadie LN. T-cell acute lymphoblastic leukaemia: subtype prevalence, clinical outcome, and emerging targeted treatments. *Leukemia.* 2025;39(6):1294-1310.
31. Dai YT, Zhang F, Fang H, et al. Transcriptome-wide subtyping of pediatric and adult T cell acute lymphoblastic leukemia in an international study of 707 cases. *Proc Natl Acad Sci U S A.* 2022;119(15):e2120787119.
32. Liu Y, Easton J, Shao Y, et al. The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. *Nat Genet.* 2017;49(8):1211-1218.

33. Muller J, Walter W, Haferlach C, et al. Correction: How T-lymphoblastic leukemia can be classified based on genetics using standard diagnostic techniques enhanced by whole genome sequencing. *Leukemia*. 2023;37(1):252.
34. Senkevitch E, Li W, Hixon JA, et al. Inhibiting Janus Kinase 1 and BCL-2 to treat T cell acute lymphoblastic leukemia with IL7-Ralpha mutations. *Oncotarget*. 2018;9(32):22605-22617.
35. van der Zwet JCG, Buijs-Gladdines J, Cordo V, et al. MAPK-ERK is a central pathway in T-cell acute lymphoblastic leukemia that drives steroid resistance. *Leukemia*. 2021;35(12):3394-3405.
36. Lauchle JO, Kim D, Le DT, et al. Response and resistance to MEK inhibition in leukaemias initiated by hyperactive Ras. *Nature*. 2009;461(7262):411-414.
37. Barata JT, Durum SK, Seddon B. Flip the coin: IL-7 and IL-7R in health and disease. *Nat Immunol*. 2019;20(12):1584-1593.
38. Barata JT. Anatomy of a crime: how IL7R and NRAS join forces to drive T-cell acute lymphoblastic leukemia. *Haematologica*. 2024;109(6):1637-1639.
39. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269-271.
40. Klinger MB, Guilbault B, Goulding RE, Kay RJ. Deregulated expression of RasGRP1 initiates thymic lymphomagenesis independently of T-cell receptors. *Oncogene*. 2005;24(16):2695-2704.
41. Herranz D, Ambesi-Impiombato A, Palomero T, et al. A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. *Nat Med*. 2014;20(10):1130-1137.
42. Weng AP, Millholland JM, Yashiro-Ohtani Y, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev*. 2006;20(15):2096-2109.

43. Carr T, McGregor S, Dias S, et al. Oncogenic and tumor suppressor functions for lymphoid enhancer factor 1 in E2a(-/-) T acute lymphoblastic leukemia. *Front Immunol.* 2022;13:845488.
44. Spaulding C, Reschly EJ, Zagort DE, et al. Notch1 co-opts lymphoid enhancer factor 1 for survival of murine T-cell lymphomas. *Blood.* 2007;110(7):2650-2658.
45. Roberts KG, Morin RD, Zhang J, et al. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. *Cancer Cell.* 2012;22(2):153-166.
46. Almeida ARM, Neto JL, Cachucho A, et al. Interleukin-7 receptor alpha mutational activation can initiate precursor B-cell acute lymphoblastic leukemia. *Nat Commun.* 2021;12(1):7268.
47. Gonzalez-Garcia S, Mosquera M, Fuentes P, et al. IL-7R is essential for leukemia-initiating cell activity of T-cell acute lymphoblastic leukemia. *Blood.* 2019;134(24):2171-2182.
48. Goossens S, Radaelli E, Blanchet O, et al. ZEB2 drives immature T-cell lymphoblastic leukaemia development via enhanced tumour-initiating potential and IL-7 receptor signalling. *Nat Commun.* 2015;6:5794.
49. Duffield AS, Mullighan CG, Borowitz MJ. International Consensus Classification of acute lymphoblastic leukemia/lymphoma. *Virchows Arch.* 2023;482(1):11-26.
50. Brady SW, Roberts KG, Gu Z, et al. The genomic landscape of pediatric acute lymphoblastic leukemia. *Nat Genet.* 2022;54(9):1376-1389.
51. Meyer LK, Huang BJ, Delgado-Martin C, et al. Glucocorticoids paradoxically facilitate steroid resistance in T cell acute lymphoblastic leukemias and thymocytes. *J Clin Invest.* 2020;130(2):863-876.
52. Silva A, Almeida ARM, Cachucho A, et al. Overexpression of wild-type IL-7Ralpha promotes T-cell acute lymphoblastic leukemia/lymphoma. *Blood.* 2021;138(12):1040-1052.

53. Vormoor B, Savage J, Kristunas C, et al. Responses can be achieved with a combination of the MEK inhibitor selumetinib and dexamethasone in patients with relapsed/refractory RAS-pathway mutated acute lymphoblastic leukemia: Results of a parallel cohort, dose-finding and expansion phase I/II trial. *EJC Paediatr Oncol*. 2026;7:100489.
54. Krause DS, Scadden DT. A hostel for the hostile: the bone marrow niche in hematologic neoplasms. *Haematologica*. 2015;100(11):1376-1387.
55. Karantanou C, Godavarthy PS, Krause DS. Targeting the bone marrow microenvironment in acute leukemia. *Leuk Lymphoma*. 2018;59(11):2535-2545.

Figure Legends.

Figure 1: The cooperation of RASGRP1 overexpression and mutIL7R results in multisystemic pathologies in a mouse model of human disease. A. Schematic

illustration of the cooperation of mutIL7R and RASGRP1. Signaling pathways converge to drive proliferation, survival, and ultimately leukemogenesis. **B.** The correlation of *RASGRP1* and *IL7R*, wild-type (blue) and mutated (gold), expression in patients. Data based on Dana-Farber Center for Patient Derived Models (CPDM) PDX database. mRNA expression was measured as Log2 values of Reads Per Kilobase of Exon per Million (RPKM), and the line of best fit was colored in red. Spearman and Pearson correlation coefficients were 0.61 ($p=4.1 \times 10^{-17}$) and 0.73 ($p=3.14 \times 10^{-27}$). **C.** The tissue distribution of mutIL7R-RASGRP1-transduced cells in the bone marrow (BM), thymus (TH), blood (BL), spleen (SP), and lymph nodes (LN) at humane endpoint. Flow cytometric analysis of the proportion of cells in the lymphocyte and singlet gates, based on forward and side scatter, that expressed GFP and mCherry. **D.** Survival curves of mice in the first and second leukemia passages and the attempted passage of mutIL7R-transduced thymocytes. Survival curves were analyzed by the log-rank test for trend ($p<0.0001$). **E.** Survival curves of mice in the first leukemia passage with the indicated cell dose. Survival curves were analyzed by the log-rank test for trend ($p=0.0011$). **F, G.** Weights of the spleens and **F.** livers isolated from control C57BL/6 mice (control), founder leukemia (FL), and passaged leukemia (PL) are expressed as relative tissue weight (tissue weight/body weight at harvest). Splenomegaly (control vs PL, $p=0.0028$; FL vs PL, $p=0.0041$) and hepatomegaly (control vs PL, $p=0.0058$; FL vs PL,

$p=0.0476$) were determined by unpaired one-way ANOVA; error bars represent standard deviations.

Figure 2: The mutIL7R+RASGRP1 leukemia is a polyclonal leukemia model with variable maturation stage-dependent aggressiveness. A. Representative photomicrographs of H&E-stained sections of lymph nodes and spleens derived from founder and passaged mutIL7R+RASGRP1 leukemia mice taken at 100x and 400x magnification. Black bars represent 200 μ m. **B.** Representative photomicrographs of H&E-stained sections of liver and kidney derived from passaged mutIL7R+RASGRP1 leukemia mice taken at 100x and 400x magnification. Black bars represent 200 μ m. **C.** The expression of CD4, CD8, TCR β , and TCR γ by founder (FL; n=9) and passaged (PL; n=13) mutIL7R+RASGRP1 leukemia at humane endpoint. Flow cytometric analysis of the proportion of cells in the lymphocyte and singlet gates, based on forward and side scatter, that expressed GFP and mCherry. **D.** Clonality of the mutIL7R+RASGRP1 founder leukemia as determined by TCR β CDR3 sequences derived from scTCR-sequencing (n=2; total of 7,026 cells). The four most dominant clones were colored blue, gold, green, and red, respectively. The fifth to tenth most numerous clones were colored purple, and all other clones were colored black. **E.** Survival curves of mice in the first leukemia passage with the indicated immunophenotypes. Survival curves were analyzed by Mantel-Cox log-rank test ($p=0.0003$). **F.** Weights of the spleens (mature T-like, n=6; immature thy-like, n=7) and livers (mature T-like, n=4; immature thy-like, n=7) isolated from passaged mutIL7R+RASGRP1 leukemia RAG1^{-/-} mice with indicated immunophenotypes expressed

as relative tissue weights (tissue weight/body weight at harvest). Splenomegaly ($p=0.0009$) and hepatomegaly ($p=0.0012$) were determined by Student's t-tests.

