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Molecular Insights on Acute Lymphoblastic Leukemia Development and Suppression

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Sir. Isaac Newton, 1676

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Publication of chapter 4 and chapter 7 in two separate original publications is currently in preparation.

Table of Contents

Original Publications ***v***

Resumo ***xv***

Summary ***xvii***

Chapter 1. Introduction ***1***

1. Lymphopoiesis 1

1.1. Early B-cell development 1

1.2. T-cell development: From the bone marrow to the thymus 2

1.3. From early thymic precursors to T-cell receptor-expressing thymocytes 2

1.4. Thymic selection 4

2. Acute Lymphoblastic Leukemia 8

2.1. Epidemiology and clinical presentation 9

2.2. Immunophenotype 10

2.3. Genetic alterations in B-ALL 11

2.4. Genetic alterations in T-ALL 13

2.4.1. Mutations and altered signaling pathways in T-ALL 14

2.5. ALL dissemination and CNS involvement 17

2.6. ALL treatment 20

2.6.1. Standard treatment 20

2.6.2. Targeted therapy and new generation therapeutic options 21

2.7. TCR expression and TCR signaling in T-ALL 23

Chapter 2. Aims ***25***

Chapter 3. Materials and Methods ***26***

Chapter 4. Tonic TCR signaling is leukemogenic while strong TCR stimulation is leukemia-suppressive ***32***

1. TCR-HY transgene expression drives leukemia development 33

2. Transgenic HY-TCR expression induces immature T-cell leukemia development 34

3. HY-TCR-induced T-ALL is not dependent on *Rag2* recombinase expression 37

4. Mixed B and T lineage hematological disease in HY-TCR transgenic females is associated with microbial status 38

5. Marilyn leukemias present *Notch1* PEST domain mutations and genetic copy number alterations 39

6. The transgenic HY-TCR is functional in Marilyn T-ALL cells and its stimulation delays leukemogenesis 41

7. Discussion 45

8. Supplementary Figures 48

Chapter 5. Qualitative differences in TCR stimulation drive distinct outcomes in leukemic cells 57

1. OT-I TCR expression delays the development of T-ALL sensitive to TCR activation 57

2. OT-I TCR stimulation with increased affinity peptides correlates with ETV6::JAK2;OT-I T-ALL cell activation and apoptosis 61

3. ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL development and TCR stimulation is not dependent on MHC-I 65

4. Discussion 68

Chapter 6. Cdkn2a inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint 72

1. The ETV6::JAK2 fusion protein induces malignant transformation of *Rag2*-deficient thymocytes blocked at the DN3 stage 73

2. *Cdkn2a* genetic inactivation accelerated T-ALL development in ETV6::JAK2; *Rag2*$^{-/-}$ mice 73

3. Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia 76

4. *Cdkn2a* inactivation and Notch1-activating mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice are frequent, independent events 76

5. Discussion 78

6. Supplementary Figures 80

Chapter 7. The ETV6::JAK2 fusion protein cooperates with Cdkn2a deletion to promote early onset precursor B-cell leukemia infiltrating the central nervous system ***83***

1. ETV6::JAK2 transgene expression cooperates with *Cdkn2a* inactivation accelerating B-ALL development in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice 83

2. B-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice was associated with extensive CNS infiltration and features of high-risk Ph-like ALL 86

3. Discussion 88

4. Supplementary Figures 90

References ***100***

Annexes ***137***

Annex 1. Original research publication 137

Annex 2. Gating strategies for flow cytometry analysis 151

Annex 3. Permissions 155

List of Figures

Figure 1. Fate of developing T-cells according to affinity of TCR-MHC interactions.*_____7
Figure 2. Signaling pathways commonly altered in T-ALL*_____15
Figure 3. HY-TCR transgene expression drives leukemia development._____34
Figure 4. Immunophenotypic analysis of HY-TCR leukemic cells. _____36
Figure 5. Transgenic HY-TCR expression in *Rag2*$^{-/-}$ mice drives development of T-ALL with thymic lymphomas. _____ 38
Figure 6. *Notch1* mutations and *Cdkn2a* copy number variations were detected in immature type HY-TCR leukemic cells._____40
Figure 7. HY-TCR T-ALL cells are responsive to TCR signaling activation._____41
Figure 8. HY-TCR T-ALL antigenic stimulation in vivo hampers leukemia development. _____43
Figure 9. ETV6::JAK2-induced T-ALL onset was delayed in high-affinity transgenic OT-I TCR mice compared to lower affinity HY-TCR females. _____58
Figure 10. Strong agonist-induced OT-I TCR stimulation in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells is leukemia suppressive._____60
Figure 11. TCR activation of OT-I TCR thymocytes leads to apoptosis in a peptide affinity-dependent manner in DP immature thymocytes but not in mature CD8 SP cells. _____62
Figure 12. Agonist-induced activation of ETV6::JAK2;OT-I T-ALL cells results in apoptosis in a peptide affinity-dependent manner. _____63
Figure 13. Strength of TCR stimulation in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells modulates the expression of T-cell activation markers. _____64
Figure 14. Leukemia development in ETV6::JAK2;OT-I mice is not MHC-dependent._____66
Figure 15. β2m-deficient ETV6::JAK2;OT-I T-ALL cells maintain TCR expression and can be activated by agonist-peptides in a MHC-independent manner._____67
Figure 16. *Cdkn2a* genetic inactivation accelerates T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice. _____ 75
Figure 17. Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia. _____ 77
Figure 18. ETV6::JAK2 transgene expression accelerates B-ALL development in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice. _____85
Figure 19. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed B-ALL with CNS infiltration and high-risk features._____87

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List of Supplementary Figures

Supplementary Figure 1. Marilyn female mice developed hematological tumors. _____48
Supplementary Figure 2. Histological analysis of HY-TCR female mice with leukemia/lymphoma. _____ 51
Supplementary Figure 5. CD69 expression in pathological HY-TCR T cells. _____52
Supplementary Figure 6. Transgenic HY-TCR expression in *Rag2*$^{-/-}$ mice develop aggressive T-ALL disseminated to several organs. _____53
Supplementary Figure 7. Stronger lipopolysacharide expression in organs from diseased Marilyn leukemic females than *Rag2*$^{-/-}$ counterparts raised in a different animal facility. _____54
Supplementary Figure 8. DNA copy number alterations detected in Marilyn T-ALL cells. _____55
Supplementary Figure 9. *Cdkn2a* loss accelerates HY-TCR T-ALL development. _____56
Supplementary Figure 10. *Cdkn2a* loss was found in *Rag2*-deficient T-ALL. _____80
Supplementary Figure 11. *Cdkn2a* haploinsufficiency does not rescue the DN3 block caused by *Rag2* deficiency. _____81
Supplementary Figure 12. ETV6::JAK2 expression and *Cdkn2a* deficiency did not rescue the B-cell developmental arrest induced by *Rag2* deficiency. _____90
Supplementary Figure 13. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells infiltrate skull and vertebral leptomeninges. _____ 91
Supplementary Figure 14. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells express CD49f and CXCR4 but not RANKL. _____92
Supplementary Figure 15. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells segregate from each other but both express Ph-like ALL signature genes. _____93

List of Tables

Table 1. Immunological ALL classification based on the European Group of the Immunological Characterization of Leukemias (EGIL). _____11

List of Supplementary Tables

Supplementary table 1. Summary of genetic alterations found in leukemic cells of the indicated genotypes. _____82
Supplementary Table 2. Features and immunophenotype of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias. _____95

List of abbreviations

Aire	Autoimmune regulator
ALL	Acute lymphoblastic leukemia
AlloHSCT	Allogenic hematopoietic stem cell transplant
APCs	Antigen-presenting cells
B-ALL	B-cell ALL
bHLH	Class II basic helix-loop-helix
BM	Bone marrow
CAR	Chimeric antigen receptor
CCL19	Chemokine (C-C motif) ligand 19
CCL21	Chemokine (C-C motif) ligand 21
CCR7	Chemokine (C-C motif) receptor 7
CCR9	Chemokine (C-C motif) receptor 9
CD40L	CD40 ligand
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CLPs	Common lymphoid progenitors
CNAs	Copy number alterations
CNS	Central nervous system
CSF	Cerebrospinal fluid
cTECs	Cortical thymic epithelial cells
CXCR3	C-X-C chemokine receptor type 3
CXCR4	C-X-C chemokine receptor type 4
DBY	Male-specific antigen
DCs	Dendritic cells
DN	Double negative
DP	Double positive
EGIL	European group for the immunologic classification of leukemia
ERK	Extracellular-signal-regulated kinase
EMA	European Medicines Agency
ETPs	Early thymic progenitors
ETP-ALL	Early T-cell precursor acute lymphoblastic leukemia
FDA	U.S. Food and Drug Administration
Fr A	Hardy fraction A
Fr B	Hardy fraction B

Fr C	Hardy fraction C
Fr C’	Hardy fraction C’
Fr D	Hardy fraction D
Fr E	Hardy fraction E
G4	OVA mutant peptide (SIIGFEKL)
HD	Heterodimerization domain
HSCs	Hematopoietic stem cells
iAMP21	Intrachromosomal amplification of chromosome 21
ICN1	Notch1 intracellular subunit
Ig	Immunoglobulin
IKZF1	IKAROS family zinc finger 1
IL-4Rα	Interleukin-4 receptor alpha
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
IL-15	Interleukin-15
Ion.	Ionomycin
ISP	Immature single positive
JAK2	Janus kinase 2
LMO	LIM-domain-only
LNs	Lymph nodes
LPS	Lipopolysaccharide
LTαβ	Lymphotoxin αβ
MHC	Major histocompatibility complex
MRD	Minimal residual disease
mTECs	Medullary thymic epithelial cells
NF-κB	Nuclear factor-kappa B
NK	Natural killer cells
OVA	Ovalbumin
PAX5alt	*PAX5*-altered
Ph	Philadelphia chromosome
Ph-like	Philadelphia chromosome-like
PI3K	Phosphoinositide 3 kinase
PMA	Phorbol 12-myristate-13-acetate
PSGL-1	P-selectin glycoprotein ligand-1
qPCR	quantitative PCR
RANKL	Receptor activator of nuclear factor-kappa B ligand

S1P1	Sphingosine-1-phosphate receptor 1
self-pMHC	self-peptide MHC
SP	Single-positive
T4	OVA mutant peptide (SIITFEKL)
T-ALL	T-cell ALL
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
TKIs	Tyrosine kinase inhibitors
TNF	Tumor necrosis factor
TRAs	Tissue-restricted antigens
Treg	Regulatory T-cells
TSPs	Thymus seeding progenitors
TSSP	Thymus-specific serine protease
WBC	White blood cell
WHO	World Health Organization
WT	Wild-type
β2m	Beta-2 microglobulin

Resumo

A leucemia linfoblástica aguda (LLA) é uma neoplasia agressiva com origem em percursores imaturos de células B (LLA-B) ou T (LLA-T). Apesar de ser possível distinguir LLA-T e LLA-B com base em marcadores moleculares, subtipos das duas entidades partilham alterações genéticas e em vias de sinalização celular tais como perda do gene *CDKN2A* e ativação da via JAK/STAT. A expressão do recetor de células T (TCR) e do pre-TCR é frequente em células de LLA-T.. No modelo murino de LLA-T por expressão da proteína de fusão ETV6::JAK2, ausência de pre-TCR atrasou o desenvolvimento de LLA-T, e nos casos em que a doença se desenvolveu, perda do gene *Cdkn2a* foi frequente. Foi também anteriormente mostrado em modelos animais que expressão do TCR pode ter um papel oncogénico em LLA-T. O nosso grupo mostrou recentemente que a estimulação do TCR em células de LLA-T resultou na indução de um programa apoptótico semelhante ao processo de seleção negativa de timócitos, o que sugere que o TCR poderá ter um duplo papel no desenvolvimento de LLA-T, tal como observado no desenvolvimento de timócitos normais, potencialmente dependente da intensidade de estimulação do TCR.

Nesta tese, tivemos como objetivo determinar o papel de ativação do TCR no desenvolvimento e progressão de LLA-T e averiguar o impacto de alterações genéticas ou em vias de sinalização celular tais como deleção do gene *Cdkn2a* e ativação da via JAK/STAT na génese e progressão de leucemia.

No capítulo 4, mostramos que expressão de um TCR transgénico foi suficiente para induzir o desenvolvimento de LLA-T caracterizada por infiltração de órgãos linfoides e não-linfóides, fenótipo imaturo e expressão de TCR. As células leucémicas apresentaram níveis intermédios de expressão de CD5 à superfície, que são indicativos de estimulação basal do TCR e que a leucemia teve origem em timócitos que sofreram seleção positiva. Mostrámos ainda que forte estimulação *in vitro* e *in vivo* do TCR de células T-ALL resultou na morte das mesmas e no aumento da sobrevida em murganhos, o que sugere que estimulação basal do TCR promove desenvolvimento de leucemia enquanto estimulação forte do TCR resulta num efeito anti-leucémico. No capítulo 5, observámos que estimulação do TCR transgénico de células LLA-T com péptidos com baixa afinidade para o TCR transgénico induziu apoptose com menor eficiência que péptidos com alta afinidade. Estimulação do TCR de células LLA-T com péptidos com afinidade crescente correlacionou-se com o aumento da taxa de apoptose, expressão de Nur77, um marcador de ativação do TCR, e diminuição da expressão de TCRβ, o que indica que apenas

interações de alta afinidade do TCR atingem o limite de sinalização essencial para indução de apoptose em LLA-T mediada por sinalização do TCR.

No capítulo 6, mostrámos o papel supressor do gene *Cdkn2a* no desenvolvimento de leucemia em timócitos imaturos, uma vez que deleção de uma cópia do gene agravou o desenvolvimento de LLA-T em murganhos ETV6::JAK2;*Rag2*$^{-/-}$, sem induzir a passagem do bloqueio da seleção-β pré-leucemia, e que o desenvolvimento de leucemia foi acompanhado de frequente perda do segundo alelo do gene *Cdkn2a*. Observámos ainda alterações genéticas adicionais como mutações *Notch1* e ganhos de número de cópia de cromossomas em células de LLA-T com perda do gene *Cdkn2a*, indicando que neste modelo são necessárias alterações genéticas secundárias para o desenvolvimento de LLA-T.