Figure 3: The mutIL7R+RASGRP1 leukemia exhibits preferential expansion of immature subpopulations, differential clonotype-dependent dominance of phenotypes, and transcriptional enrichment and functional hyperactivation of the RAS-ERK and STAT5 signaling pathways. **A.** Representative immunofluorescence photomicrographs of lymph nodes derived from the founder and passaged leukemia mice labeled for DAPI (blue), GFP (green), and TdT (magenta). The percentages of cells that were immunoreactive for both GFP and TdT (gold) in the founder and passaged leukemia were quantified as a percentage of all DAPI-positive cells. Black bars represent 50 μm . **B.** The 6 most numerous cell-type annotations in the founder leukemia by clonotype. The top 4 dominant clones (blue) were compared to all other clones (gold). **C.** Ten selected enriched (gold) and underrepresented (blue) GSEA signatures, based on Hallmark Gene Sets derived from the Molecular Signature Database (MSigDB), between founder mutIL7R+RASGRP1 leukemia and mutIL7R-driven leukocytosis. Three key signaling pathways of interest were noted (red arrows). **D.** The phosphorylation of ERK1/2 under unstimulated conditions was determined by flow cytometry. The founder leukemia (gold; $n=5$) had significantly higher p-ERK1/2 compared to mutIL7R-driven leukocytosis (blue; $p=0.0009$; $n=7$) and control splenocytes from C57BL/6 mice (black; $p=0.0019$; $n=2$). Two biological replicates were performed, and relative mean fluorescent intensity (MFI) was calculated as the fold change relative to the control spleen from a C57BL/6 mouse in the respective replicate. **E.** The

phosphorylation of STAT5 under unstimulated conditions was determined by flow cytometry. Compared to control splenocytes from C57BL/6 mice (black; n=1), the founder leukemia (gold; n=3) had an observable, non-significant elevation in p-STAT5 ($p=0.1035$), and the mutIL7R-driven leukocytosis (blue; n=3) had significantly higher ($p=0.0198$) p-STAT5.

Figure 4: The combination of ruxolitinib and trametinib significantly suppresses the aggressive immature thymocyte-like mutIL7R+RASGRP1 leukemia and prolongs survival. A, B, C. At time-matched euthanasia of the passaged immature thymocyte-like leukemia, only low-dose (LD) ruxolitinib + LD trametinib and high-dose (HD) ruxolitinib + HD trametinib rescue resulted in consistent significant suppression of the aggressive immature thymocyte-like mutIL7R+RASGRP1 leukemia in the blood ($p=0.0009$, $p=0.0024$), bone marrow ($p<0.0001$, $p<0.0001$), and spleen ($p<0.0001$, $p<0.0001$) compared to vehicle control. One-way ANOVA was performed for multiple comparisons, and error bars represent standard deviation. **D.** Drug treatment significantly extended survival compared to vehicle control ($p<0.0001$). Survival curves were analyzed by the Mantel-Cox log-rank test.

Figure 1.

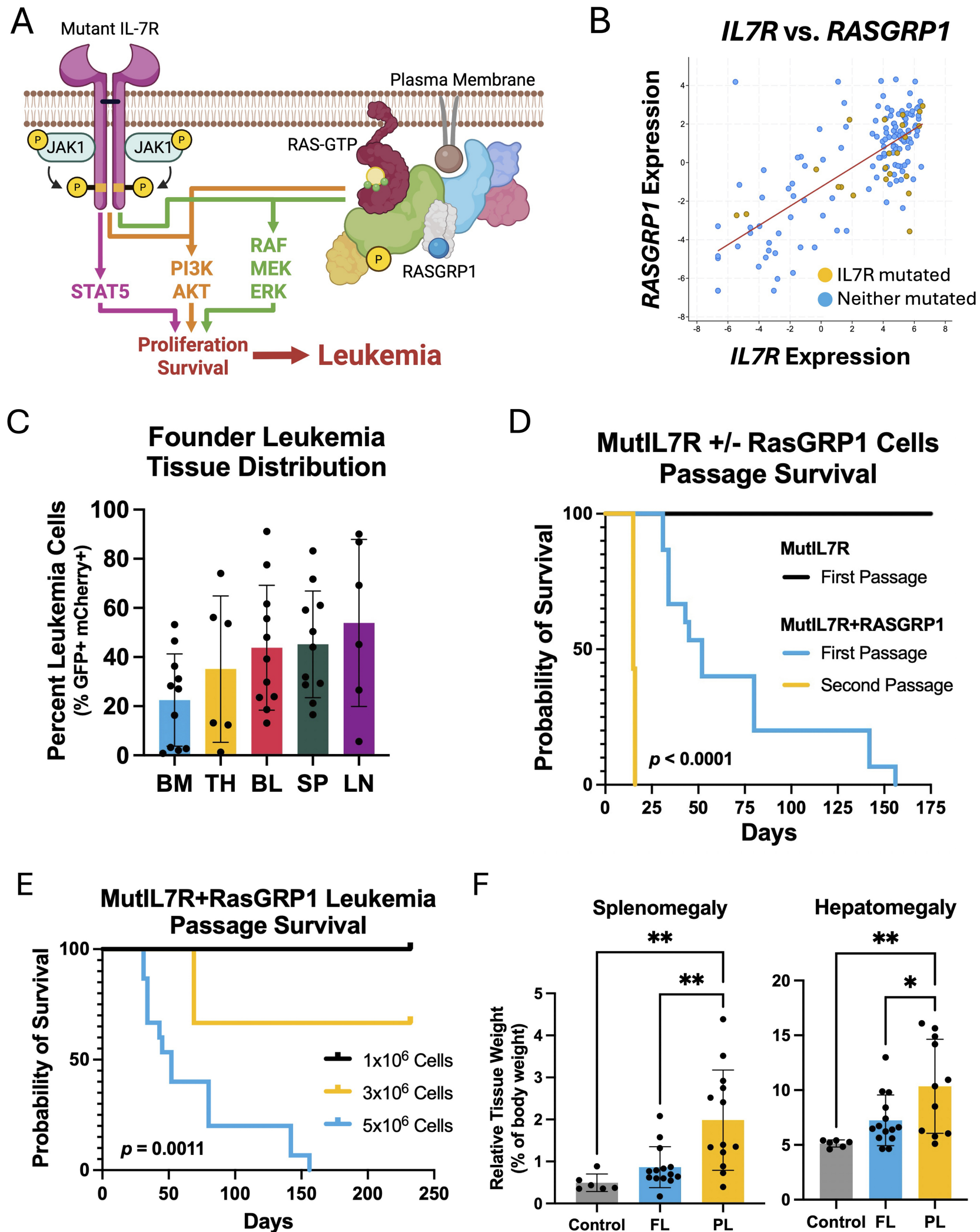


Figure 2.