No capítulo 7, verificámos que a deleção total do gene *Cdkn2a* em murganhos ETV6::JAK2;*Rag2*$^{-/-}$ resultou no desenvolvimento precoce de LLA-B com sinais de invasão do (sistema nervoso central) SNC, em comparação com murganhos *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ da mesma ninhada. Infiltração das leptomeninges do crânio e das vertebras, assim como expressão de moléculas e ativação de vias de sinalização envolvidas em infiltração do SNC foram confirmadas. Estas observações indicam que a ativação constitutiva de JAK2 coopera com a deleção de *Cdkn2a* na promoção do desenvolvimento de LLA-B precoce e de alto risco. Adicionalmente, em células leucémicas de murganhos ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mas também *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$, foi observada a expressão de marcadores usados para a identificação de LLA-B de alto risco do subtipo *Ph-like*, evidenciando o potencial destes modelos animais como ferramentas para o estudo do desenvolvimento e progressão de LLA-B de alto risco assim como de mecanismos de invasão do SNC por células leucémicas.

Summary

Acute lymphoblastic leukemia/lymphoma (ALL/LBL) is an aggressive hematological malignancy originating in T- or B-cell precursors. While B-ALL and T-ALL can be distinguished based on molecular markers, several genetic and signaling pathway alterations are shared between subgroups of the two, as is the case of *CDKN2A* loss and JAK/STAT signaling activation. The pre-T-cell receptor (TCR) and TCR are frequently expressed in T-ALL. In the ETV6::JAK2 transgenic T-ALL transgenic mouse model, absence of pre-TCR resulted in delay of T-ALL development, and when T-ALL developed *Cdkn2a* loss was frequently found. Additionally, it was previously shown in mouse models that transgenic TCR expression can play a tumor-promoting role in leukemogenesis. Our group has previously shown that TCR stimulation in T-ALL cells results in an apoptotic program reminiscent of thymocyte negative selection, suggesting that TCR may play a dual role in leukemogenesis, as it does in normal thymocytes development, putatively associated with TCR signaling intensity.

In this thesis our aim was to determine the role of TCR activation in T-ALL leukemogenesis and progression and to assess the impact of genetic and signaling pathway alterations such as *Cdkn2a* deletion and JAK/STAT signaling activation in leukemia development and progression.

In chapter 4, we showed that transgenic TCR expression alone was sufficient to trigger T-ALL development. The disease was characterized by infiltration of lymphoid and non-lymphoid organs, immature phenotype and TCR expression. Leukemic cells expressed intermediate CD5 levels at the surface, which are indicative of basal TCR stimulation and that leukemia arose from positively selected thymocytes. Furthermore, strong *in vitro* and *in vivo* TCR stimulation of T-ALL cells resulted in T-ALL cell death and increased mouse survival, suggesting that tonic TCR signaling is leukemogenic, while strong TCR signaling is leukemia-suppressive. In chapter 5, we observed that stimulation of transgenic TCR in T-ALL cells with lower affinity peptides induced T-ALL apoptosis less efficiently than higher affinity peptides. TCR stimulation with peptides of increasing affinity correlated with increased apoptotic rate, expression of the TCR activation marker Nur77, and downregulation of TCRβ, suggesting that TCR activation is not sufficient to induce apoptosis of T-ALL cells and that only high-affinity interactions meet the threshold for TCR-mediated induction of T-ALL apoptosis.

In chapter 6, we showed that *Cdkn2a* suppressed leukemia development in immature thymocytes, as *Cdkn2a* haploinsufficiency aggravated T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice, without pre-leukemia bypass of the β-selection block, and T-ALL development was often accompanied by somatic loss of the second *Cdkn2a* allele. Further genetic alterations such as Notch1 mutations and chromosomal copy number gains were found in *Cdkn2a*-deficient T-ALL cells, indicating that secondary alterations are required for leukemogenesis in this model.

In chapter 7, we showed that total loss of *Cdkn2a* in ETV6::JAK2;*Rag2*$^{-/-}$ mice resulted in early-onset B-ALL development with signs of central nervous system (CNS) colonization in comparison to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ littermates. Infiltration of skull and vertebral leptomeninges and expression of molecules and activation of pathways involved in CNS infiltration were confirmed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice. These observations suggest that constitutive JAK2 activation cooperates with *Cdkn2a* loss to promote early onset high-risk B-ALL. Moreover, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ but also *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells expressed markers used to identify the human B-ALL high-risk Ph-like subtype, highlighting the potential of these models as tools to study high-risk B-ALL development and progression and the mechanisms for CNS invasion by leukemic cells.

Chapter 1. Introduction

1. Lymphopoiesis

Hematopoietic stem cells (HSCs) reside in the bone marrow (BM) and have the potential to generate all blood cells. This process is regulated by cell-cell interactions and growth factors, driving the expression of lineage commitment transcription factors and results in progressive differentiation and gradual loss of self-renewal capacity (Lai & Kondo, 2008). Throughout this process, HSCs differentiate into multipotent progenitors and subsequently to oligopotent progenitors that then generate lineage-committed precursor cells, including common lymphoid progenitors (CLPs) (Seita & Weissman, 2010). CLPs are able to generate T, B, natural killer (NK), innate lymphoid cells and dendritic cells (DCs).

1.1. Early B-cell development

The BM microenvironment, where all stages of B-cell development (from early B-cell progenitors to mature B-cells) can be found, is necessary for early steps of B-cell development. Pre-pro-B cells are the earliest identified stage of B-cell lineage development and in the mouse are characterized by B220 and CD43 expression, lack of CD19 and low CD24 expression levels. These cells are referred to as Hardy fraction A (Fr A) ($B220^{+}$,$CD43^{+}$,$CD24^{low}$,$BP\text{-}1^{-}$) and precede the start of immunoglobulin (Ig) rearrangement (Hardy & Hayakawa, 2001; Tung et al., 2004). Importantly, during early stages of B-cell development, expression of the transcription factors E2A, EBF1 and PAX5 play a central role in B-cell lineage specification by activation of B-cell-specific transcriptional programs or reinforcement of B-cell lineage commitment by repression of non-B linage genes (Nutt & Kee, 2007).

In the pro-B stage, corresponding to Fr B ($B220^{+}$,$CD43^{+}$,$CD24^{+}$,$BP\text{-}1^{-}$) and Fr C ($B220^{+}$,$CD43^{+}$,$CD24^{+}$,$BP\text{-}1^{+}$), developing B-cells undergo active Ig heavy chain rearrangements. This process is critical for assembly of the pre-B-cell receptor (pre-BCR) and B-cell development progression. Inability for Ig rearrangement (as observed in *Rag1*- or *Rag2*- deficient mice) results in a developmental block at the pro-B stage (Hardy & Hayakawa, 2001; Pieper et al., 2013; Tung et al., 2004). Heavy chain rearranged pre-B cells undergo a proliferative burst, thought to be associated with pre-BCR assembly and signaling (Fr C'; $B220^{+}$,$CD43^{+}$,$CD24^{high}$,$BP\text{-}1^{+}$) and downregulate CD43 expression (Fr D;

$B220^{+}$,$CD43^{low}$), where light chain rearrangement occurs, followed by differentiation into immature B-cells, which express surface IgM (Fr E; $B220^{+}$,$CD43^{low}$,IgM^{+}) (Hardy & Hayakawa, 2001; Tung et al., 2004; Winkler & Mårtensson, 2018). At this stage, antigen-induced BCR signaling downregulates the expression of chemokine receptor C-X-C chemokine receptor type 4 (CXCR4) and immature B-cells egress from the BM to the spleen where the B-cell maturation process continues (Beck et al., 2014; Tung et al., 2004).

1.2. T-cell development: From the bone marrow to the thymus

While the first steps of B-cell development occur in the BM, T-cells develop in the thymus. In order to home in the thymus and to differentiate into T-cells, CLPs differentiate into thymic seeding progenitors (TSPs) which express P-selectin glycoprotein ligand-1 (PSGL-1), chemokine (C-C motif) receptor 7 (CCR7) and 9 (CCR9), required for this process (Cordes et al., 2022; Krueger et al., 2010; Rossi et al., 2005; Sultana et al., 2012; Zlotoff et al., 2010). Once in the thymus, TSPs give rise to early thymic progenitors (ETPs) that then undergo T-lineage specification and differentiate further to complete T-cell development (Cordes et al., 2022; Seita & Weissman, 2010). ETPs have a limited capacity to generate T-cells, hence continuous recruitment of precursors is thought to be necessary to sustain continuous generation of T-cells (Donskoy & Goldschneider, 1992; Foss et al., 2001; Schwarz & Bhandoola, 2006).

1.3. From early thymic precursors to T-cell receptor-expressing thymocytes

The thymus provides the conductive microenvironment for T-cell development and can be histologically divided in central medulla, peripheral cortex and cortico-medullary junction. ETPs enter the thymus through cortico-medullary junction blood vessels and then, by receiving instructive signals from the microevironment (e.g. Interleukin-7 (IL-7) and NOTCH ligands), undergo T-lineage specification and proceed with T-cell differentiation (Cordes et al., 2022; Lind et al., 2001). During the first steps of mouse thymocyte development, these cells lack expression of CD4 and CD8 and are designated double-negative (DN) cells, which can be further subdivided in the populations DN1-to-4 based on CD44 and CD25 expression.

The DN1 population ($CD44^{+}$, $CD25^{-}$, $CD4^{-}$, $CD8^{-}$) is constituted by a heterogeneous group of cells that are still not committed to the T-cell lineage and can be further subdivided, based on CD127 and CD24 expression into DN1a-to-e (Cordes et al., 2022; Lee & Love, 2016;

Shah & Zúñiga-Pflücker, 2014). After differentiation into DN1 cells, expression of CD25 characterizes the progression to the DN2 stage ($CD44^+$, $CD25^+$, $CD4^-$, $CD8^-$), in which cells migrate to the cortex, expand by active proliferation, and partially lose potential to differentiate in other lineages (Lee & Love, 2016; Shah & Zúñiga-Pflücker, 2014).

RAG1- and RAG2-mediated rearrangements of T-cell receptor (TCR)β, TCRγ and TCRδ gene segments begin in the DN2 stage and are completed in the DN3 ($CD44^-$, $CD25^+$, $CD4^-$, $CD8^-$) stage (Capone et al., 1998; Shah & Zúñiga-Pflücker, 2014). Full T-cell lineage commitment occurs during DN2 to DN3 transition. In DN3 thymocytes, successfully rearranged TCRβ assemble with the invariant pre-TCRα and CD3 proteins constituting the pre-TCR signaling complex, which together with Notch signals, mediates T-cell development progression from DN3 stage through the β-selection checkpoint, and provides thymocyte survival and proliferation stimuli (Kashiwagi et al., 2022; Lee & Love, 2016; Liu et al., 2021; Shah & Zúñiga-Pflücker, 2014). Of note, prior to the DN3 stage, cortical thymic epithelial cells (cTECs) provide NOTCH ligands and IL-7 sustaining T-cell development (Balciunaite et al., 2005; Kashiwagi et al., 2022). At this stage, alternative expression of γδ TCR resulting from γ and δ chain rearrangements generate γδ T-cells that diverge from the more common αβ T-cell lineage and are involved in innate-like immune responses. These cells go through γδ-selection, a process equivalent to β-selection in αβ T-cells (Ciofani & Zúñiga-Pflücker, 2010).

While expression of the CXCR4, CCR7 and CCR9 chemokine receptors is important for migration of DN3 thymocytes to the subcapsular region (Benz et al., 2004; Misslitz et al., 2004; Plotkin et al., 2003; Trampont et al., 2010), the pre-TCR, CXCR4 and Notch signals drive DN3 active proliferation (Maillard et al., 2006; Trampont et al., 2010), downregulation of CD25 expression, and subsequent progression to the DN4 ($CD44^-$, $CD25^-$, $CD4^-$, $CD8^-$) stage (Lee & Love, 2016; Shah & Zúñiga-Pflücker, 2014). DN4 cells then upregulate CD4 and CD8 expression, generating double positive (DP) cells. The process of differentiation from DN4 to DP cells includes a transitional immature CD8 single-positive (ISP) stage, where rearrangements of the TCRα locus begin (Lee & Love, 2016; Rodríguez-Caparrós et al., 2020; Shah & Zúñiga-Pflücker, 2014).

DP cells express surface αβTCR and are highly motile in the cortex, where their TCRs undergo a selection process (positive selection) by interacting with self-peptides bound to major histocompatibility complex (MHC) molecules expressed in thymic stromal cells (cTECs and DCs). Successful interactions of TCR and TCR-associated co-receptors CD8

or CD4 with MHC class I or MHC class II molecules respectively, drive the maturation of DP thymocytes into CD4 or CD8 single-positive (SP) cells (Takahama, 2006). TCR interactions with MHC molecules in DP thymocytes also result in overexpression of CCR7 and subsequent chemotaxis towards the medulla, where CCR7 ligands (chemokine (C-C motif) ligand 19 (CCL19) and 21 (CCL21)) are produced by medullary thymic epithelial cells (mTECs) (Takahama, 2006; Ueno et al., 2004).

Once in the thymic medulla, positively selected thymocytes express tumor necrosis factor (TNF) family ligands such as CD40 ligand (CD40L), receptor activator of nuclear factor-kappa B ligand (RANKL) and lymphotoxin $\alpha\beta$ (LT$\alpha\beta$), stimulating mTECs maturation by binding to the CD40, RANK and LTβR receptors and activating nuclear factor-kappa B (NF-κB)-induced signaling (Akiyama et al., 2008; Boehm et al., 2003; Hikosaka et al., 2008; Irla et al., 2008). Here, positively selected thymocytes mature and go through a second round of selection (negative selection) that eliminates self-reactive thymocytes. The process of TCR selection through positive and negative selection will be further discussed in the next section.

During thymocyte maturation, SP cells upregulate G-protein-coupled receptors such as sphingosine-1-phosphate receptor 1 (S1P1), which are crucial for thymocyte egress (Allende et al., 2004; Matloubian et al., 2004). In addition, interleukin-4 receptor alpha (IL-4Rα) and CCR7 expressed on SP cells were also shown as important mediators of the thymic exiting (Ueno et al., 2002; White et al., 2017). At this stage, mature thymocytes exit the thymus through perivascular spaces and blood vessels and are able to reach lymphoid and non-lymphoid organs (Takahama, 2006).

1.4. Thymic selection

Successful interactions between TCR and self-peptide MHC (self-pMHC) ligands are pivotal for T-cell development. In fact, the strength of these interactions dictates thymocyte fate. According to the TCR affinity model for thymocyte selection, weak TCR interactions with self-pMHC presented by thymic antigen-presenting cells (APCs) result in survival stimuli and promote thymocyte positive selection, while strong interactions result in cell death by negative selection (Alam et al., 1996; Hogquist et al., 1994; Klein et al., 2014). Interestingly, it has been demonstrated that small changes in the strength of TCR-pMHC interaction impact positive versus negative selection, indicating a narrow window for positive selection of thymocytes, and that developing thymocytes are more sensitive to TCR stimuli than

mature T cells (Daniels et al., 2006; Davey et al., 1998). Of note, in the thymic cortex, the great majority (about 90%) of DP thymocytes express TCRs that are unable to establish proper interactions with pMHC molecules, resulting in thymocyte elimination through a process named 'death by neglect' (Klein et al., 2014; Merkenschlager et al., 1997).