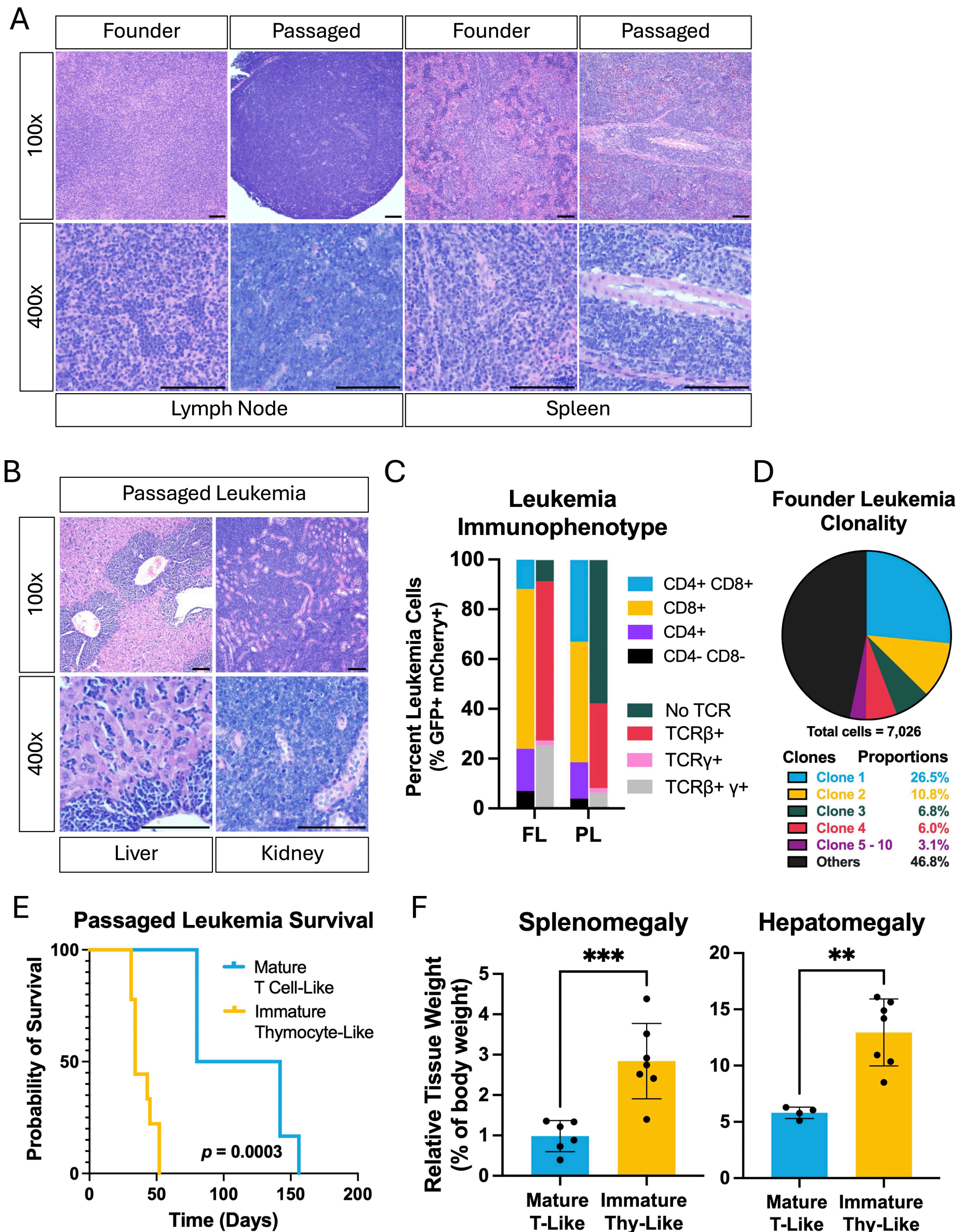
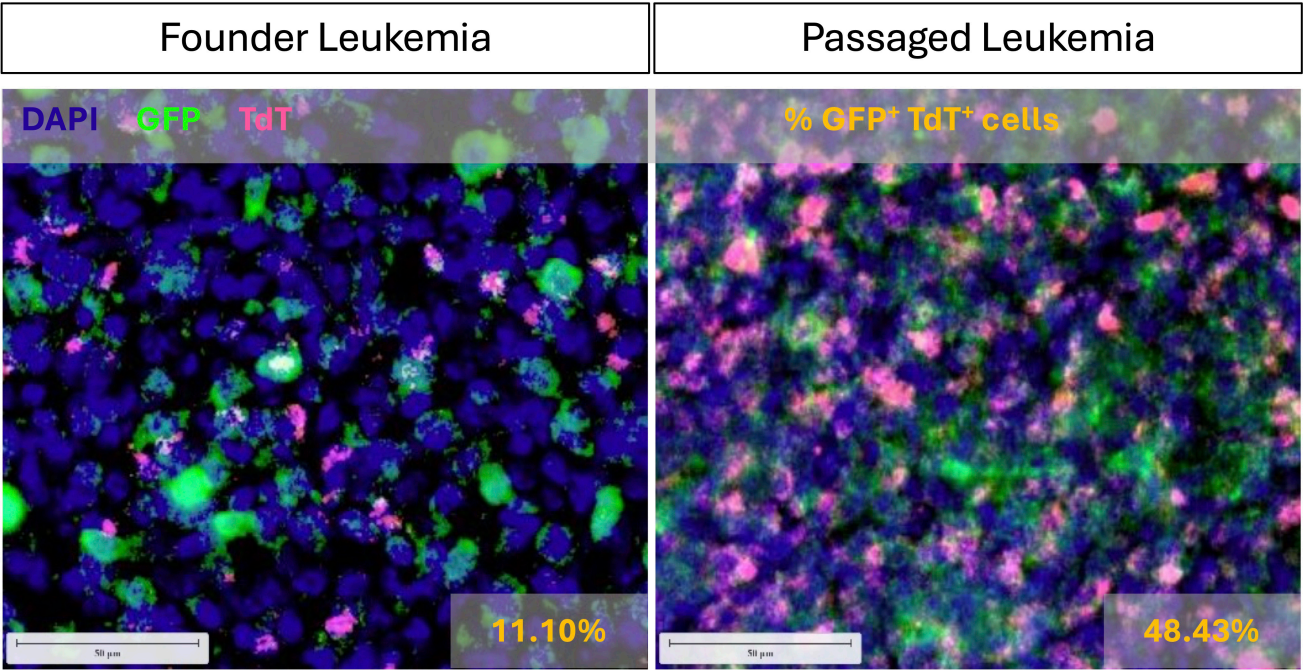
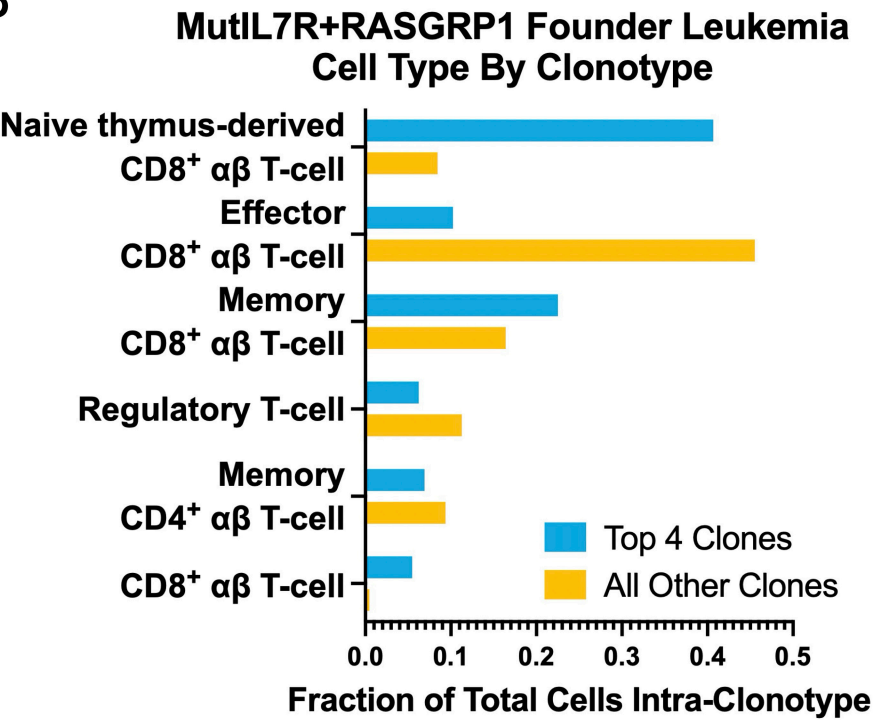


Figure 3.

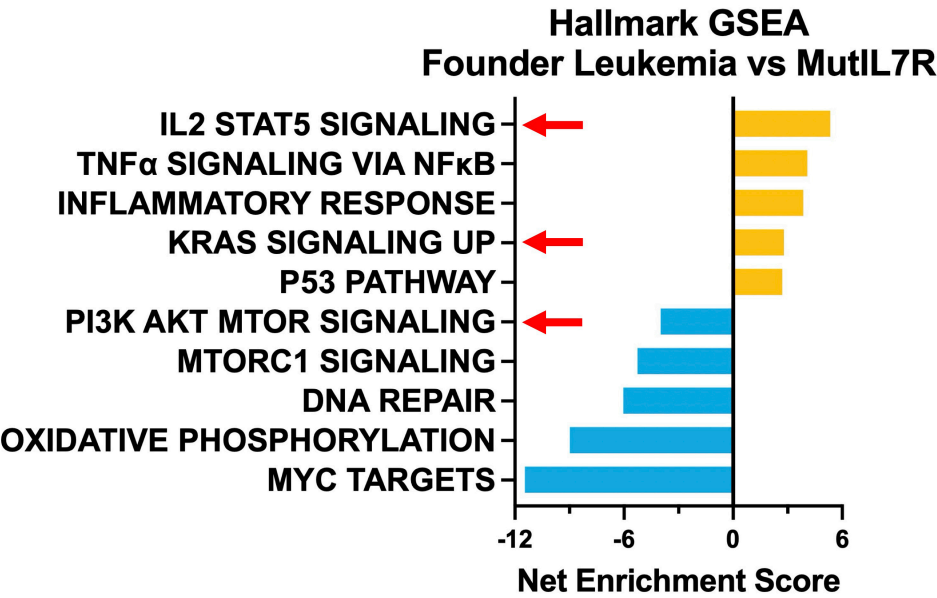
A



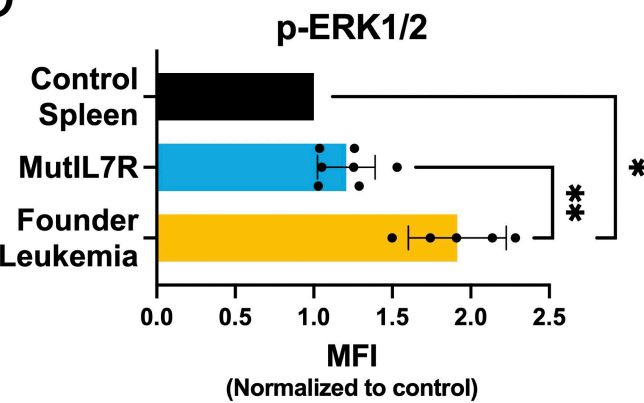
B



C



D



E

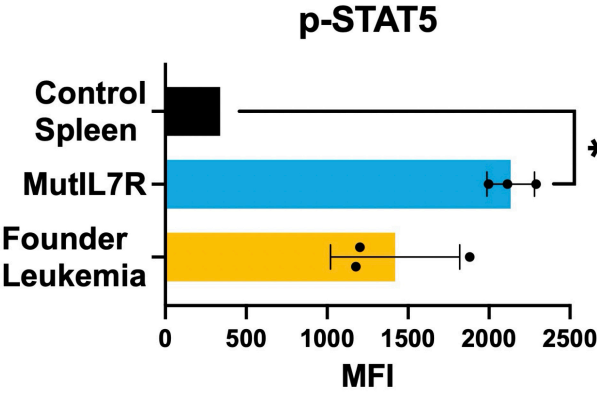
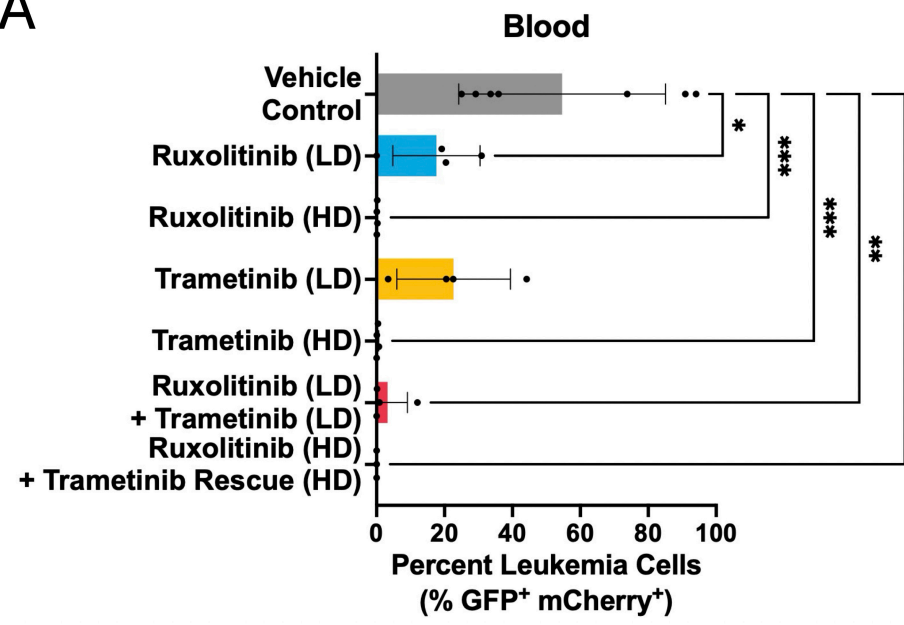
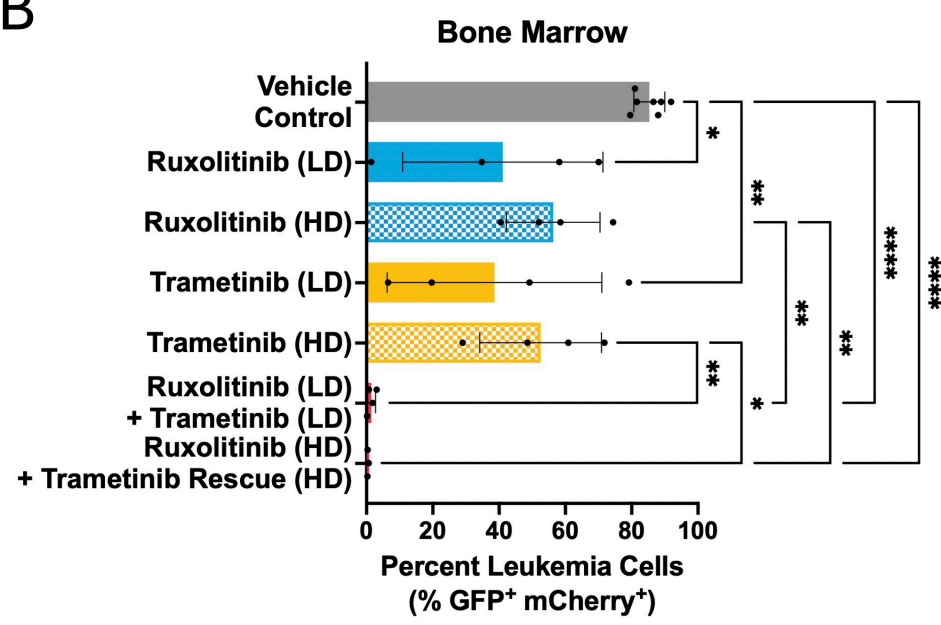


Figure 4.