Immature thymocytes undergo positive selection in the cortex, while negative selection and subsequent thymocyte maturation occurs in the thymic medulla, aided by specialized thymic epithelial cells that play a central role in each of these steps: cTECs and mTECs, respectively. cTECs are key mediators in the positive selection step as they express specialized machinery for antigen-processing: β5t, a unique proteasome catalytic subunit, important for MHC-I-mediated selection of CD8 SP thymocytes (Murata et al., 2007), and the thymus-specific serine proteases, cathepsin L and thymus-specific serine protease (TSSP), involved in MHC-II-mediated selection of CD4 SP thymocytes (Gommeaux et al., 2009; Nakagawa et al., 1998). Absence of either β5t or cathepsin L/TSSP severely impacts positive selection of CD8 and CD4 SP thymocytes, respectively (Gommeaux et al., 2009; Murata et al., 2007; Nakagawa et al., 1998).

In theory, positive selecting- TCR-self-pMHC interactions imprint a degree of self-reactivity in developing T-cells. Therefore, tuning the TCR activation threshold is an important mechanism to limit unwanted TCR activation. In that sense, another important mediator of the positive selection process is CD5, a negative regulator of TCR signaling expressed at the membrane of thymocytes (Azzam et al., 2001; Grossman & Paul, 2001). CD5 expression levels in thymocytes are set during positive selection, and maintained in mature T-cell, correlating with the intensity of TCR interactions with self-pMHC experienced during thymic selection (Azzam et al., 1998, 2001; Tarakhovsky et al., 1995). Thus, $CD5^{low}$ mature T-cells are less responsive to cytokines in homeostatic conditions and show lower basal TCR phosphorylation than $CD5^{high}$ counterparts (Mandl et al., 2013). Different CD5 levels have also been associated with differences in T-cell survival and function in response to antigenic stimulation or pathogens (Fulton et al., 2015; Matson et al., 2020; Persaud et al., 2014). These observations suggest that upregulation of CD5 during positive selection modulates TCR sensitivity to self-pMHC interactions and allows cells with higher antigen affinity to avoid too strong TCR signaling and thus evade negative selection. This hypothesis is supported by the observed deficient positive and negative selection seen in mice with CD5 deletion or overexpression, respectively (Azzam et al., 2001; Cho & Sprent, 2018; Tarakhovsky et al., 1995).

In the medulla, SP thymocytes undergo another round of selection that eliminates T-cells with autoreactive TCRs in a process named 'negative selection' and mediated by mTECs and DCs. A particular feature of mTECs is their ability to promiscuously express proteins that are expressed in other tissues, designated tissue-restricted antigens (TRAs), important for elimination of autoreactive TCRs (Derbinski et al., 2001). In turn, DCs can also mediate negative selection through transfer of TRAs from mTECs to DCs (Hubert et al., 2011). Key mediators of this process are the transcription factors Fezf2 and autoimmune regulator (Aire), expressed in mTECs but not cTECs (Derbinski et al., 2005; Eldershaw et al., 2011; Takaba et al., 2015). In fact, Aire deficiency was shown not only to result in disruption of tolerance, through abnormal T-cell selection and consecutive autoimmunity in humans and mice (Anderson et al., 2002; Meyer et al., 2016), but also in impairment of mTEC maturation and production of CCR7 ligands that mediate thymocyte migration to the thymic medulla (Laan et al., 2009).

A subset of autoreactive CD4 T-cells escapes negative selection and differentiates into regulatory T-cells (Treg), characterized by Foxp3 expression. Tregs are central players in regulating tolerance and immune responses in the periphery, as attested by the severe autoimmunity developing when these cells are absent (Savage et al., 2020).

The molecular mechanisms through each T-cells discriminate between low- or high- affinity TCR-pMHC interactions that induce positive or negative selection are still not fully elucidated. It has been proposed that high- and low- affinity stimuli might trigger qualitatively different responses, influencing positive or negative selection, rather than triggering the same signals in different quantities, in which case a positive/negative selection threshold would dictate thymocyte fate. In line with the "threshold signaling" hypothesis, it was shown that low-affinity TCR-pMHC interactions induce partial CD3 phosphorylation and positive selection, while high-affinity interactions trigger signal transduction and result in negative selection (Palmer & Naeher, 2009; Reis e Sousa et al., 1996; Teague et al., 2008) (Figure 1).

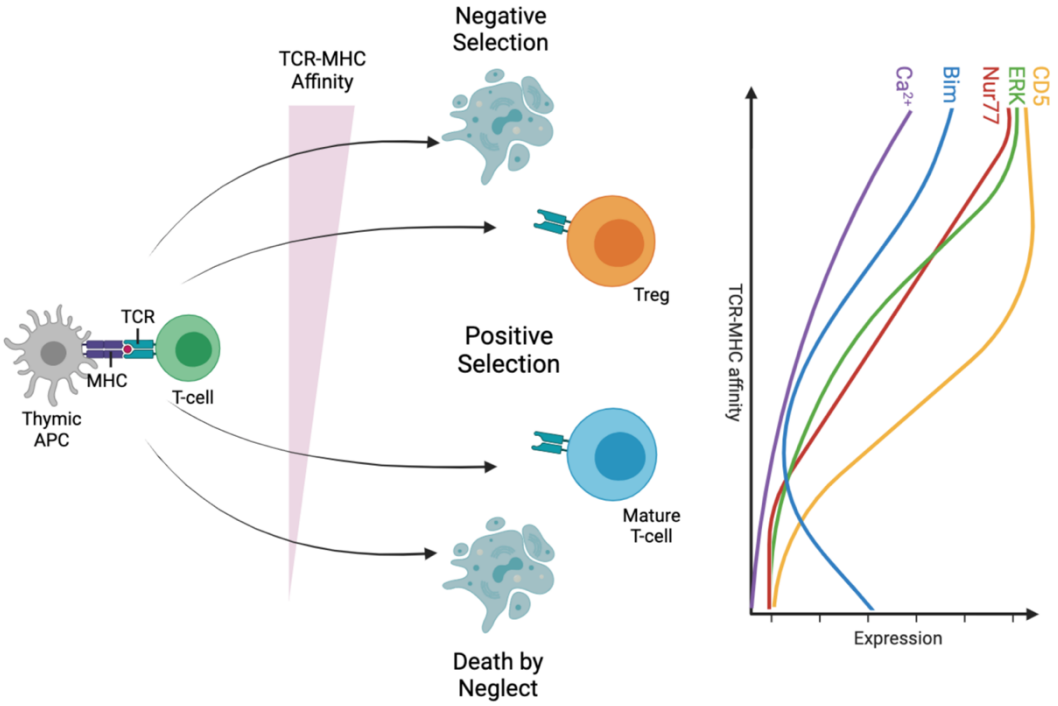


Figure 1. Fate of developing T-cells according to affinity of TCR-MHC interactions. In the thymus, successful interactions between TCR and MHC molecules presented by thymic APCs are critical for T-cell survival (positive selection). T-cells that fail to establish interactions with MHC lack survival stimuli and undergo apoptosis (death by neglect). High-affinity TCR-MHC interactions in turn lead to differentiation into Treg cells or to TCR-mediated apoptosis (negative selection). During this process, expression of key molecules regulates T-cell fate and are associated with the different outcomes.

Regarding downstream signals, although calcium influx might be quantitatively associated to positive versus negative selection (Kim et al., 2021; Mariathasan et al., 1998), negative selection signals induce fast and robust activation of extracellular-signal-regulated (ERK) kinase, while positively selecting stimuli induce lower but sustained ERK activation (McNeil et al., 2005) (Figure 1).

Furthermore, Nur77, a NR4A family protein, and Bim are considered the main effectors of thymocyte deletion during negative selection and are consistently upregulated during this process (Xing & Hogquist, 2012). Nur77 can act as a transcription factor and expression levels correlate with antigen-specific signaling intensity (Ashouri & Weiss, 2017), and NR4A family proteins have been associated with Treg function and regulation of tolerance (Hiwa et al., 2021). In addition, Nur77 in particular, interacts with Bcl-2 to expose the Bcl-2 pro-apoptotic domain BH3 in the mitochondria and consequently lead to thymocyte deletion (J. Thompson & Winoto, 2008). In turn, the pro-apoptotic molecule Bim was shown to mediate

autoreactive T-cell deletion (negative selection) in response to superantigen, TRAs or ubiquitous self-antigens (Bouillet et al., 2002; Hu et al., 2009; Kovalovsky et al., 2010; Moran et al., 2011; Villunger et al., 2004; Xing & Hogquist, 2012). Of note, Bim was shown to be downregulated during positive selection (J. Wang et al., 2017). Tolerance was impaired in Bim-deficient mice (Bouillet et al., 2002; Moran et al., 2011; Villunger et al., 2004) (Figure 1).

Interestingly, a recent study reported that TCR stimulation of mature splenocytes with varying concentrations of exogenous agonist peptide and for varying periods of time, resulted in the activation of distinct transcriptional programs and that upregulation of specific TCR inhibitory proteins might be time- and peptide concentration-sensitive (Elliot et al., 2021). In addition to fast upregulation of Nur77 and CD69, high antigen concentration led to the upregulation of TCR co-inhibitory molecules such as PD1, CTLA4, Lag3 and Tigit (Elliot et al., 2021). Despite providing convincing evidence that varying TCR signaling might lead to the induction of specific TCR signaling programs, whether and how these target proteins are involved in the integration of quantitatively and qualitatively different TCR stimuli in developing thymocytes as well as their impact in positive or negative selection remains to be clarified.

2. Acute Lymphoblastic Leukemia

According to the World Health Organization (WHO), hematological malignancies comprise a heterogeneous group of neoplasms originated from hematopoietic and lymphoid tissues that can be classified considering morphology, immunophenotype, genetic and molecular signatures and clinical presentation. These malignancies can be subdivided in different categories according to criteria that include cell lineage (lymphoid or myeloid), differentiation (immature or mature), and disease progression (acute or chronic) (Campo et al., 2011).

Acute lymphoblastic leukemia (ALL) refers to a group of hematological malignancies characterized by the proliferation of immature lymphoblasts that can be further subdivided in two categories according to their origin: precursor B-cell ALL/lymphoma (B-ALL/B-LBL) or precursor T-cell ALL/lymphoma (T-ALL/T-LBL) (Onciu, 2009).

2.1. Epidemiology and clinical presentation

Most ALL cases occur in children (about 80%), representing the most frequent pediatric malignancy (Terwilliger & Abdul-Hay, 2017). Although less frequent, adult ALL is particularly concerning since it is associated with disease aggressiveness and resistance to therapy (Onciu, 2009; Terwilliger & Abdul-Hay, 2017). ALL is more frequent in males than females, and T-ALL is less frequent than B-ALL representing 10-15% of childhood ALL cases and approximately 25% of adult ALL cases (Onciu, 2009; You et al., 2015).

Risk factors for ALL development include genetic susceptibility and environmental factors. Congenital syndromes, such as Down's syndrome, ataxia telangiectasia or Bloom syndrome, inherited gene variants in *IKZF1*, *CDKN2A*, *CDKN2B*, *ETV6* and other genes, Robertsonian translocation between chromosomes 15 and 21, rob(15;21)(q10;q10), and single nucleotide polymorphisms, have all been found to influence the risk for ALL development. Although these alterations have shown an additive effect in disease predisposition, alone they rarely result in ALL, indicating a polygenic basis for disease development (Karrman & Johansson, 2017; Malard & Mohty, 2020). Environmental factors associated with ALL include pesticide exposure, ionizing radiation and childhood infections (Karrman et al., 2009; Malard & Mohty, 2020; Wakeford, 2008).

ALL is frequently detected in peripheral blood and bone marrow but its presence in extramedullary sites is frequent (Terwilliger & Abdul-Hay, 2017). In fact, clinical presentation of ALL results from the accumulation of malignant immature lymphoblasts in these organs and include hyperleukocytosis (characterized by high white blood cell (WBC) count) and signs of BM failure, associated with symptoms such as fever, lethargy, dyspnea, recurrent infections, frequent bleeding and bone pain (Karrman & Johansson, 2017; Terwilliger & Abdul-Hay, 2017). Accumulation of ALL lymphoblasts in extramedullary sites is common and can cause lymphadenopathy, splenomegaly and hepatomegaly (Terwilliger & Abdul-Hay, 2017). Central nervous system (CNS) involvement is found in about 5% of adult ALL patients at diagnosis and can be asymptomatic or cause neurological symptoms such as headache, seizures, nausea and paresthesias (del Principe et al., 2014). Particularly in T-ALL, a mediastinal mass can be found (in approximately 60% of the cases), consequence of accumulation of malignant lymphoblasts in the thymus, that can cause dyspnea, headache, and nausea (Karrman & Johansson, 2017; Terwilliger & Abdul-Hay, 2017).

2.2. Immunophenotype

Since B-ALL and T-ALL cells are morphologically indistinguishable, identification of ALL lineages requires immunophenotypic analysis using cell surface or intracellular antigens.

B cell-specific antigens are frequently found in B-ALL, including PAX5, CD19, CD22 and CD79a. CD10, a marker expressed by normal B cell progenitors, is frequently found in B-ALL. Also, most B-ALL show dim expression of CD45 (common leukocyte antigen) and often express CD34 and terminal deoxynucleotidyl transferase (TdT), early-stage B cell precursor markers (Bene et al., 1995; Onciu, 2009). According to the European Group for the Immunologic classification of Leukemia (EGIL), B-ALL can be classified according to differentiation markers and the pattern of Ig chain expression: early Pre-B-ALL or Pro-B-ALL lack Ig expression; Pre-B-ALL express cytoplasmic μ chains but not light chain; and transitional Pre-B-ALL express surface μ chain without light chain expression (Bene et al., 1995; Chiaretti et al., 2014; Onciu, 2009). B-ALL subtypes and characteristic antigen expression are summarized in Table 1.

T-ALL cells are characterized by the expression of T-cell lineage markers, particularly CD3 expression in the cytoplasm or on cell surface (Karrman & Johansson, 2017). CD3 associates with TCRs on the membrane and this complex is necessary for T-cell survival in the thymus. TdT is commonly expressed in T-ALL and the expression of other T-cell antigens such as CD2, CD5, CD7 and CD8 is variable in T-ALL (Karrman & Johansson, 2017; Onciu, 2009). Taking in consideration the expression of these differentiation markers, EGIL subclassifies T-ALL in Pro-T-ALL, Pre-T-ALL, cortical-T-ALL and mature-T-ALL (Bene et al., 1995; Chiaretti et al., 2014; Karrman & Johansson, 2017) as listed in Table 1. Of note, both B-ALL and T-ALL may express CD11b, CD13, CD15 and CD33, common myeloid marker, in particular subtypes (Chiaretti et al., 2014; Karrman & Johansson, 2017). An additional infiltrative subtype of T-ALL with phenotypic resemblance to early T-cell progenitors (ETP) has been identified as a very high-risk subtype in terms of aggressiveness and poor prognosis, designated ETP-ALL (Coustan-Smith et al., 2009). ETP-ALL expresses CD3 in the cytoplasm as well as CD7, being considered part of the pro-T-ALL subtype, and represents 5 to 10% of T-ALL cases (Coustan-Smith et al., 2009; Karrman & Johansson, 2017; You et al., 2015).