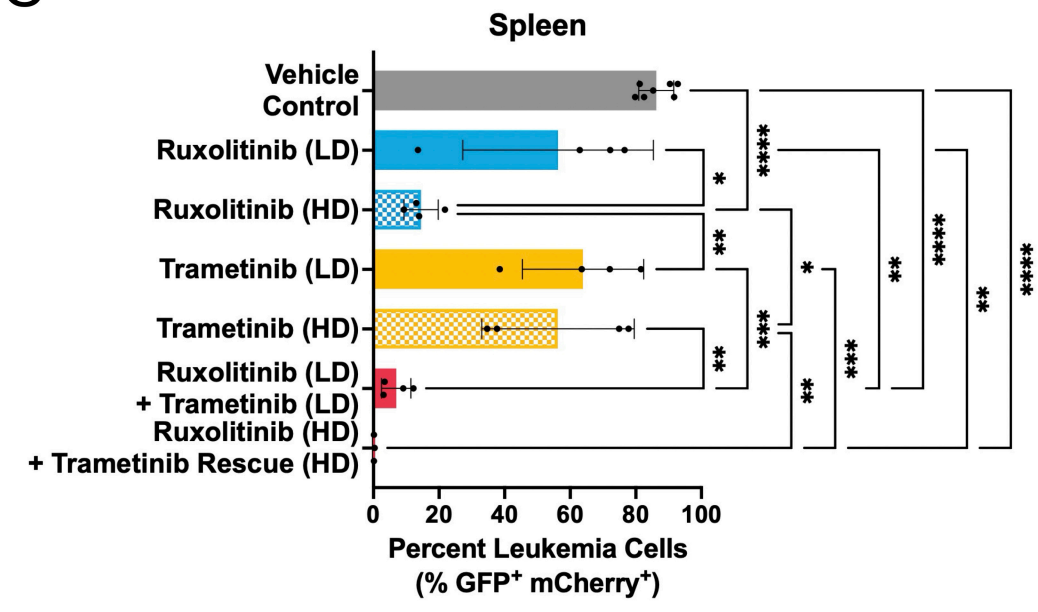
A



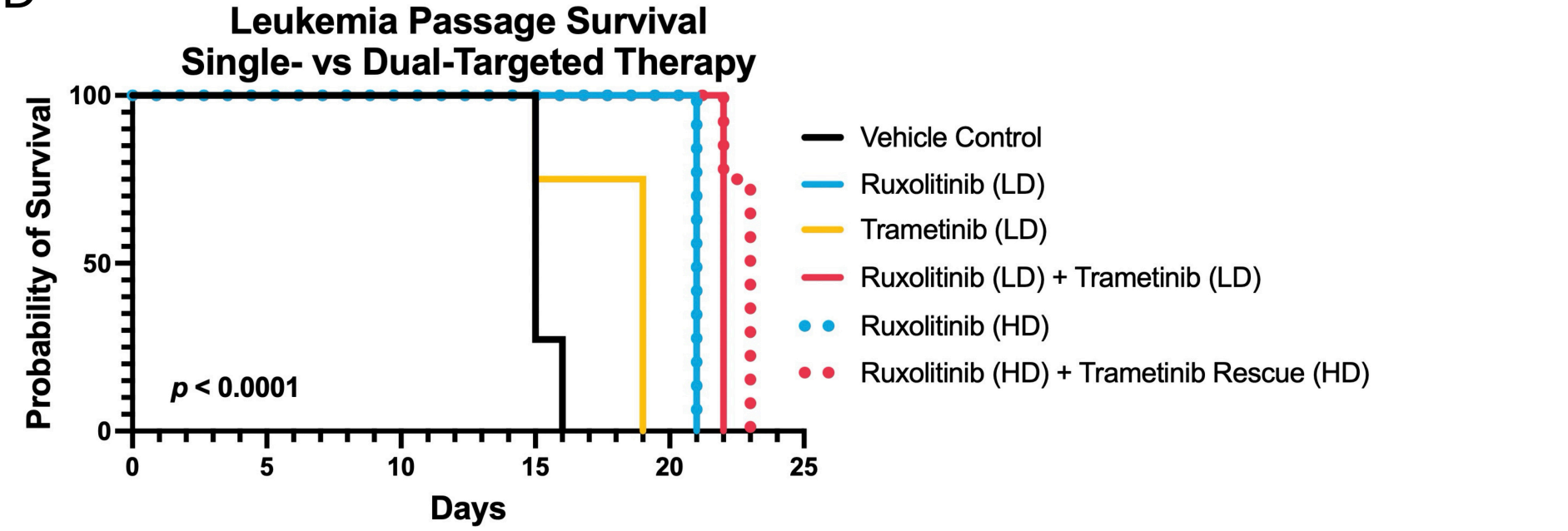
B



C

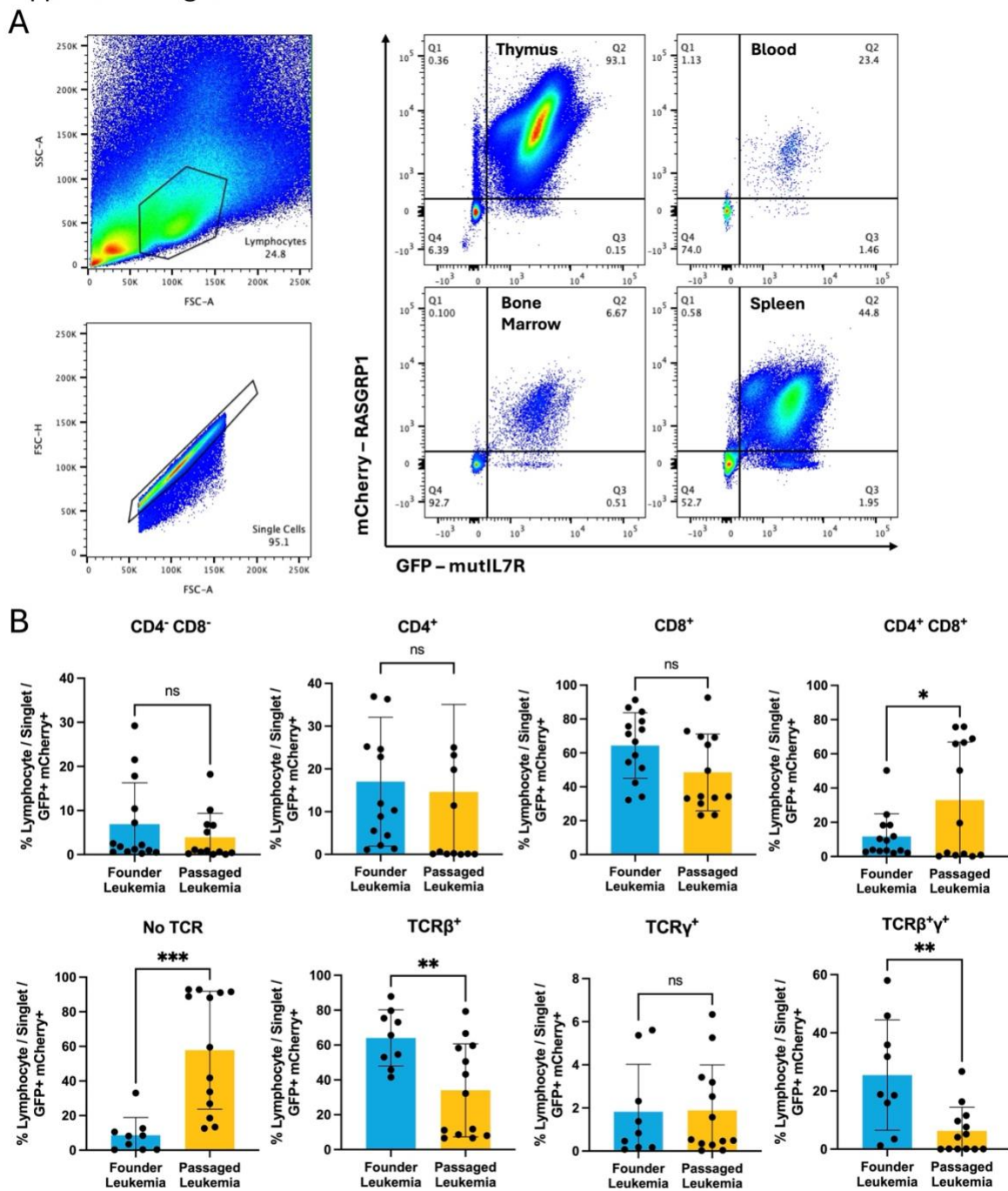


D



Supplementary figures and legends

Supplemental Figure 1.

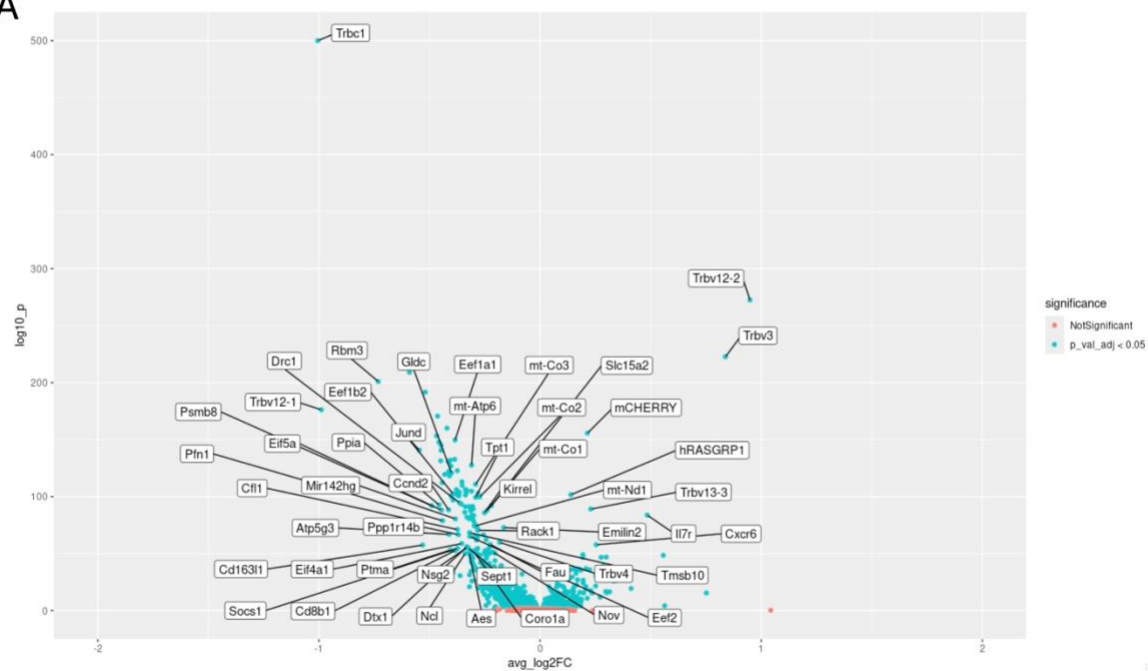


Supplemental Figure 1: Flow cytometric gating strategy and immunophenotypic characterization of founder and passaged mutIL7R+RASGRP1 leukemia. A.

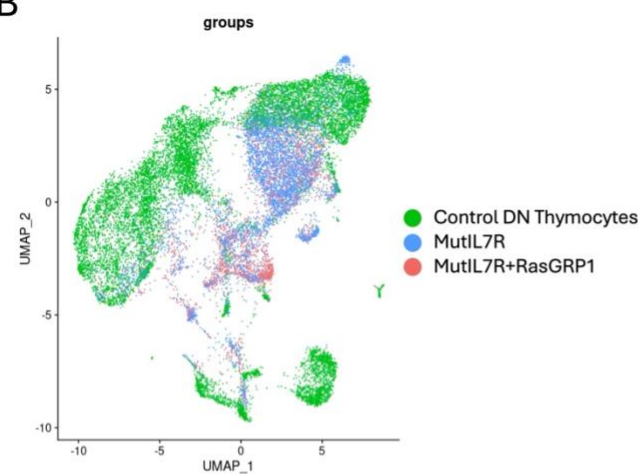
Representative gating strategy for flow cytometric analysis. Forward scatter (FSC-A) and side scatter (SSC-A) were used to define the lymphocyte gate, followed by singlet discrimination. GFP and mCherry expression were then used to identify mutIL7R+RASGRP1 leukemia cells in representative thymus, blood, bone marrow, and spleen samples from a mouse with founder leukemia. **B.** The expression of CD4, CD8, TCR β , and TCR γ by founder (FL; n=9) and passaged (PL; n=13) mutIL7R+RASGRP1 leukemia at humane endpoint (consolidated in Figure 2B). Flow cytometric analysis of the proportion of cells in the lymphocyte and singlet gates, based on forward and side scatter, that expressed GFP and mCherry.

Supplemental Figure 2.