ALL subtypes	**Differentiation markers**
B lineage ALL	**CD19$^+$ and/or CD79a$^+$ and/or CD22**
Pro-B-ALL (B-I)	-
Common B-ALL (B-II)	CD10$^+$
Pre-B-ALL (B-III)	IgM$^+$ cytoplasmic
Transitional B-ALL (B-IV)	Ig kappa or lambda cytoplasmic or surface
T cell lineage ALL	**CD3$^+$ cytoplasmic or surface**
ETP-T-ALL (T-I)	CD7$^+$, stem cell or myeloid markers
Pro-T-ALL (T-I)	CD7$^+$
Pre-T-ALL (T-II)	CD2$^+$ and/or CD5$^+$ and/or CD8$^+$
Cortical-T-ALL (T-III)	CD1a$^+$
Mature T-ALL (T-IV)	CD3$^+$, CD1a$^-$
α/β T ALL (group a)	TCR α/β^+
γ/δ T ALL (group b)	TCR γ/δ^+

Table 1. Immunological ALL classification based on the European Group of the Immunological Characterization of Leukemias (EGIL) (Bene et al., 1995; Chiaretti et al., 2014; Coustan-Smith et al., 2009; Onciu, 2009; You et al., 2015).

2.3. Genetic alterations in B-ALL

Genetic alterations are common in ALL and comprise structural chromosomal alterations, alterations of DNA copy number and sequence mutations that usually lead to the deregulation of oncogenes or expression of chimeric fusion genes. Importantly, several key genetic alterations are pathognomic for distinct subtypes of ALL, being important for diagnosis, prognosis, and in some cases for targeted therapy. The frequency of particular genetic alterations occurs variably in childhood and adult ALL. However, the mechanisms underlying ALL development are thought to be similar (Iacobucci & Mullighan, 2017; Pui et al., 2004). This subject has been extensively reviewed by Iacobucci and Mullighan (Iacobucci & Mullighan, 2017) and Girardi and colleagues (Girardi et al., 2017).

In B-ALL, cytogenetic alterations are common and have been used to distinguish B-ALL subtypes with implications in prognosis. High hyperdiploidy B-ALL (characterized by the gain of at least 5 chromosomes) with *ETV6::RUNX1* fusion is frequent in childhood ALLs, but accounts for less than 3% in adults and is associated with good prognosis. Rearrangements of *KMT2A* (MLL), most frequently those caused by t(4;11)(q21;q23)

translocations but also those resulting from the fusion of 11q23 with more than 80 partner genes (Winters & Bernt, 2017), are frequent in infants and associated with aggressive behaviour and poor outcome (el Chaer et al., 2020; Iacobucci & Mullighan, 2017; Pui et al., 2004). *BCR::ABL1*-positive (Philadelphia (Ph) chromosome) B-ALL is more frequent in adult than childhood ALL (representing 25% versus 2-5% of ALL cases respectively). Although this subgroup was initially associated with poor prognosis, treatment with tyrosine kinase inhibitors (TKIs) drastically improved patient survival. Secondary alterations, such as deletions of IKAROS family Zinc Finger 1 (*IKZF1*) and *PAX5*, regulators of B-cell development, and the Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and *CDKN2B* cell cycle regulators, are frequently found in this subgroup (Iacobucci & Mullighan, 2017; Pui et al., 2004). Translocations involving TCF3, particularly translocation t(1;19)(q23;p13), which generates a *TCF3::PBX1* gene fusion, are generally associated with favorable outcome after standard chemotherapies. This subgroup represents approximately 5% of B-ALL cases in both children and adults (Burmeister et al., 2010; Iacobucci & Mullighan, 2017; Jia et al., 2021; Kager et al., 2007). Other genetic alterations occurring in B-ALL include intrachromosomal amplification of chromosome 21 (iAMP21) (Iacobucci & Mullighan, 2017; Moorman et al., 2013) and hypodiploidy (less than 44 chromosomes). This group encompasses subtypes with different molecular profiles and genetic alterations, and includes cases with Ras-activating mutations and IKFZ3 alterations or IKZF2 alterations and TP53 mutations (Holmfeldt et al., 2013; Iacobucci & Mullighan, 2017).

Secondary genetic alterations are common in B-ALL and are thought to be acquired and selected for during leukemia progression. These alterations include lymphoid transcription factors (*IKZF1*, *PAX5* and *EBF1*), cell-cycle regulators (*CDKN2A*, *CDKN2B* and *RB1*), genes involved in transcription regulation and coactivation (*ETV6* and *ERG*), as well as epigenetic alterations (Mullighan et al., 2007).

Although the genetic landscape of ALL has been extensively studied, a considerable proportion of childhood and particularly adult B-ALL cases lack cytogenetically detectable chromosomal alterations. More recently, characterization of B-ALL transcriptomic profiles led to identification of new subtypes of B-ALL. One such subtype is the BCR::ABL1-like or Ph-like B-ALL, which harbors a similar gene expression profile to that of BCR::ABL1-positive B-ALL but lack the BCR::ABL1 fusion (Arber et al., 2016; Roberts, Li, et al., 2014). The frequency of Ph-like B-ALL increases with age (from 10% of ALL cases in children to 25% in adults) and is associated with high-risk clinical features and poor prognosis (Roberts, Pei, et al., 2014). Recurring genetic alterations in Ph-like ALL include *IKZF1* deletions, rearrangements or mutations of the cytokine receptor *CRLF2*, rearrangements of

ABL-class tyrosine kinase genes, rearrangements of Janus kinase 2 (JAK2) or JAK/STAT signaling-activating mutations, Ras signaling mutations, as well as *CDKN2A* and *PAX5* deletions (Downes et al., 2022; Iacobucci & Mullighan, 2017; Roberts et al., 2018; Roberts, Pei, et al., 2014). Further reported B-ALL subtypes are the DUX4-rearranged ALL with common deregulation of the transcription factor *ERG* (Yasuda et al., 2016; Zhang et al., 2016), and *MEF2D*- and *ZNF384*- rearranged ALL (Gu et al., 2016; Suzuki et al., 2016).

In a 2019 study, Gu and colleagues described additional B-ALL subtypes including *PAX5*-altered (*PAX5*alt), characterized by *PAX5* rearrangements, sequence mutations and intragenic amplifications; *PAX5* P80R B-ALL subtype, characterized by a biallelic *PAX5* p.Pro80Arg point mutation in PAX5 that leads to activation of Ras and JAK/STAT signaling pathways; and the *IKZF1* N159Y, defined by a heterozygous *IKZF1* p.Asn159Tyr missense mutation, and characterized by a distinct gene expression profile (Gu et al., 2019). Both *PAX5*-alt and *PAX5* P80R were associated with intermediate outcomes (Gu et al., 2019). The IKZF1 N159Y mutation is thought to act as a B-ALL initiating rather than secondary event, as is the case in other B-ALL subtypes. Of note, the BCL2/MYC B-ALL subtype, characterized by concurrent BCL2 and MYC translocations or other abnormalities, has been identified and associated with a very poor outcome and with uniformly early treatment failure (Cucco et al., 2020; Gu et al., 2019; Johnson et al., 2009; Li et al., 2015).

2.4. Genetic alterations in T-ALL

As is the case for B-ALL, major T-ALL subtypes are defined by the presence of specific chromosomal alterations, which in this case lead to the ectopic expression of particular transcription factors. The most frequent chromosomal alterations in T-ALL are rearrangements of TCR loci, frequently recombining with homeobox transcription factor genes, such as LIM-domain-only (LMO) and class II basic helix-loop-helix (bHLH). These rearrangements juxtapose the TCR promoters and enhancer with partner genes and result in aberrant gene expression (Campana et al., 1991; Furley et al., 1986; Girardi et al., 2017; Karrman & Johansson, 2017). Moreover, T-ALL subgroups can be defined by the unique gene expression signatures associated with overexpression of particular genes, such as *TAL1*, *TLX1*, *TLX3*, *HOXA*, *NKX2-1*, *LMO1/2* and *LYL1* (Ferrando et al., 2002; Girardi et al., 2017; van Vlierberghe et al., 2008). Five main T-ALL subgroups can be transcriptomically defined: TAL1, TLX, HOXA, NKX2-1 and LMO2/LYL1.

Although T-ALL rarely presents a complex karyotype (most often only one or a few structural chromosomal abnormalities), genomic and cytogenetic analyses detected more than 70

fusion genes in T-ALL, resulting most often in transcription factor and tyrosine kinase activation (Karrman & Johansson, 2017). Genes frequently involved in gene fusions found in T-ALL include the transcription factor *ETV6*, chromatin regulator *KMT2A*, and tyrosine kinase *ABL1* (Karrman & Johansson, 2017).

Genetic analysis of pediatric T-ALL showed frequent copy number alterations (CNAs) and uniparental isodisomies, particularly in *CDKN2A* (Karrman et al., 2015). Gene deletions are the most common CNA in T-ALL and usually target tumor suppressor genes such as *CDKN2A* (deleted in over 70% of T-ALL cases), often cooccurring with *CDKN2B* deletions (Girardi et al., 2017; Karrman et al., 2015). Other gene deletions found in T-ALL include the *PTEN* phosphatase (Gutierrez et al., 2009), the *WT1* transcription factor (Tosello et al., 2009), the *RB1* cell cycle regulator (van Vlierberghe et al., 2013), the *BCL11B* (Gutierrez et al., 2011) and *LEF1* (Gutierrez et al., 2010) transcription factors, the tyrosine phosphatase *PTPN2* (Kleppe et al., 2010), and the epigenetic regulators *EZH2* (Ntziachristos et al., 2012) and *PHF6* (van Vlierberghe et al., 2010). Besides resulting in loss of gene function, deletions can alternatively lead to gene expression. Examples of this are the overexpression of *TAL1*, consequence of promoter swapping (Ferrando et al., 2002; Karrman & Johansson, 2017), or of *LMO2,* caused by cryptic deletions that lead to loss of negative regulatory elements (Karrman & Johansson, 2017; van Vlierberghe et al., 2006). Additionally, *MYB* locus tandem duplications have been identified in approximately 10% of T-ALL cases (Clappier et al., 2007).

2.4.1. Mutations and altered signaling pathways in T-ALL

Many genes have been shown to be mutated in T-ALL, although most at relatively low frequencies (Belver & Ferrando, 2016). A notable exception are activating *NOTCH1* mutations, which are particularly frequent in T-ALL, found in more than 50% of the cases (Karrman & Johansson, 2017; Weng et al., 2004), and often associated with disease progression (Albertí-Servera et al., 2021). NOTCH1 is a transmembrane heterodimeric receptor crucial for T-cell development and differentiation (Deftos & Bevan, 2000). Upon extracellular domain interaction with ligands such as Delta-like 1, 3 and 4 or Jagged 1 and 2, NOTCH1 is cleaved by gamma-secretases, resulting in the translocation of its intracellular subunit (ICN1) to the nucleus, where it forms a transcriptional activation complex with DNA-binding proteins. Through this mechanism, NOTCH1, which does not possess a DNA-binding domain, regulates the expression of genes involved in proliferation, cell cycle and metabolism (Deftos & Bevan, 2000; Karrman & Johansson, 2017). In T-ALL, NOTCH1 modulates the expression of genes such as *MYC*, *HES1* and *IL7RA* leading to

activation of the PI3K-AKT-mTOR pathway and proliferation (Paganin & Ferrando, 2011), and promotes T-ALL survival by modulation of NF-κB-related genes (dos Santos et al., 2010) (Figure 2).

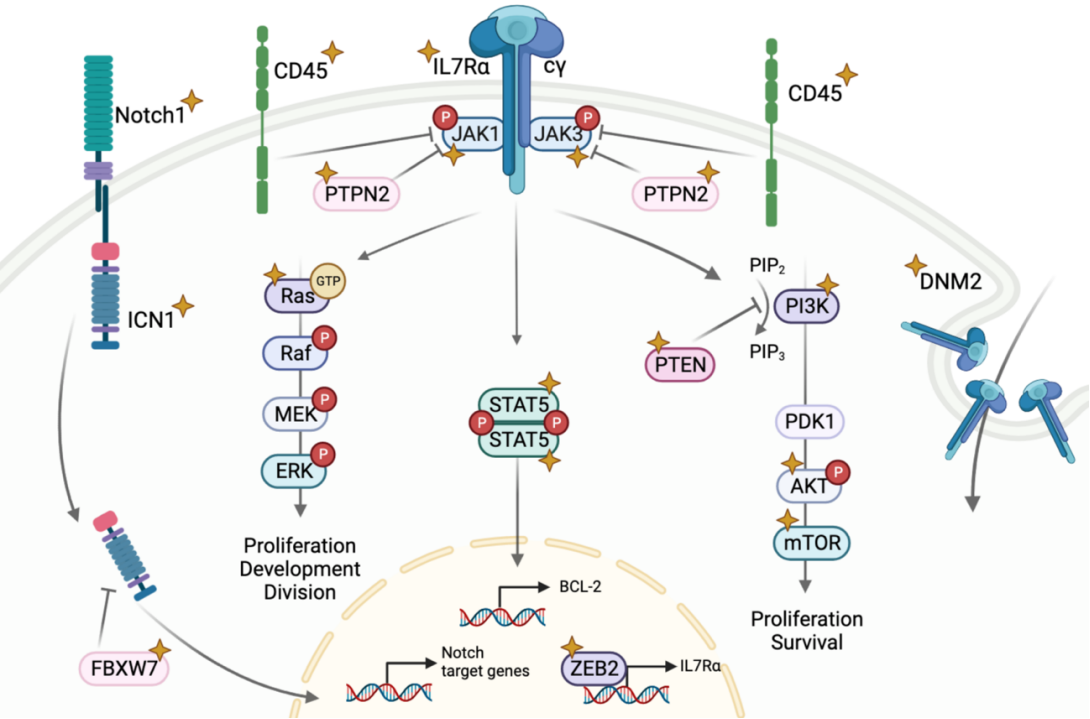


Figure 2. Signaling pathways commonly altered in T-ALL. Representation of recurrent oncogenic events in T-ALL leukemogenesis. Activation of Notch1 leads to cleavage and subsequent release of the intracellular domain ICN1 followed by ICN1 nuclear translocation where it promotes transcription of Notch1 target genes. FBXW7 inhibits this process by inducingf Notch1 degradation. Upon binding of IL-7, IL-7R promotes JAK1 and JAK3 phosphorylation and STAT5 recruitment. After STAT5 activation and dimerization it translocates to the nucleus promoting BCL-2 transcription. IL-7R activation can also lead to RAS/MAPK pathway activation through recruitment and activation of Ras and subsequent activation of Raf, MEK and ERK which is translocated to the nucleus and mediates proliferation, development and cell division. Additionally, IL-7R activation can induce PI3K/AKT signaling by activation of PI3K and subsequent engagement of PDK1 and AKT, activating mTOR and resulting in survival and proliferation stimuli. PTEN inhibits PI3K activation. Overexpression of ZEB2, which promotes IL-7Rα transcription, or impaired IL-7R regulation by phosphatases CD45 and PTPN2 or by DNM2-mediated receptor internalization also results in increased IL-7R expression and signaling. ✦ mutated in T-ALL. Adapted from (Girardi et al., 2017).

NOTCH1 is degraded by the proteasome after PEST domain phosphorylation and recruitment of the ubiquitin ligase FBXW7 (Karrman & Johansson, 2017). Most NOTCH1 mutations in T-ALL are found in the heterodimerization domain (HD; approximately 40%), where the extracellular and the membrane/intracellular units interact, or in the PEST domain (about 15%). HD mutations result in ligand-independent NOTCH1 activation while PEST

mutations impair NOTCH1 degradation. NOTCH1 degradation can also be impaired by *FBXW7*-inactivating mutations, found in 15% of T-ALL cases (Ferrando et al., 2002; Karrman & Johansson, 2017). Of note, dual NOTCH1 mutations, involving HD and PEST or HD and FBXW7, are reported in 20% of T-ALL cases and are thought to synergistically increase NOTCH1 signaling (Paganin & Ferrando, 2011; B. J. Thompson et al., 2007).

Mutations in the Interleukin-7 receptor (IL-7R)/JAK/STAT pathway are also common in T-ALL, occurring in 20-30% of T-ALL cases, and include activating mutations in *IL7R*, *JAK1, JAK3* and/or *STAT5* (Girardi et al., 2017; Vicente et al., 2015). IL-7 signaling, activated by interaction of the heterodimeric IL-7R with its ligand, is crucial for T-cell development (Hong et al., 2012). This interaction leads to the reciprocal phosphorylation of JAK1 and JAK3, and subsequent recruitment and activation of STAT5 and phosphoinositide 3 kinases (PI3Ks) (Hong et al., 2012). Phosphorylation of STAT5 in particular leads to homodimerization and translocation to the nucleus, with consequent transcription induction of target genes, which include pro-survival BCL-2 protein family members (Hong et al., 2012). In T-ALL, IL-7-mediated STAT5 activation is also reported to upregulate expression of *PIM1*, an oncogenic cell cycle regulator that also promotes T-ALL cell survival (Ribeiro et al., 2018). Of note, *IL7R*, *JAK1/3* and/or *STAT5*-activating mutations are particularly represented in TLX and HOXA T-ALL subgroups (Zenatti et al., 2011). Additionally, deletion of *PTPN2* and mutation of *PTPRC* (encoding the CD45 protein), both phosphatases that regulate JAK kinases, have also been found in a small percentage of T-ALL cases (Kleppe et al., 2010; Porcu et al., 2012).

In vivo, IL-7 levels are considered to be constant in steady-state conditions, indicating that IL-7 signaling at the cellular level is controlled by the expression of IL-7R (Hong et al., 2012). In fact, overexpression of wild-type (WT) IL-7Rα was shown to be sufficient to induce mouse T-ALL (Silva et al., 2021). The IL-7R/JAK/STAT signaling pathway can be hyperactivated in patients with no mutations in *IL7R*, *JAK* or *STAT5* genes, indicating it can be activated by alternative mechanisms. That is the case of *DNM2* loss-of-function mutations, which lead to increased levels of IL-7R by impairment of receptor endocytosis (C. S. Tremblay et al., 2016), ZEB2 transcription factor overexpression, which induces in IL-7R expression (Goossens et al., 2015), and gene fusions involving ETV6 (also designated TEL) and *JAK2* (Figure 2). Although found in T-ALL with low frequency (<1%) (Lacronique et al., 1997), ETV6::JAK2 fusion proteins were shown to activate STAT5 and to be sufficient to induce T-ALL development in mice (Carron et al., 2000).

Furthermore, PI3K/AKT, RAS/mitogen-activated protein kinase (MAPK) and NF-κB pathways are also commonly found activated in T-ALL (Bongiovanni et al., 2017; dos Santos et al., 2010). Hyperactivation of the PI3K/AKT pathway leads to alterations in cell metabolism, proliferation and apoptosis. In T-ALL, the main PI3K/AKT pathway alterations is inactivation of the PTEN negative regulator, mainly by inactivating mutations or deletions (Girardi et al., 2017; Mendes et al., 2014; Silva et al., 2008; Zuurbier et al., 2012). However, mutations in the AKT and mTOR downstream effectors of the pathway have been also found (Girardi et al., 2017; Gutierrez et al., 2009). In addition, mutations in the oncogene *RAS* and/or in the tumor suppressor *NF1* found in T-ALL patients result in RAS/MAPK hyperactivation (Bongiovanni et al., 2017) (Figure 2). Regarding the NF-κB pathway, both canonical and noncanonical pathways were found to be activated in human and mouse T-ALL. Mutations in members of the pathway are rare in T-ALL, indicating that NF-κB activation is caused by other factors (dos Santos et al., 2008; Espinosa et al., 2010; Kordes et al., 2000; Vilimas et al., 2007). In T-ALL, Notch1 is the main activator of the NF-κB pathway, fundamental for maintenance of Notch1-induced mouse T-ALL (Espinosa et al., 2010; Vilimas et al., 2007).

Additionally, epigenetic modifications have been reported to contribute for leukemogenesis, with genetic alterations being identified in epigenetic regulators such as *PHF6*, *CREBBP* and *EP300* (Huether et al., 2014; Karrman & Johansson, 2017), as is the case for microRNAs targeting the expression of genes such as *PTEN*, *BIM*, *PHF6*, *NF1* and *FBXW7* (Mavrakis et al., 2011; Vicente et al., 2015).

2.5. ALL dissemination and CNS involvement

When a malignant lymphoblast clone emerges due to genetic or epigenetic alterations it usually proliferates uncontrollably and propagates through lymphoid and non-lymphoid organs, most frequently BM, peripheral blood, LNs, and CNS. Expansion and propagation of lymphoblasts ultimately compromises organ function, usually resulting in typical ALL symptoms. Most notably, accumulation of lymphoblasts in the BM impairs its hematopoietic function.

Current chemotherapy regimens are efficient at eliminating extramedullary ALL infiltration. Nonetheless, CNS infiltration by ALL lymphoblasts poses an additional problem, because the CNS is not easily targeted by systemic administration of most chemotherapeutic agents. In those cases, intrathecal chemotherapy is applied to patients. Although ALL is present in

the CNS in only 5-10% of cases at diagnosis, CNS infiltration is commonly found in ALL relapse and is associated with poor prognosis.

The CNS is protected by the meninges, an intricate three-layer membranous covering that lies between the brain and skull, where the outermost layer is the tough and fibrous *dura mater*, followed by the more delicate inner membranes *arachnoid mater* (the intermediate), and *pia mater* (the innermost layer), which together constitute the leptomeninges (Frishman-Levy & Izraeli, 2017; Morris et al., 2022). The space between *arachnoid* and *pia mater* is known as the leptomeningeal/subarachnoid space and is filled with cerebrospinal fluid (CSF), mainly produced by the choroid plexus. CSF fills the subarachnoid space from over the brain surface throughout the length of the spinal cord (Frishman-Levy & Izraeli, 2017; Morris et al., 2022).

ALL invasion of the CNS is mainly found in the meninges (*dura mater*, leptomeninges or both) or CSF, and meningeal infiltration can be diffuse or focal (Ginsberg & Leeds, 1995). Leukemic cells can be found in superficial arachnoid veins in the first steps of ALL CNS infiltration, and are thought to then invade surrounding tissues. Although not present in the CSF at first, when arachnoid trabeculae are destroyed by ALL invasion, leukemic cells are released into the CSF (Frishman-Levy & Izraeli, 2017). Brain parenchyma ALL invasion is rare and is thought to occur after pia mater destruction caused by ALL infiltration (Frishman-Levy & Izraeli, 2017). The process of CNS infiltration by leukemic cells is still unclear but two main hypothesis are proposed: CNS invasion via systemic circulation, supported by the presence of leukemic cells in the walls of arachnoid veins found in autopsy studies (Frishman-Levy & Izraeli, 2017); and CNS colonization via skull BM vasculature, aided by vascular leakiness induced by ALL proliferation and hypoxia in the BM (Frishman-Levy & Izraeli, 2017; Passaro et al., 2017) or possible skull bone destruction (Rajakumar et al., 2021).

Extravasation if one of the first steps leukemic cells go through in order to disseminate and invade other organs. Several molecules important for extravasation have been reported in T-ALL or T-ALL cell lines, including LFA-1 (Pyszniak et al., 1994; Tanaka et al., 1998), PSGL-1 (Pereira et al., 2021), CD44 (Cavalcanti Júnior et al., 1994) and CD43 (Caracciolo et al., 2021). In B-ALL, molecules associated with extravasation have not been extensively studied. Nevertheless, both CD44 (Bendall et al., 2003) and CD43 (Nonomura et al., 2008) are expressed in B-ALL lymphoblasts and are thought to play a similar role in facilitating B-ALL adhesion and extravasation.

Chemokines play a central role in ALL invasion and differences in the pattern of expression of chemokines and adhesion molecules might influence the migratory capacity of ALL cells. High expression of CXCR4 has been reported in T- and B-ALL and to be associated with ALL migration towards CXCL12 and infiltration of the spleen, liver and CNS (Arnaud et al., 2015; Crazzolara et al., 2001; Dürig et al., 2001; Jost et al., 2016). On the other hand, expression of adhesion molecules such as LFA-1 and ICAM-1 has been shown to mediate T-ALL cell survival induced by co-cultured BM cells (Winter et al., 2001). Similarly, CCR7 expression by T-ALL cells (Alsadeq et al., 2017), has been reported to promote ALL migration towards CCL19 and invasion of the CNS (Alsadeq et al., 2017; Buonamici et al., 2009).

Interestingly, additional molecules have been shown to be involved in ALL migration. That is the case for integrin α6, which has been shown to mediate B-ALL CNS colonization by interacting with laminin (Gang et al., 2020; Yao et al., 2018) and RANKL, thought to mediate B-ALL infiltration from the bone marrow to the CNS by bone destruction (Rajakumar et al., 2020, 2021). Of note, expression of other adhesion proteins is also found in ALL cells (Deak et al., 2021), as is the case of integrin β1 (Cacciola et al., 1997). Although Integrin β1 association with ALL invasion/migration has not been reported to date, its expression has been associated with extramedullary migration in acute myeloid leukemia (Pillozzi et al., 2007).

Moreover, besides being frequently altered in T- and B-ALL, activation of the JAK/STAT pathway by either Interleukin-15 (IL-15) or IL-7R, has also been described as a risk factor for B-ALL CNS involvement in preclinical models (Alsadeq et al., 2017; Williams et al., 2014). Of note, in one of these studies, JAK/STAT pathway activation through IL-15 induced the expression of the CXCR3 chemokine receptor concomitantly with CNS invasion (Williams et al., 2014). Although CXCL10, the CXCR3 ligand, was found in the CNS of B-ALL patients (Williams et al., 2014), whether or not CXCR3 plays a direct role mediating CNS infiltration remains unclear. Thus, detailed characterization of the landscape of chemokines and adhesion molecules expressed in ALL cells and the impact of specific molecules or signaling pathways in ALL migration and invasion is a topic that requires further investigation having the potential to impact ALL treatment by either targeting common mechanisms of extramedullary invasion or subtype-specific mediators.

2.6. ALL treatment

The standard measure for evaluation of response to treatment and prognosis in ALL is minimal residual disease (MRD). MRD can be assessed by multiparametric flow cytometry or molecular methods (quantitative PCR or next-generation sequencing) to detect leukemic cells at the end of each treatment cycle. Low MRD after first or second phase treatment is associated with favorable outcome, particularly in childhood ALL. Moreover, MRD is a valuable tool to detect early preclinical disease relapse (Malard & Mohty, 2020).

2.6.1. Standard treatment

Standard treatment regimens for ALL usually consists in the systemic administration of a combination of chemotherapy agents in four phases usually over the course of 2-3 years: induction, consolidation, intensification and maintenance (Malard & Mohty, 2020). In high-risk cases, prophylactic treatment is administered to prevent CNS relapse, as standard therapy does not reach the CNS (Pinnix et al., 2018).

Induction chemotherapy usually includes the administration of a glucocorticoid, vincristine, L-asparaginase and an anthracycline and the aim of this phase is to significantly decrease disease burden and restore normal hematopoiesis. The second step of standard treatment regimen, consolidation, consists of sequential short courses of chemotherapy, usually with a glucocorticoid, high-dose methotrexate, low-dose cytarabine and asparaginase, and is followed by the intensification phase. The intensification phase, also named reinduction therapy phase, is similar to the induction therapy phase (Malard & Mohty, 2020). Maintenance therapy is based on treatment with mercaptopurine and methotrexate, with or without combination with glucocorticoid and vincristine (Malard & Mohty, 2020). This therapeutic strategy contributes for the 90% 5-year overall survival in childhood ALL. Although not as efficient in adults, pediatric-inspired therapy regimen in adults has significantly improved 5-year survival rates (Gökbuget et al., 2013; Huguet et al., 2009; Malard & Mohty, 2020; Siegel, Advani, et al., 2018; Siegel, Stock, et al., 2018). ALL relapse can occur several months after first time remission, but that can be prevented by prolonged maintenance treatment (2-3 years following induction) (Childhood ALL Collaborative Group, 1996). Of note, ALL treatment outcome is variable across different ALL subtypes possibly due to different molecular mechanisms influencing ALL progression. Treatment outcome also varies among different age groups. In particular, older adults (>60 years old) show a less favorable outcome (Dores et al., 2012).