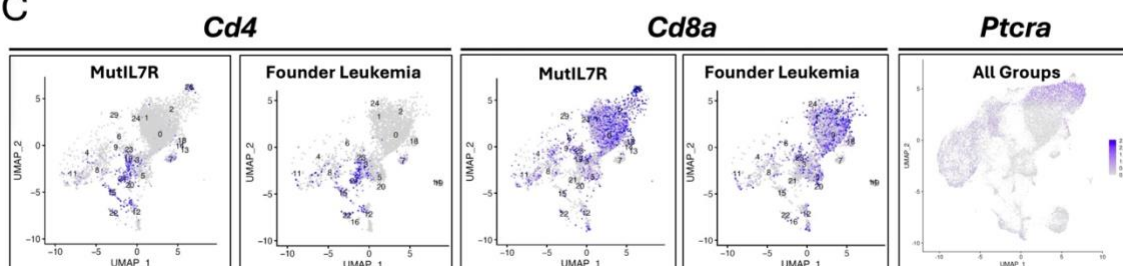
A



B



C

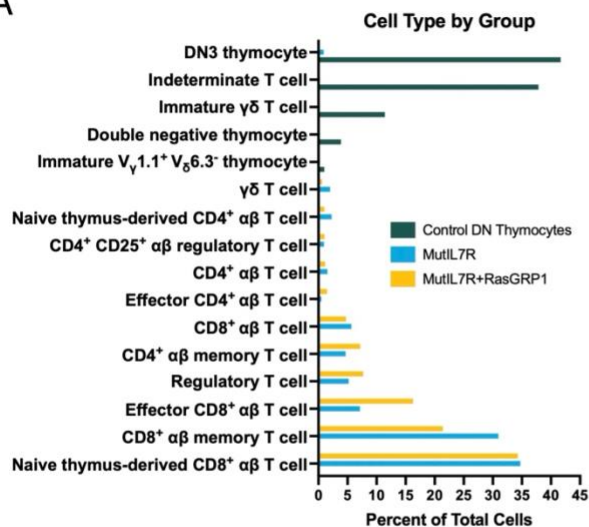


Supplemental Figure 2: The differentially expressed genes and transcription profile comparison between the mutIL7R+RASGRP1 leukemia and mutIL7R-transduced cells.

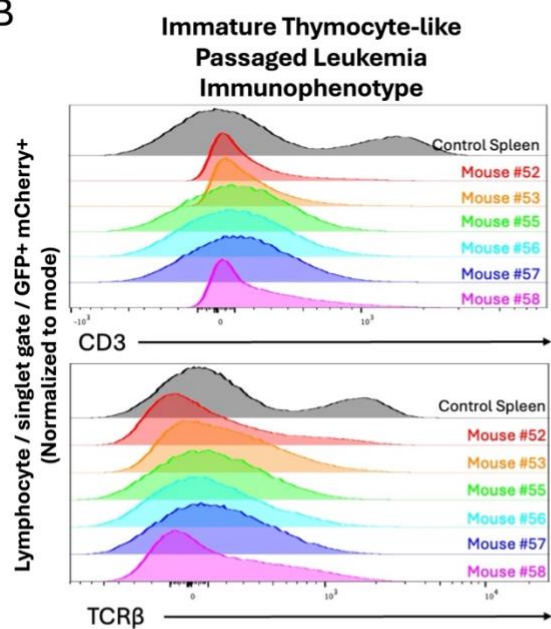
A. Volcano plot showing differentially expressed genes between mutIL7R+RASGRP1 founder leukemia and mutIL7R-driven leukocytosis. Differential expression analysis was performed using the MAST algorithm. Each point represents a single gene, with the x-axis indicating the average log2 fold change (avg_log2FC) and the y-axis showing the $-\log_{10}$ adjusted P-value. Significantly differentially expressed genes (adjusted $P < 0.05$) are shown in blue, and the top 50 differentially expressed genes are labeled. **B.** UMAP embedding plot of control DN thymocytes, mutIL7R, and mutIL7R+RasGRP1 groups were colored in green, blue, and red. **C.** Expression of *Cd4*, *Cd8a*, and *Ptcra* by group. An individual dot on the UMAP embedding plots represented a single cell, and color indicated the normalized gene expression density. The numbers on the UMAP embedding plots denoted the cell clusters.

Supplemental Figure 3.

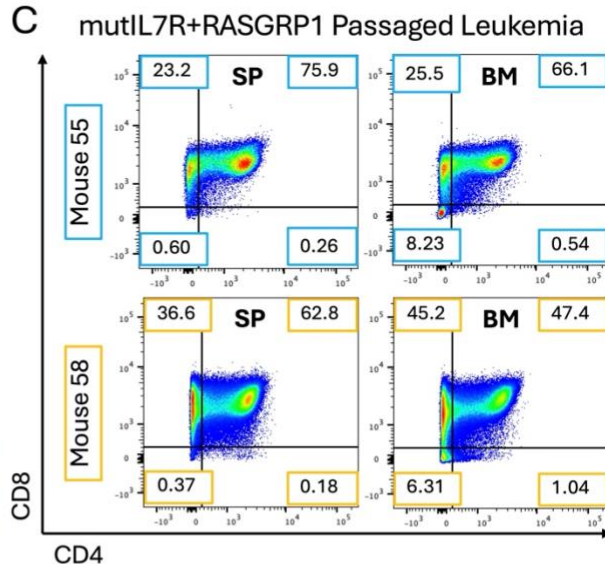
A



B

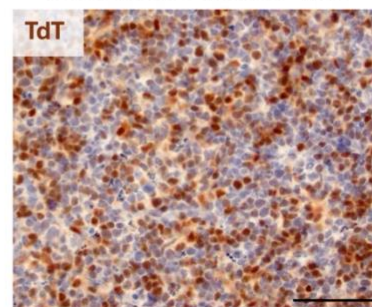


C



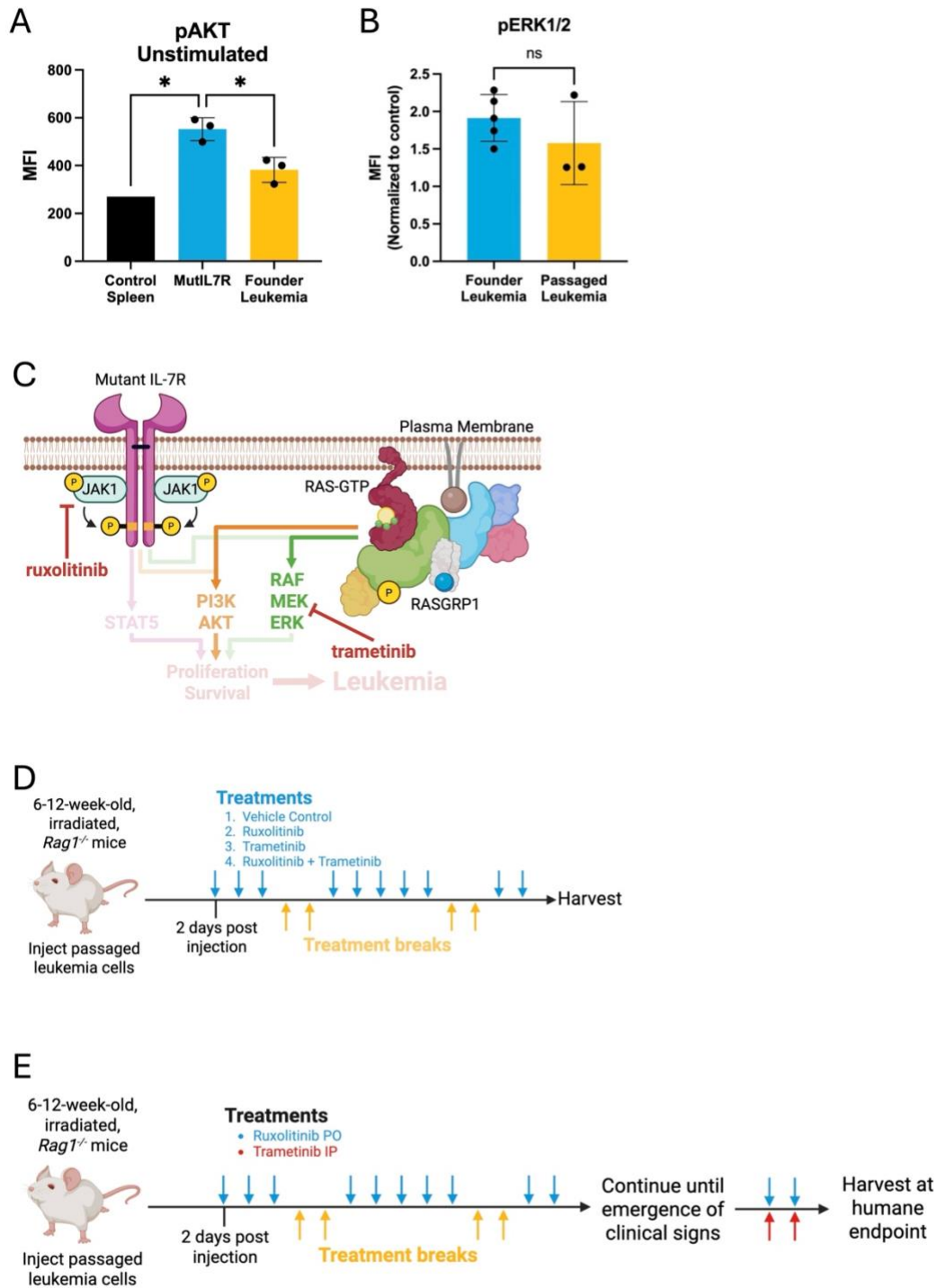
D

Passaged Leukemia - IHC



Supplemental Figure 3: From populations of founder mutIL7R+RASGRP1 leukemia, transcriptionally characterized as naïve and effector T-cell, arise passaged leukemias that phenotypically resemble thymocytes at various stages of maturation. **A.** The sixteen most numerous cell type annotations of control DN thymocytes (green), mutIL7R leukocytosis (blue), and mutIL7R+RASGRP1 founder leukemia (gold). **B.** The expression of CD3 and TCRB by immature thymocyte-like passaged leukemia cells in the spleen (colored) and control splenocytes derived from a C57BL/6 mouse (black) at the humane endpoint. **C.** The expression of CD4 and CD8 by the leukemia cells in the spleen (SP) and bone marrow (BM) derived from two mice with immature thymocyte-like passaged leukemia. Lymphocyte and singlet gates were set based on forward and side scatter, and GFP and mCherry were used to gate on the leukemia cells. **D.** Representative photomicrograph of an immunohistochemically stained section of lymph nodes derived from a mouse with passaged leukemia for TdT (brown). Black bars represent 200 μm .