B-ALL and T-ALL lymphoblasts are able to infiltrate the meningeal membranes and the cerebrospinal fluid. Although CNS involvement is found in about 5% of ALL cases at diagnosis, the incidence of ALL CNS infiltration significantly increases with ALL relapse (Frishman-Levy & Izraeli, 2017; Krishnan et al., 2010). CNS prophylactic treatment has been shown to dramatically reduce CNS detection of ALL, hence becoming a fundamental part of the ALL therapeutic regimen regardless of CNS involvement at diagnosis (Frishman-Levy & Izraeli, 2017; Künz et al., 2022). For CNS prophylaxis, several treatment strategies can be used: systemic treatment, intrathecal therapy and cranial radiation therapy. Although effective, cranial radiation is associated with severe neurological toxicity, secondary neoplasms, neurocognitive and neuroendocrine dysfunctions (Armstrong et al., 2013; Krull et al., 2013; Pui et al., 2003). For this reason, its practice has been discontinued in most clinical centers worldwide. The limitation of systemic chemotherapy for targeting of CNS disease is that its effectiveness depends on the ability of the therapy to cross the blood-brain barrier. Hence, systemic chemotherapy alone is not appropriate as a CNS prophylactic treatment option. Current treatment mostly consists in intrathecal chemotherapy (methotrexate and/or cytarabine) coupled with high-dose systemic chemotherapy. Despite being safer than cranial radiotherapy, CNS-directed chemotherapy is also associated with short- and long- term neurotoxicity (Frishman-Levy & Izraeli, 2017; Jacola et al., 2016; Richards et al., 2013).

In high-risk ALL cases, allogenic hematopoietic stem cell transplant (alloHSCT) is the standard consolidation treatment when a suitable donor is available (Giebel et al., 2019; Hoelzer et al., 2016; Ribera et al., 2020). In BCR::ABL-positive B-ALL cases, alloHSCT is recommended as first-line consolidation therapy, but it might also be an option for BCR::ABL1-negative cases with positive MRD after first or second phase of treatment. Moreover, this therapeutic strategy is recommended based on MRD in adult patients after pediatric-inspired intensive chemotherapy, and in cases of second remission after relapsed or refractory ALL due to very poor prognosis (Giebel et al., 2019).

2.6.2. Targeted therapy and new generation therapeutic options

Administration of standard treatment regimens including CNS prophylaxis has substantially impacted ALL survival rates in both pediatric and adult patients. Yet, severe toxicity, frequent relapse and emergence of refractory disease have emphasized the need for alternative therapeutic options. Despite the aggressiveness of *BCR::ABL1*-positive ALL, treatment of these patients with TKIs became a textbook example of how particular genetic alterations found in tumors can be used to develop targeted therapies. In fact, treatment of

BCR::ABL1 B-ALL with the TKI imatinib in combination with chemotherapy is responsible for a complete remission rate of approximately 90% (Daver et al., 2015; de Labarthe et al., 2007). Comparably, dasatinib, a second generation TKI, used in combination with chemotherapy achieved similar complete remission rates (Ravandi et al., 2015). However, since dasatinib is better than imatinib at penetrating the CNS, CNS relapse is less frequent than in imatinib-treated patients (Porkka et al., 2008; Takayama et al., 2002). Additionally, a third generation TKI, ponatinib, was developed to target mutations in the *ABL* kinase domain, a frequent cause of ALL relapse, achieving a very significant cytogenetic response in patients (Cortes et al., 2013).

Despite lacking the BCR::ABL1 fusion, Ph-like ALL patients have also shown clinical response to treatment with TKIs, for example dasatinib, particularly when kinase-activating alterations were detected (Roberts, Li, et al., 2014; Tanasi et al., 2019). Moreover, JAK/STAT signaling pathway genetic alterations, which are a key feature of this B-ALL subset and confer poor prognosis (Roberts et al., 2017; Roberts, Li, et al., 2014), make the use of JAK inhibitors promising therapeutic tools. In fact, the use of ruxolitinib and other JAK2 inhibitors showed promising effects in B-ALL preclinical models when combined with standard chemotherapy (Böhm et al., 2021; Downes et al., 2022; Steeghs et al., 2017; Wu et al., 2015). Given the high frequency of JAK2 genetic alterations in high-risk B-ALL and T-ALL, further characterization of the role of JAK2 in leukemia development and progression is of pressing importance. Mouse models that recapitulate molecular and clinical features of human leukemia will be crucial in this research.

T-ALL harbors a higher genetic heterogeneity than B-ALL, with frequent translocations leading to the expression of oncogenic transcription factors and a large number of mutated genes. Nonetheless, the high frequency of alterations in particular signaling pathways in T-ALL has provided promising new therapeutic options. One such pathway is NOTCH1, with prevention of Notch cleavage by γ-secretase inhibitors showing promising results (Gavai et al., 2015; Real et al., 2009). Additionally, blocking the PI3K-AKT signaling pathway by PI3K (Subramaniam et al., 2012), AKT (Falà et al., 2008) or dual mTOR/PI3K (Lang et al., 2020) inhibitors showed promising antileukemic effects *ex vivo*, in preclinical T-ALL models and in a phase-I clinical trial. This studies highlighted the importance of this signaling pathway for leukemia survival and for the development of novel targeted therapies.

Novel therapeutic strategies have also been focusing on targeting surface antigens, particularly for B-ALL. These include targeting of CD20 (Chevallier et al., 2012; Jabbour et al., 2020), CD22 (H. M. Kantarjian et al., 2019) and CD19 (H. Kantarjian et al., 2017; Topp

et al., 2015), with monoclonal antibodies, all showing promising results in patients with refractory B-ALL. Additionally, because of the frequent expression of CD19 in B-ALL, this surface protein was considered an ideal target for the development of cellular immunotherapy with CD19-directed chimeric antigen receptor (CAR) T-cells. Administration of autologous anti-CD19 CAR T-cell resulted in 83% complete remission in patients with relapsed or refractory disease (Park et al., 2018) and has been approved by the EMA – European Medicines Agency and FDA – U.S. Food and Drug Administration. Similar CAR-T strategies, directed against the CD7 surface marker, are being developed to target T-ALL/LBL (Lu et al., 2022).

2.7. TCR expression and TCR signaling in T-ALL

TCR loci rearrangements (commonly affecting TCRβ, TCRγ and TCRδ, alone or in different combinations) are the most frequent alterations in T-ALL (Campana et al., 1991; Furley et al., 1986). At the same time, surface TCR and CD3 expression is found in approximately 50% of T-ALL cases (Asnafi et al., 2003). Of note, despite frequent mutations in several signaling pathways including NOTCH1 and IL-7R/JAK/STAT, mutations in TCR signaling components are rare in T-ALL. Although TCR expression is common in T-ALL, its impact in T-ALL development and progression is not fully understood, as reviewed by dos Santos et al. (2009).

Transgenic TCR expression in different mouse models resulted in spontaneous T-ALL development even in the absence of self-pMHC interactions (Brabb et al., 1997; Cui et al., 2015; Serwold et al., 2010; Strzadala et al., 1997). On the other hand, transgenic TCR expression was shown to accelerate leukemia development in mouse models of T-ALL driven by oncogenic events (e.g. SV40 large T antigen-induced p53 inactivation, STAT5 overexpression or Tpl2 deficiency) (Kelly et al., 2003; Liao et al., 1998; Tsatsanis et al., 2008). In turn, genetic inactivation of TCRα, CD3ε, pTα or MHC delayed T-ALL development in mouse models induced by expression of intracellular NOTCH1 (Campese et al., 2006), Notch3 (Bellavia et al., 2002), Myc (Smith et al., 2005), ETV6-JAK2 (dos Santos et al., 2007) and TAL/LMO (M. Tremblay et al., 2010) proteins. However, although mature TCR$\alpha\beta$ expression can be found in the previous T-ALL mouse models, the presence of mature TCR$\alpha\beta$ or MHC-dependent antigen presentation do not seem to be universally required for leukemogenesis, as neither TCRα inactivation in the ETV6::JAK2-induced and RasGRP1-induced mouse T-ALL (dos Santos et al., 2007; Klinger et al., 2005) nor impairment of MHC-I/MHC-II interactions in the TAL/LMO model (M. Tremblay et al., 2010)

impacted T-ALL onset. Together, these observations suggest a pro-oncogenic role for basal, agonits stimulus-independent TCR signals. On the other hand, in the Pten-deficient T-ALL mouse model, while TCR inactivation accelerated, transgenic TCR expression delayed T-ALL onset (Gon et al., 2018). Additionally, in this model, transgenic TCR expression was counterselected in pre-leukemic cells, except when positive selection was prevented (Gon et al., 2018), overall suggesting a tumor-suppressive role for TCR in this model. Overall, these apparently contradicting observations suggest that in fact the potential for TCR to promote or suppress leukemogenesis might be dependent on cellular context and TCR affinity with particular peptides-MHC complexes.

In contrast with the observations of basal TCR signaling promoting leukemogenesis, strong TCR signaling either by CD3 or cognate peptide was shown to induce a strong anti-leukemic effect: ETV6::JAK2 T-ALL cells expressing the transgenic HY-TCR, recognizing a male-specific antigen (DBY peptide), failed to generate leukemia in transplanted males. Moreover, stimulation of human T-ALL samples with CD3 monoclonal antibody resulted in apoptosis *in vitro* and impaired leukemogenesis *in vivo* (Trinquand et al., 2016). Anti-CD3 treatment of T-ALL cells resulted in the expression of a transcriptional program similar to that involved in negative selection of thymocytes. Indicating that the CD3 antibodies suppress leukemia progression by acting directly in leukemic cells and not by Fc-mediated recruitment of immune cells, the anti-leukemic effect of TCR stimulation was strongly inhibited by LAT expression knockdown (Trinquand et al., 2016). Furthermore, treatment with non-Fcγ receptor-binding anti-CD3 antibodies significantly increased mouse survival in preclinical models of T-ALL (Tran Quang et al., 2020).

TCR expression in human T-ALL cases can be used as a target for CAR-T cell therapy. The constant region of TCRβ chain is encoded by either *TRBC1* or *TRBC2*, two closely related genes. Because T-ALL cells express either TRBC1 or TRBC2 in a monoclonal manner, and not both as normal polyclonal T-cell populations do (Maciocia et al., 2017), it becomes possible to target TCR-positive T-ALL with CAR T-cells without the risk of eliminating all normal T-cells. This approach gave promising results in preclinical T-ALL models (Maciocia et al., 2017).

Chapter 2. Aims

As discussed above, while TCR expression was shown to favor T-ALL development in several mouse models (Campese et al., 2006; dos Santos et al., 2007; Liao et al., 1998; Tsatsanis et al., 2008), it delayed or had no impact in leukemia development in others (Gon et al., 2018; Klinger et al., 2005). These observations, and the fact that a very significant percentage of T-ALL cases express TCR, highlight the potential of targeting TCR and TCR signaling as an attractive approach for new T-ALL therapies. However, since the role for TCR and TCR signaling in leukemogenesis and leukemia progression might be context-dependent and might be influenced by TCR affinity, how TCR stimulation affinity and particular TCR signaling regulators might shape T-ALL response to TCR signaling required further investigation.

Alterations frequently found in T-ALL patients, such as *CDKN2A* inactivation, *NOTCH1* mutations or JAK/STAT pathway activation have been associated with leukemia progression (Albertí-Servera et al., 2021; Karrman et al., 2015; Sulong et al., 2009; Vicente et al., 2015; Weng et al., 2004). Additionally, a study from the group has shown that, in preTCR- and TCR-deficient ETV6::JAK2;*Rag2*$^{-/-}$ mice, leukemia development was significantly delayed, in comparison with TCR-proficient counterparts, and that loss of *Cdkn2a* could occur (dos Santos et al., 2007). These data indicated that lack of pre-TCR/TCR expression results in delayed oncogene-induced T-ALL onset, and that in these cases further genetic alterations might contribute to malignant transformation.

The main aims of this thesis were:
1) evaluate whether TCR signaling could play a dual role in T-ALL,
2) to address how fine-tuning TCR signaling affinity could impact T-ALL cell fate, and
3) to evaluate the how key genetic alterations (JAK2 activation and loss of *Cdkn2a*) might contribute to ALL development in the absence of TCR expression.

Chapter 3. Materials and Methods

1. Mice

EμSRα-ETV6::JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) transgenic mice (dos Santos et al., 2007) were crossed with *Rag2* (B6.129S6-Rag2tm1Fwa) and *Cdkn2a* (B6.129-Cdkn2a^{tm1Rdp}/NCI) knockout mice on the C57BL/6 background. The ETV6::JAK2 transgene was kept in hemizygosity. The *Cdkn2a* knock-out mice (Serrano et al., 1996) were obtained from the NCI mouse repository (strain number 01XB1). HY-TCR Marilyn transgenic mice (B6.Cg-Tg(TcraH-Y,TcrbH-Y)1Pas), on a CD45.1 background, were obtained from Jocelyne Demengeot (IGC, Oeiras), OT-I TCR transgenic mice (B6-Tg(TcraTcrb)1100Mjb/J), were obtained from Nuno Alves (i3S, Porto), maintained in a *Rag2*$^{-/-}$ background, and Beta-2 microglobulin (β2m) knockout mice (B6.129P2-B2m^{tm1Unc}/DcrJ) were obtained from Nuno Alves (i3S, Porto). Nude CBA mice (NU/J) were obtained from i3S animal facility. Mice were generated and maintained at the i3S barrier animal facility (HEPA filtration of incoming air, differential pressure and disinfection or sterilization of room equipment and supplies), under 12-h light/dark cycles and with food (2014S diet; Envigo) and water *ad libitum*. HY-TCR Marilyn mice were also generated and maintained at the CBMR animal facility in equivalent conditions. Microorganism screening detected opportunistic pathogens (*Helicobacter* spp., *Pasteurella pneumotropica* and murine norovirus) in the CBMR, but not i3S, experimental rooms. All experimental procedures were approved by the i3S and CBMR ethics committees and Portuguese authorities (*Direção-Geral de Agricultura e Veterinária*) and followed recommendations from the European Commission (Directive 2010/63/UE) and the local Portuguese authorities (*Decreto-Lei* n°113/2013). Both female and male mice were used for all experiments. Mice were monitored for signs of disease (e.g. dyspnea, lethargy, enlarged lymph nodes, enlarged abdomen, and paraplegia) and killed by CO_2 inhalation when reaching predefined experimental endpoints. Mice of different genotypes from the same litter were kept together in the same cages and monitoring for signs of disease was done blindly. Adult mice that were euthanized without leukemia were censored in Kaplan-Meier survival curves.

2. Mice genotyping

Rag2 knockout mice were genotyped using the following primers: 5'-TGTCCCTGCAGATGGTAACA-3' and 5'-CCTTTGTATGAGCAAGTAGC-3' for WT allele

and 5'-CTATTCGGCTATGACTGGG-3' and 5'-AAGGCGATAGAAGGCGATG-3' for the mutated allele. *Cdkn2a* knockout mice were genotyped using 5'-GTGATCCCTCTACTTTTTCTTCTGACTT-3' primer in combination with 5'-CGGAACGCAAATATCGCAC-3' or 5'-GAGACTAGTGAGACGTGCTACTTCCA-3' for the detection of the WT or mutated alleles, respectively. HY-TCR Marilyn mice were genotyped using the following primers: 5'-CGAGAGGAACCTGGGAGCTGT-3' and 5'-TGCTGTCTGTACCACCAGAAATAC-3'. OTI-TCR transgenic mice were genotyped using the following primer pairs: 5'-AAGGTGGAGAGAGACAAAGGATTC-3' and 5'-TTGAGAGCTGTCTCC-3' for transgene detection and 5'-CAAATGTTGCTTGTCTGGTG-3' and 5'-GTCAGTCGAGTGCACAGTTT-3' for internal control. Beta-2 microglobulin knockout mice were genotyped using the following primer 5'-CCTGCCGAGAAAGTATCCA-3' in combination with 5'-CTGAGCTCTGTTTTCGTCTG-3' or 5'-CTTAACTCTGCAGGCGTATG-3' for the detection of the WT or mutated allele.

3. *In vivo* experiments

Rag2$^{-/-}$ mice infused with ETV6::JAK2;OT-I T-ALL cells, as indicated in figure legends, were treated intraperitoneally with 0.01 mg of ovalbumin SIINFEKL (OVA) or OVA mutant peptides SIITFEKL (T4) and SIIGFEKL (G4) intraperitoneally, all purchased from Eurogentec.

Rag2$^{-/-}$ mice infused with ETV6::JAK2; Marilyn T-ALL cells as indicated in figure legends, were treated intraperitoneally with 0.01 mg of DBY peptide (HYab epitope) from Eurogentec intraperitoneally. Ten mg/kg Dasatinib (LC Laboratories) or DMSO were diluted in 80mM citrate buffer (pH 3.1) and administered by oral gavage. Treatment initiation and duration is specified in figure legends.

4. Flow cytometry

Single-cell suspensions were prepared from lymphoid organs using cell strainers, washed with FACS buffer (phosphate-buffered saline (PBS) with 3% fetal bovine serum (FBS) and 10 mM NaN_3), and stained for 30-45 min with fluorochrome-labeled antibodies in FACS buffer. The following Biolegend antibodies were used: CD25-fluoresceine isothiocyanate (FITC) (clone PC61), TCRβ-phycoerithrin (PE) (clone H57-597), CD44-PE/Cyanine5 (clone IM7), CD90.2-PE-Cyanine7 (clone 30-H12), CD24-allophycocyanin (APC) (clone M1/69), CD4-APC/Cyanine7 (clone GK1.5), CD8α-Pacific Blue (clone 53-6.7), BP-1-FITC (clone 6C3), Gr1-PE (clone RB6-8C5), CD127-PE/Cyanine5 (clone A7R34), CD43-PE/Cyanine7 (clone S11), c-Kit-APC (clone 2B8), B220-APC/Cyanine7 (clone RA3-6B2), CD24-Pacific

Blue (clone M1/69), IgM-PE (clone RMM-1), CD19-Pacific Blue (clone 5D6), CD49f-FITC (clone GoH3), RANKL-PE (clone IK22/5), and CXCR4-APC (clone L276F12), CD90.2-FITC (clone 30-H12), TCR-Vb6-PE (clone RR4-7), TCR-Vb5.1, 5.2-PE (clone MR9-4), CD5-APC (clone 53-7.3), CD69-FITC (clone H1.2F3), CD62L-APC (clone MEL-14), CD45.1-APC (clone A20), CD45.2-Peridinin Chlorophyll Protein (PerCP)-Cyanine5.5 (clone 104) and CD151(CTLA4)-PE (clone UC10-4B9). Nur77-PE (clone 12.14) and PD1-PE (clone J43) were purchased from eBioscience. Immunostained cells were washed twice with FACS buffer and incubated in PBS with 10 mM NaN_3. Cell viability was determined using the Zombie Aqua Fixable Viability Kit (Invitrogen).

For assessment of apoptosis, after staining for surface markers, cells were washed and resuspended in Annexin-binding buffer, and then incubated with Annexin V-APC and 7-AAD, all from Biolegend, for 15 minutes according to the manufacturer's instructions. The percentage of live cells was normalized to the control condition.

For intracellular staining with Nur77-PE antibody, after immunostaining for surface markers, cells were washed twice with in PBS with 10% FBS and 10 mM NaN_3 and fixed for 20 minutes in a 0.5% paraformaldehyde solution. Next, cells were washed and incubated on ice with a PBS 0.5% BSA and 0.1% saponin solution containing Nur77-PE antibody. Immunostained cells were washed twice with FACS buffer and incubated in PBS with 10 mM NaN_3. Samples were acquired using BD FACS CANTO II or BD FACS Accuri C6 and analyzed using FlowJo software.

5. *Ex-vivo* T-ALL cell culture

T-ALL cells of the different genotypes were cultured ex-vivo for 18 hours in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin and 50 μM of 2-mercaptoethanol all from Gibco. For *ex vivo* stimulation of Marilyn T-ALL cells, plates were coated with 10 μg/ml anti-CD3 for 2 hours at 37°C, and then plates were then washed with PBS. Ten ng/ml of phorbol 12-myristate-13-acetate (PMA) and 250 ng/ml Ionomycin treatments were performed for 18 hours. For *ex vivo* stimulation of non-leukemic OT-I thymocytes and ETV6::JAK2;OT-I T-ALL, cells were treated with 10 μM of OVA or OVA mutant peptides dissolved in PBS. ETV6::JAK2;OT-I;$\beta 2m^{-/-}$ T-ALL cells were maintained in monoculture or co-culture with WT C57BL/6 or CBA Nude splenocytes.

6. Low-coverage whole genome sequencing

For low-coverage whole genome sequencing, Ion Xpress Plus Fragment Library kit (Thermo Fisher Scientific) was used for library preparation. Sequencing was performed using the Ion S5XL system (Thermo Fisher Scientific). For copy number analysis, sequencing data was aligned with the mouse genome (mm10) as reference and further analyzed using QDNAseq (RRID:SCR_003174) package in R software. A bin size of 30kb was used.

7. Quantitative PCR

Genomic DNA was isolated from $2x10^6$ thymic cell pellet using GeneJet genomic DNA purification kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Quantitative PCR was performed using the SsoFast Evagreen Supermix (BioRad) under the following conditions: denaturation and polymerase activation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 1 min, and annealing and elongation at 60°C for 30 sec. All samples were tested in triplicate and the results expressed as the mean plus SD. Quantitative PCR results were analyzed with BioRad CFX Manager Software, version 1.6, using the comparative CT method (2-ΔΔC (T) method). DNA copy number alterations were detected relative to the amplification of DNA from the *Vcpip* and *Spna2* loci (chromosomes 1 and 2, respectively), which were used as a normalization control. The following primers were used for each locus.

Vcpip1:
5'-AGTGACATGGGCCTTCAAAC-3'
5'-GGCCCAGATCCAGAATAACA-3';
Spna2:
5'-AGGAGGAGGCTTGGATCAAT-3'
5'-CGAGGGTACTGCCACTGTCT-3';
Cdkn2a exon 1a:
5'-ATCTGGAGCAGCATGGAGTC-3'
5'-CTCCTTGCCTACCTGAATCG-3';
Cdkn2a exon 2:
5'-TCAACTACGGTGCAGATTCG-3'
5'-TCGCACGATGTCTTGATGTC-3';

Cdkn2a exon 3:
5'-GGTGGCTAACGGGTATCAGA-3'
5'-CTGAGGCCGGATTTAGCTCT-3'.

8. *Notch1* mutation detection

Genomic DNA from mouse leukemia samples were used for PCR amplification of two segments of *Notch1* exon 34: primer pair 5'-GCTCCCTCATGTACCTCCTG-3' and 5'-TAGTGGCCCCATCATGCTAT-3', generating a predicted amplicon of 904 bp, and primer pair 5'-ATAGCATGATGGGGCCACTA-3' and 5'-CTTCACCCTGACCAGGAAAA-3', generating a predicted amplicon of 893 bp. PCR products were Sanger sequenced using the same primers at i3S Genomics (Porto, Portugal) or CCMAR *Serviços de Biologia Molecular* (Faro, Portugal).

9. Histology and immunohistochemistry

For histological and immunohistochemistry analysis, formalin-fixed paraffin-embedded tissues were sectioned with 4 μm thickness. Hematoxylin and eosin staining was performed using standard procedures. For immunohistochemistry, heat-mediated antigen retrieval was performed with citrate-based antigen retrieving solution (cat. no. H-3300, Vector Laboratories; for PAX5 or cleaved-caspase 3, Ki67 and LPS immunodetection) or 10 μM EDTA, pH 8.0 and 0.05% Tween 20 in PBS (for B220 or CD3 immunodetection) for 35-40 min. Endogenous peroxidase was inactivated with 3% H_2O_2 in methanol and nonspecific antibody binding was blocked using Ultravision Protein-block (Thermo Fisher Scientific). Sections were incubated overnight with rat anti-B220 (1:200, clone RA3-6B2, Biolegend), anti-PAX5 hybridoma (1:20, PCRP-PAX5-1B7), anti-CD3 (1:200, ab5690, Abcam), anti-cleaved-caspase 3 (1:200, 9661, Cell Signaling), Ki67 (1:500, ab15580, Abcam) or LPS (1:100, WN1222-5, Deltaclon) at 4°C. The PCRP-PAX5-1B7 hybridoma was deposited to the Developmental Studies Hybridoma Bank, by Common Fund – Protein Capture Reagents Program. For B220 detection, slides were incubated with avidin/biotin blocking system (Biolegend), next goat anti-rabbit secondary antibody (Biovision), followed by incubation with streptavidin-horseradish peroxidase (HRP) (Enzo). For PAX5, CD3, Ki67 and LPS detection, HRP-conjugated rabbit/mouse secondary antibody (DAKO) was used. 3,3′-Diaminobenzidine chromogen (DAKO) was used as detection reagent.

10. RNA sequencing

$5x10^6$ single cell suspensions from spleen were pelleted and stored at -80°C in Purezol RNA Isolation reagent (Bio-rad). RNA was later isolated using Direct-zol RNA kit (Zymo Research) following the manufacturer's instructions. For RNA sequencing, libraries were prepared using Ion AmpliSeq Transcriptome Mouse Gene Expression Kit (Thermo Fisher Scientific) and sequenced in a Ion S5XL system (Thermo Fisher Scientific). The downstream analysis to identify pathways that are highly enriched and are activated and inhibited was performed using core analysis in QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA). Scatter plots and heatmaps were generated using Transcriptome Analysis Console (TAC) software (Thermo Fisher Scientific). Mouse homologs of genes reported to be predictive of human Ph-like ALL (Chiaretti et al., 2018; Harvey et al., 2013) were used for heatmap generation with TAC software. Principal component analysis and hierarchical unsupervised clustering was performed using the ClustVis web tool.

11. Statistics

Statistical analysis was performed with GraphPad Prism 6.0 software (RRID:SCR_002798). Log-rank test was used to compare survival of different groups. Unpaired student's t-test was used for comparisons between two groups. Welch's correction was used when variances were different. One-way ANOVA was used to compare differences between more than two groups, multiple comparisons were performed comparing each group to control (PBS). Sample numbers are indicated in figure legends. $P<0.05$ was considered statistically significant.

12. Data availability

Raw unaligned sequencing reads (fastq-format) that support the findings of this study have been deposited in the SRA under the accession numbers PRJNA843967 and PRJNA844038.

Chapter 4. Tonic TCR signaling is leukemogenic while strong TCR stimulation is leukemia-suppressive

T-cell receptor (TCR) expression is a key feature of T-cells and plays a critical role during T-cell development. During the DN3 stage of thymocyte development, assembly of the pre-TCR complex, composed by a rearranged TCRβ chain with the invariant TCRα and CD3, mediates the progression in T-cell development through the β-selection checkpoint, by providing survival and proliferation stimuli (Liu et al., 2021; Shah & Zúñiga-Pflücker, 2014). After successful rearrangement of the TCRα chain and expression of the αβTCR at the surface, thymocytes go through additional checkpoints to establish MHC restriction (positive selection) and to minimize autoreactivity (negative selection) (Klein et al., 2014). Briefly, DP thymocytes with TCRs unable to interact with self-peptide MHC complexes are eliminated by the lack of survival stimuli in a process named death by neglect. Thymocytes experiencing successful low affinity TCR-pMHC interactions are positively selected and survive, whereas high affinity interactions caused by self-pMHC recognition triggers TCR signaling-mediated apoptosis and negative selection (Klein et al., 2014).

TCR and/or CD3 surface expression are found in approximately 50% of T-ALL cases. Although a number of genetic alterations have been recurrently found in T-ALL, including *CDKN2A* inactivation, *NOTCH1* mutations, and JAK/STAT signaling pathway mutations, mutations in TCR signaling machinery were rarely observed (Asnafi et al., 2003; Girardi et al., 2017). Nevertheless, the Lck kinase, a key member of the pre-TCR and TCR signaling pathways, has been shown to be active and a potential therapeutic target in T-ALL (Buffière et al., 2018; Gocho et al., 2021; Laukkanen et al., 2022; Serafin et al., 2017). However, the impact of TCR expression and TCR signaling in T-ALL development and progression remains somewhat controversial. While, transgenic TCR expression accelerated leukemia development in mouse models of T-ALL (Kelly et al., 2003; Liao et al., 1998; Tsatsanis et al., 2008), genetic inactivation of pre-TCR signaling machinery (e.g. CD3ε, pTα or Rag1/2 deficiency) delayed mouse T-ALL development driven by intracellular NOTCH1 (Campese et al., 2006), transgenic Notch3 expression (Bellavia et al., 2002), Myc (Smith et al., 2005), ETV6::JAK2 transgene expression (dos Santos et al., 2007) or TAL/LMO (M. Tremblay et al., 2010). Mature TCRαβ expression or MHC-dependent antigen presentation was not required for leukemogenesis in the ETV6::JAK2 or TAL/LMO models (dos Santos et al., 2007; M. Tremblay et al., 2010). Yet, in the *Pten*-deficient T-ALL mouse model, TCR inactivation accelerated leukemia development while transgenic TCR expression delayed

T-ALL onset (Gon et al., 2018). Overall these observations suggest that depending on the context, TCR might play a tumor-supportive or tumor-suppressive role.

Recently, our group reported that strong TCR stimulation of human or mouse ETV6::JAK2-driven T-ALL cells by anti-CD3 or cognate peptide induced a strong anti-leukemic effect and triggered the expression of a transcriptional program similar to that involved in thymocyte negative selection (Trinquand et al., 2016), highlighting the potential of TCR as a target for the development of novel T-ALL therapeutic approaches. To this end, a better understanding of how TCR stimulation regulates T-ALL cell life and death decisions is pivotal. Here our aim was to investigate the role of basal and strong TCR signaling in T-ALL development and progression.