Supplemental Figure 4.



Supplemental Figure 4: The mechanistic rationale and study designs for the combination of

ruxolitinib and trametinib in the treatment of the mutIL7R+RASGRP1 leukemia. A. The

phosphorylation of AKT under unstimulated conditions was determined by flow cytometry. The founder leukemia (gold; n=3) did not have significantly higher p-AKT compared to mutIL7R-

driven leukocytosis (blue; n=3) and control splenocytes from C57BL/6 mice (black; n=1). **B.** The

phosphorylation of ERK1/2 under unstimulated conditions was determined by flow cytometry.

No difference in p-ERK1/2 was observed between the founder leukemia (blue, n=5) and

passaged leukemia (gold, n=3). **C.** Schematic illustration of the rationale for the combination of

ruxolitinib and trametinib. Ruxolitinib and trametinib inhibit the JAK-STAT and RAS-MEK-ERK signaling pathways, respectively. These two pathways drive proliferation, survival, and

leukemogenesis in the mutIL7R+RASGRP1 leukemia. **D.** Schematic illustration of the ruxolitinib

and trametinib treatment design. Treatment days (blue) and breaks (gold) are marked with

arrows. All mice were harvested after 10 total treatment days. **E.** Schematic illustration of the

ruxolitinib and trametinib treatment survival study design. Treatment days with ruxolitinib

(blue), trametinib (red), and breaks (gold) are marked with arrows. Mice were harvested at

humane endpoints.

Supplemental Table 1.

A

	Control DN Thymocytes	MutIL7R	MutIL7R+ RasGRP1
NK cells	12	14	49
NKT	229	204	399
T cells	878	4,128	2,758
Tgd	12,236	700	114
Total	13,355	5,056	3,320

Supplemental Table 1: Cell type annotation of control DN thymocytes, mutIL7R-transduced cells, and the mutIL7R+RASGRP1 founder leukemia. A. Tabulation of cells characterized as NK, NKT, T, and gd T cells by groups. The number of cells characterized based on the ImmGen database as NK, NKT, T, and gd T cells was tallied, and the total number of these characterized cells was summed for each group.

Supplementary material and methods

Retroviral vector construction

Human mutIL7R and RASGRP1 cDNAs were subcloned into pMIG and IRES-mCherry (Addgene, Watertown, MA, USA) retroviral vectors by PCR cloning, respectively. The mutIL7R sequence contained an in-frame insertion of c.731_732insTTGTCCCAC, based on subject P2¹, and the hRASGRP1 sequence was wild-type. Phoenix cells were transfected with lipofectamine (Invitrogen, Waltham, MA, USA), OPTI-MEM I reduced serum medium (Invitrogen), pCL-Eco (Addgene) helper plasmid, and the retroviral vector pMIG-mutIL7R or IRES-mCherry-RASGRP1. Retroviruses were harvested from Phoenix cell media and used for transduction. Thymi were harvested from 3- to 6-week-old C57BL/6J female mice, processed, depleted of CD4⁺ and CD8⁺ cells, and transduced as previously described². After transduction, thymocytes were maintained on OP9-DL4 cells prior to injection. Founder and passaged leukemia generation Transduced thymocytes were injected into sub-lethally irradiated (3 Gy), 6- to 12-week-old, female Rag1^{-/-} (B6.129S7-Rag1tm1Mom/J) mice via the tail vein (5 x 10⁵ cells/200μl/mouse). Six biological replicate studies that

combined mutIL7R and RASGRP1 were performed (n=3), and a total of 14 mice had engraftment. Peripheral blood was collected biweekly (submandibular), and flow cytometry was used to monitor for engraftment (GFP+ mCherry+ cells). Clinical signs, such as hunched posture, reduced movement, inappetence, weight loss, rough hair coat, and dyspnea, were monitored daily. Mice that showed severe signs of morbidity were euthanized, and the blood, spleen, lymph nodes, long bones, and liver were harvested. For passage studies, spleen-derived mutIL7R+RASGRP1 founder leukemic cells (GFP+ mCherry+) were similarly injected into sub-lethally irradiated *Rag1*^{-/-} mice via the tail vein (1×10^6 , 3×10^6 , or 5×10^6 cells/200 μ l/mouse). Two biological replicate studies were performed at 5×10^6 cells/200 μ l/mouse (n=6 and 9), and all 15 mice showed engraftment. Criteria for euthanasia and the list of tissues collected were as previously described.

Drug treatment study

Spleen-derived mutIL7R+RASGRP1 leukemic cells from a passaged leukemia *Rag1*^{-/-} mouse were injected into sub-lethally irradiated *Rag1*^{-/-} mice (n=30) via the tail vein (5×10^6 cells/200 μ l/mouse). Forty-eight hours after injection, mice were treated daily (skipped weekends) for 10 days with vehicle control, ruxolitinib, trametinib (Selleckchem, Houston, TX, USA), or ruxolitinib and trametinib. Ruxolitinib was synthesized by the NCI Chemical Biology Laboratory, Chemistry Support Group (R enantiomer with 98% enantiomeric excess), and delivered per os (P.O.) via Nutra-gel (Bio-Serv, Flemington, NJ, USA)³, *ad libitum*, at 0.4 g/kg (high dose [HD]; n=4) or 0.2 g/kg (low dose [LD]; n=4) Nutra-

gel. Trametinib was dissolved in 4% DMSO in corn oil and administered intraperitoneally (I.P.) at 1 mg/kg (high dose; n=4) or 0.5 mg/kg (low dose; n=4). The ruxolitinib and trametinib combination groups of mice (n=7) received the respective drugs as previously described. The vehicle group mice (n=7) received plain Nutra-gel, P.O., *ad libitum*, and were administered I.P. with 4% DMSO in corn oil. All animal experimental procedures were conducted according to the US NIH guidelines for the Care and Use of Laboratory Animals and were approved by the NCI Animal Care and Use Committee.

Flow cytometric analysis

Cells derived from the blood, spleen, bone marrow, lymph node, and thymus were incubated with CD16/32 Fc receptor block (BioLegend, San Diego, CA, USA) and stained with combinations of anti-CD3 Alexa Fluor 700 (500A2; eBiosciences, Waltham, MA, USA), anti-CD4 PE (RM4.5; Pharmingen, Franklin Lakes, NJ, USA), anti-CD8a Brilliant Violet 785 (53-6.7; BioLegend), anti-TCRVa2 PerCP-Cy5.5 (B20.1; BioLegend), anti-TCRb APC/Cyanine7 (H57-597; BioLegend), and anti-TCRg/d Alexa Fluor 647 (GL3; BioLegend). Cells were incubated for 30 minutes at 4°C and protected from light. Cells were sequentially washed with FACS buffer and PBS and fixed in 1% PFA/PBS. Flow cytometric analysis was performed on an LSRIISORP flow cytometer (BD) with FACS DIVA software, and data analysis was performed on FlowJo (v10.10.0; Tree Star, Inc., Ashland, OR, USA). To determine the phosphorylation of STAT5, AKT, and ERK1/2, 5×10^6 spleen-derived cells were resuspended in RPMI1640 complete medium (Gibco, Waltham, MA, USA) per mouse. Cells from each mouse were transferred to individual wells of 6-well plates. At the end of

the incubation, cells were washed and resuspended in PBS, and an equal amount of intracellular fixation buffer (Biolegend) was added. Cells were fixed at room temperature and permeabilized with methanol. Cells were labeled with anti-p-STAT5 PE (47, pY694; Pharmingen), anti-p-AKT PE (M89-61, pS473; BD), and anti-p-ERK1/2 PE (MILAN8R, pT202/Y204; eBioscience) for 45 minutes at room temperature and protected from light. At the end of the incubation period, wash cells with PBS + 5% FBS at 1,000 x g for 5 minutes, twice. After the final wash, resuspend cells in PBS + 5% FBS. Flow cytometric analysis was performed on an LSRIISORP flow cytometer with FACS DIVA software, and data analysis was performed on FlowJo.

Histopathologic analysis

All tissues were fixed in 10% neutral-buffered formalin. The fixed tissues were dehydrated through graded alcohols, cleared in xylene, and infiltrated with paraffin. All tissues were then embedded in paraffin after processing. Paraffin blocks were cut on a microtome to a thickness of 4 µm, and the sections were mounted on frosted glass slides and stained with hematoxylin and eosin (H&E). H&E staining was performed on Tissue-Tek Prisma Plus (model 6170; Sakura Finetek, Torrance, CA, USA). Formalin-fixed paraffin-embedded tissue slides were deparaffinized with xylene and then hydrated through graded alcohols up to water. The slides were stained with Carazzi's hematoxylin, washed in tap water, and placed in 95% ethanol. They were then counterstained with eosin-phloxine, dehydrated through graded alcohols, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Hampton, NH, USA) as the mounting media. Histopathologic evaluation was performed by

light microscopy, and photomicrographs were taken at low (40x), medium (100x), and high (400x) magnification.

Analysis of leukemic burden in blood and tissues

Harvested tissues were dissociated into single-cell suspensions. Erythrocytes in the spleen and peripheral blood were lysed with ACK lysis buffer (Lonza, Walkersville, MD, USA). The cellularity of each sample was obtained on an automated hematology analyzer (Sysmex, Lincolnshire, IL, USA). Cells were fixed in 1% paraformaldehyde. The percentage of GFP⁺ mCherry⁺ cells per tissue, relative to total cells in the lymphocyte and singlet gate, was measured on an LSRIISORP cytometer with FACS DIVA software.

scRNA- and scTCR-sequencing data processing

Six 10X Genomics Single Cell Chromium 5' mRNA libraries (PN-1000263, 10X Genomics, Pleasanton, CA, USA) and six single cell TCR libraries were made and sequenced on one NextSeq 2000 and one NovaSeq6000 run, respectively. The six 5' mRNA libraries were created according to the manufacturer's instructions, and the six TCR libraries were created with the addition of two sets of three *Tcrg*- and *Tcrd*-specific primers (0.5 μ M each) to the *Tcra*- and *Tcrb*-specific primers in the Chromium Single Cell Mouse TCR Amplification Kit (PN-1000254, 10X Genomics). The primer sequences were obtained from Lee and colleagues and adjusted to ensure compatibility with the 10X Chromium Genomics Single Cell 5' v2 dual index chemistry reagent kit ⁴ (v1.1 was used by Lee et al.). The *Tcrg*- and *Tcrd*-specific primer sequences for PCR reaction 1 were 5'-

TCGAATCTCCATACTGACCAAGCTTG-3', 5'-GTCTTCAGCGTATCCCCTTCCTG-3', 5'-CTTTCAGGCACAGTAAGCCAGC-3', and 5'-TCTTCAGTCACCGTCAGCCAAC-3', and PCR reaction 2 were 5'-CCACAATCTTCTTGGATGATCTGAGACT-3' and 5'-GTCCCAGTCTTATGGAGATTGTTTCAG-3'. Both sequencing runs were set up as 26 cycles + 90 cycles non-symmetric runs. Demultiplexing was done, and one mismatch was allowed in the barcodes. Cells with an extremely low number of UMI counts were filtered out. The analysis was performed with the Cell Ranger v7.1.0 software using the default parameters. Downstream single-cell RNA-sequencing quality control and analysis were, for the most part, performed using the Seurat package (v4.3) in R (v4.2) ⁵. Per sample quality control was performed with the MiQC package, using default parameters to identify and remove low-quality cells on the basis of mitochondrial read fraction and number of unique genes found ⁶. Sample read counts were individually normalized and scaled using the SCTransform tool through the Seurat package ⁷. Doublets were identified and removed using the DoubletFinder package (v2.0.2) ⁸. After cell filtering, samples were combined within Seurat and batch corrected using the Reciprocal Principal Components Analysis technique ⁵. High confidence cells for the mutIL7R and mutIL7R+RasGRP1 groups were identified and subsetted based on mutant hIL7R and/or eGFP expression, and mutant *hIL7R* and/or eGFP and *RASGRP1* and/or mCherry expression, respectively, and k-nearest neighbors and Uniform Manifold Approximation and Projections were recalculated as necessary ⁹.

T-cell lineage transcriptional classification

Preliminary cell type identification (Supplemental Table 1) was performed with the SingleR package (v1.0.5) and based on the ImmGen database as a reference ^{10,11}. Further T cell lineage subclassification was performed with the Celldex R package to annotate cell clusters ¹⁰. The DEG and GSEA wrapper functions in Seurat used the MAST algorithm to perform differential gene expression analysis (Supplemental Figure 2A) ¹². Significantly differentially expressed genes were identified as having an absolute log2 fold change (logFC) value greater than 0.5, gene expression in at least 10% of the contrast groups, and an adjusted p-value less than 0.05. Gene set enrichment analysis was performed using the GSEA pre-ranked tool, using a scoring metric based on the fold change, p-value, and the expression fraction:

$$Score_{gene} = \begin{cases} -\log_{10}(pVal) * \text{sign}(FC) * \max(pct.1, pct.2) + \log_2(FC), & pVal > 0 \\ 500 * \text{sign}(FC) * \max(pct.1, pct.2) + \log_2(FC), & pVal = 0 \end{cases}$$

Reference gene sets and pathways were obtained from MSigDB ¹³⁻¹⁵. FeaturePlot functions from Seurat were used to produce the images.

Clonotype identification by CDR3 Sequence

The Cell Ranger (v7.1.0) V(D)J T cell pipeline was used to determine the TCRa and TCRb CDR3 amino acid sequences, clonotypes, frequency, and proportion of cells in mutIL7 and mutIL7R+RasGRP1 groups. Only the top 20 clones were tabulated in the pipeline and used as a cursory overview. Further clonality analyses were performed on the scTCR-seq data.

Vdj_GRCm38_alts_ensembl-7.0.0 was used as the reference V(D)J recombination. A clone

was defined as cells with an identical TCR CDR3 nucleic acid sequence. The top 4 clones in the mutIL7R+RasGRP1 leukemia were analyzed individually; all other clones were binned into “other,” and cells with no CDR3 sequences were binned into “none.” For visualization, the top 4 clones were plotted individually, the top fifth to tenth clones were combined into one group, and all other clones were combined into one group. The gene expression profiles of these clonal cells were determined after the single-cell TCR and single-cell RNA datasets were integrated through cell barcodes.

Supplementary References

1. Zenatti PP, Ribeiro D, Li W, et al. Oncogenic IL7R gain-of-function mutations in childhood T-cell acute lymphoblastic leukemia. *Nat Genet.* 2011;43(10):932–939.
2. Rodrigues GOL, Li W, Cramer SD, et al. Co-Culture and Transduction of Murine Thymocytes on Delta-Like 4-Expressing Stromal Cells to Study Oncogenes in T-Cell Leukemia. *J Vis Exp.* 2023;(196).
3. Senkevitch E, Li W, Hixon JA, et al. Inhibiting Janus Kinase 1 and BCL-2 to treat T cell acute lymphoblastic leukemia with IL7-Ralpha mutations. *Oncotarget.* 2018;9(32):22605–22617.
4. Lee M, Lee E, Han SK, et al. Single-cell RNA sequencing identifies shared differentiation paths of mouse thymic innate T cells. *Nat Commun.* 2020;11(1):4367.
5. Hao Y, Hao S, Andersen-Nissen E, et al. Integrated analysis of multimodal single-cell data. *Cell.* 2021;184(13):3573–3587 e3529.
6. Hippen AA, Falco MM, Weber LM, et al. miQC: An adaptive probabilistic framework for quality control of single-cell RNA-sequencing data. *PLoS Comput Biol.* 2021;17(8):e1009290.

7. Hafemeister C, Satija R. Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* 2019;20(1):296.
8. McGinnis CS, Murrow LM, Gartner ZJ. DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. *Cell Syst.* 2019;8(4):329–337 e324.
9. Becht E, McInnes L, Healy J, et al. Dimensionality reduction for visualizing single-cell data using UMAP. *Nat Biotechnol.* 2018.
10. Aran D, Looney AP, Liu L, et al. Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol.* 2019;20(2):163–172.
11. Heng TS, Painter MW, Immunological Genome Project C. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol.* 2008;9(10):1091–1094.
12. Finak G, McDavid A, Yajima M, et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Genome Biol.* 2015;16:278.
13. Aibar S, Gonzalez-Blas CB, Moerman T, et al. SCENIC: single-cell regulatory network inference and clustering. *Nat Methods.* 2017;14(11):1083–1086.
14. Hanzelmann S, Castelo R, Guinney J. GSEA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics.* 2013;14:7.
15. Liberzon A, Birger C, Thorvaldsdottir H, et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* 2015;1(6):417–425.