1. TCR-HY transgene expression drives leukemia development

To study the effect of TCR expression in leukemia development, we resorted to the Marilyn transgenic mouse model, expressing the MHC-II-restricted transgenic TCR-HY (TCR-Vα1.1/Vβ6) specific for the male antigen (Lantz et al., 2000). In this model, thymocytes in males (HY-positive) are exposed to their cognate antigen (DBY peptide) through MHC-II molecules and are eliminated by negative selection, female thymocytes (HY-negative) undergo positive selection and differentiate as mature CD4 T-cells. It was previously described that ETV6::JAK2 transgenic protein expression in Marilyn females drives HY-TCR-expressing T-ALL development (Trinquand et al., 2016). Interestingly, following-up on this work, we found that HY-TCR expression accelerated T-ALL development in ETV6::JAK2 mice (median survival of 10.5 weeks versus 14 weeks; Figure 3A), suggesting HY-TCR signaling cooperates with the ETV6::JAK2 transgene in driving leukemogenesis. To determine whether HY-TCR expression alone could promote T-cell malignant transformation, we followed a cohort of Marilyn males and females and observed that while females developed hematological disease, none of the males did (Figure 3B). Macroscopic analyses showed that Marilyn females developed thymic lymphomas with massive infiltration of lymphoid (e.g. spleen and lymph nodes (LNs)) and non-lymphoid organs (liver and kidneys) (Figure 3C; Supplementary Figures 1 and 2), increased whole blood cell counts, and presence of lymphoblasts in blood smears (Figure 3D). Interestingly, females with thymic lymphomas (TL) presented higher total lymphoid organ disease burden than females with splenomegaly and lymphadenopathy and no thymic lymphomas (non-TL). Additionally, TL Marilyn females tended to developed leukemia faster than non-TL counterparts (Supplementary Figure 3A) and showed increased LNs weight (Figure 3E;

Supplementary Figure 3B). Collectively, these data indicate that TCR transgenic expression is sufficient to induce leukemia development.

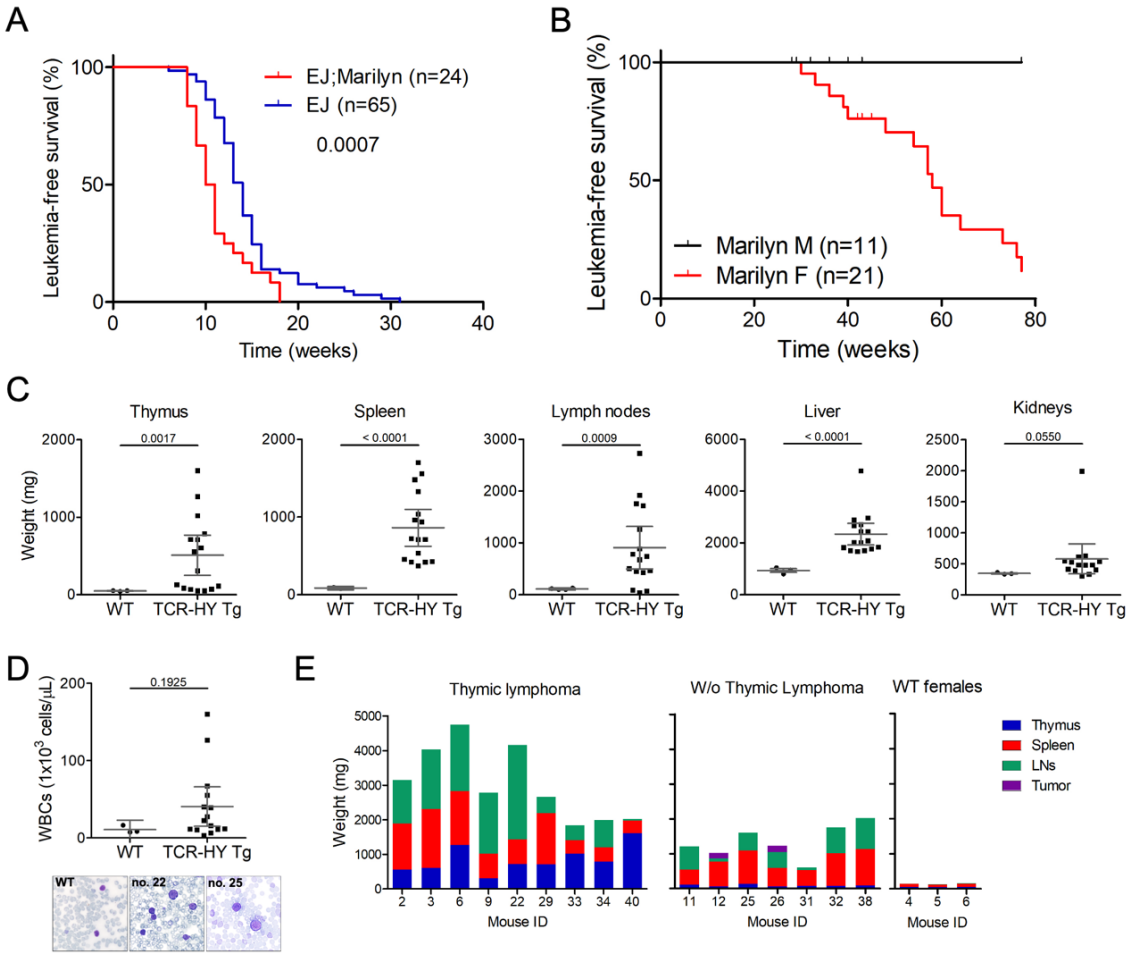


Figure 3. HY-TCR transgene expression drives leukemia development. (A-B) Kaplan-Meier leukemia-free survival curves of ETV6::JAK2 and ETV6::JAK2;Marilyn transgenic mice (A) and male and female HY-TCR transgenic mice (B). Log-rank test *P* values are shown in graphs. (C-D) Organ weights (C) and white blood cell (WBC) counts (D) of Marilyn females with leukemia/lymphoma compared to those of wild-type females (WT). Indicated *P* values were obtained from unpaired *t* tests. Mean and SEM are shown. (E) Representation of tumor burden in lymphoid organs (thymus, spleen, and lymph nodes (LNs)) of Marilyn females with or without thymic lymphoma. Female no. 12 had a mediastinal lymphoid tumor, while no. 26 had lymphoid tumors on the duodenum wall. Each column represents a mouse. Wild-type (WT) female mice were used as control.

2. Transgenic HY-TCR expression induces immature T-cell leukemia development

To identify the disease developing in Marilyn females, we performed flow cytometry immunophenotypic analysis of leukemic cells from different lymphoid organs. We detected

three different leukemia immunophenotypes based on the expression of key differentiation markers on thymic leukemic cells. Mixed B- and T- cell leukemias presented populations of B220-positive cells together with cells with surface expression of the CD90.2, HY-TCR (detected by TCR-Vβ6), CD44 and variable levels of CD24 (Figure 4). Such mixed B- and T-cell populations were present in the thymus, spleen and LNs (Supplementary Figure 4). T-cell leukemias (based on CD90.2 positivity and no B220 expression) expressed CD4, CD8, TCR-Vβ6 and CD5, albeit at variable levels, suggesting the malignant transformation occurred in post-positive selection thymocytes. By assessing CD24 and CD44 expression, we could define two different phenotypes. Mature T-cell leukemias expressed CD44 and low CD24 levels while immature T-cell leukemias were CD44 negative and presented high expression of CD24 (Figure 4). Furthermore, leukemic cells from spleen and LNs recapitulated the immunophenotype of thymic leukemic cells (Supplementary Figure 4). Analysis of CD69 expression, indicative of TCR stimulation, showed that while TCR-Vβ6$^+$ cells in immature leukemias did not express CD69, indicating they did not experience TCR stimulation, TCR-Vβ6$^+$ cells from mixed B and T leukemias showed CD69 positivity (Supplementary Figure 5). This suggests that splenomegaly and mixed B and T disease in a subset of Marilyn mice was driven by stimulation of the HY-TCR transgene in mature T-cells.

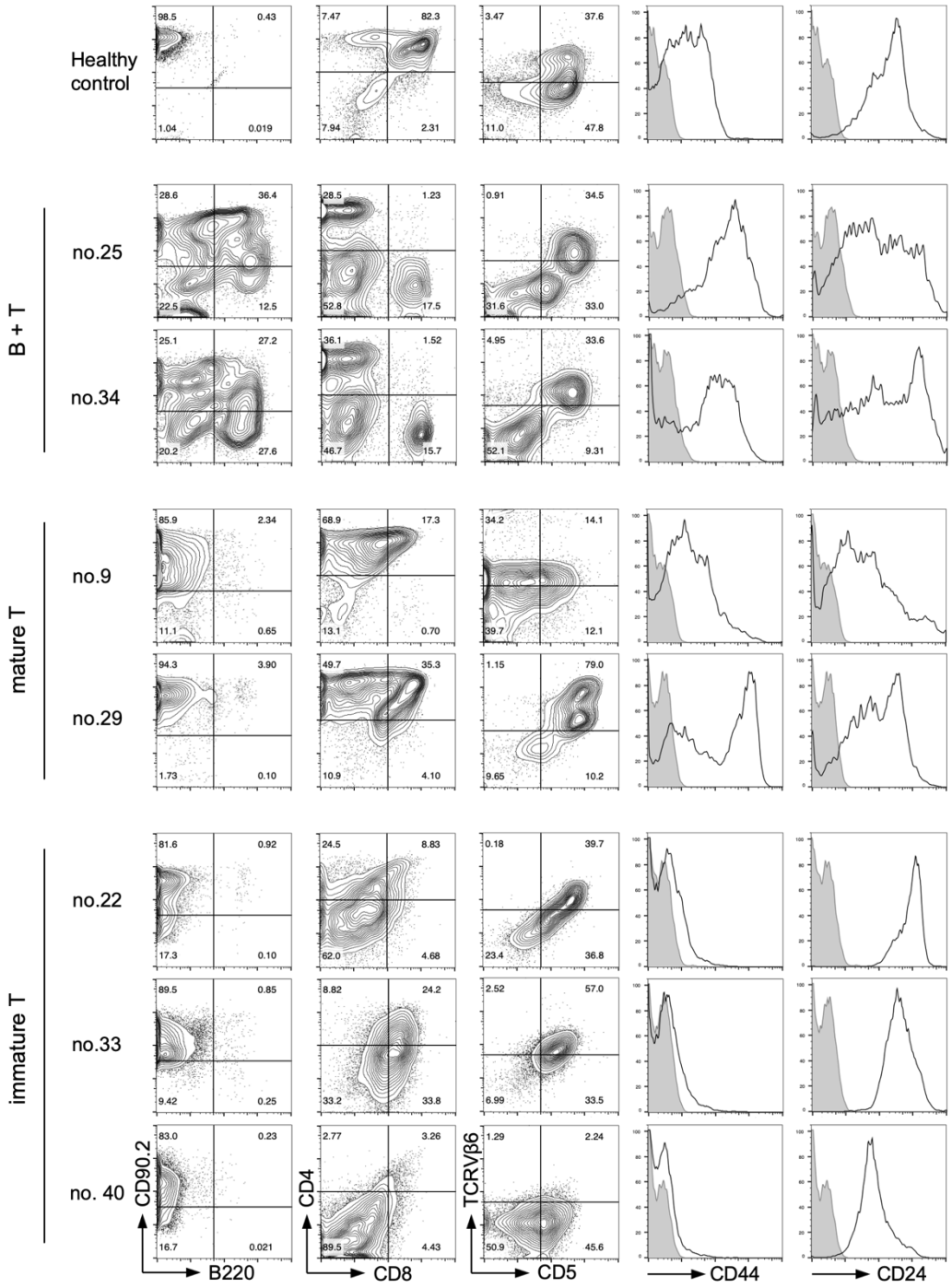


Figure 4. Immunophenotypic analysis of HY-TCR leukemic cells. Flow cytometry analysis of thymus-derived leukemic cells from Marilyn mice presenting mixed B- and T-cell phenotype, based on B220 and CD90.2 expression (nos. 25 and 34), mature T-cell phenotype, based on CD44 positivity and CD24 intermediate/low expression (nos. 9 and 29), and immature T-cell phenotype, based on CD44 negativity and CD24 high expression (nos. 22, 33 and 40). In CD44 and CD24 histogram plots, unstained cells were used as negative control (grey shades). Healthy HY-TCR female thymocyte immunostaining is shown as control.

3. HY-TCR-induced T-ALL is not dependent on *Rag2* recombinase expression

Since Marilyn females housed at CBMR/Faro animal facility developed heterogenous hematological disease, with some mice unexpectedly displaying increased B-cell populations, we generated a new Marilyn cohort (Marilyn;*Rag2*$^{+/-}$) in a different animal facility (i3S/Porto) and a cohort of Marilyn mice crossed with *Rag2* constitutive knockout mice (Marilyn;*Rag2*$^{-/-}$). In this setting, B-cell development is blocked while T-cell development is rescued by transgenic TCR expression. Although a few Marilyn;*Rag2*$^{-/-}$ males developed hematological disease after 1 year of age (median survival of 70 weeks), leukemia onset was much faster in Marilyn;*Rag2*$^{-/-}$ females (median survival of 46 weeks; Figure 5A). Diseased Marilyn;*Rag2*$^{-/-}$ females presented thymic lymphomas, frequently accompanied by increased spleen, LNs, liver and kidney weights (Figure 5B; Supplementary Figure 6A). Leukemias from Marilyn;*Rag2*$^{-/-}$ females had immature T-cell phenotype expressing CD8 and CD4, TCR-Vβ6, high levels of CD24 and variable expression of CD25 and CD5. A similar immunophenotype was present across different affected organs, suggesting that diseased cells disseminated from the thymus to other organs (Figure 5Ci-ii). To assess the degree of infiltration in different organs, we performed histological analysis of Marilyn;*Rag2*$^{-/-}$ leukemic mice. Lymphoblasts were abundant in thymus and spleen sections and infiltrated, with variable extent, the liver, kidneys, lungs and bone marrow (Supplementary Figure 6B). Interestingly, immunohistochemical staining of thymus histological sections showed that while Marilyn;*Rag2*$^{-/-}$ T-ALL cells were highly proliferative (Ki67 staining), they maintained a high level of apoptosis (cleaved caspase 3 staining; Figure 5D). *Rag2*-positive Marilyn thymic lymphomas also displayed a high level of cleaved caspase 3 (Figure 5D). Together these data show that Rag recombinase recombination is not required for transgenic TCR-driven T-ALL.

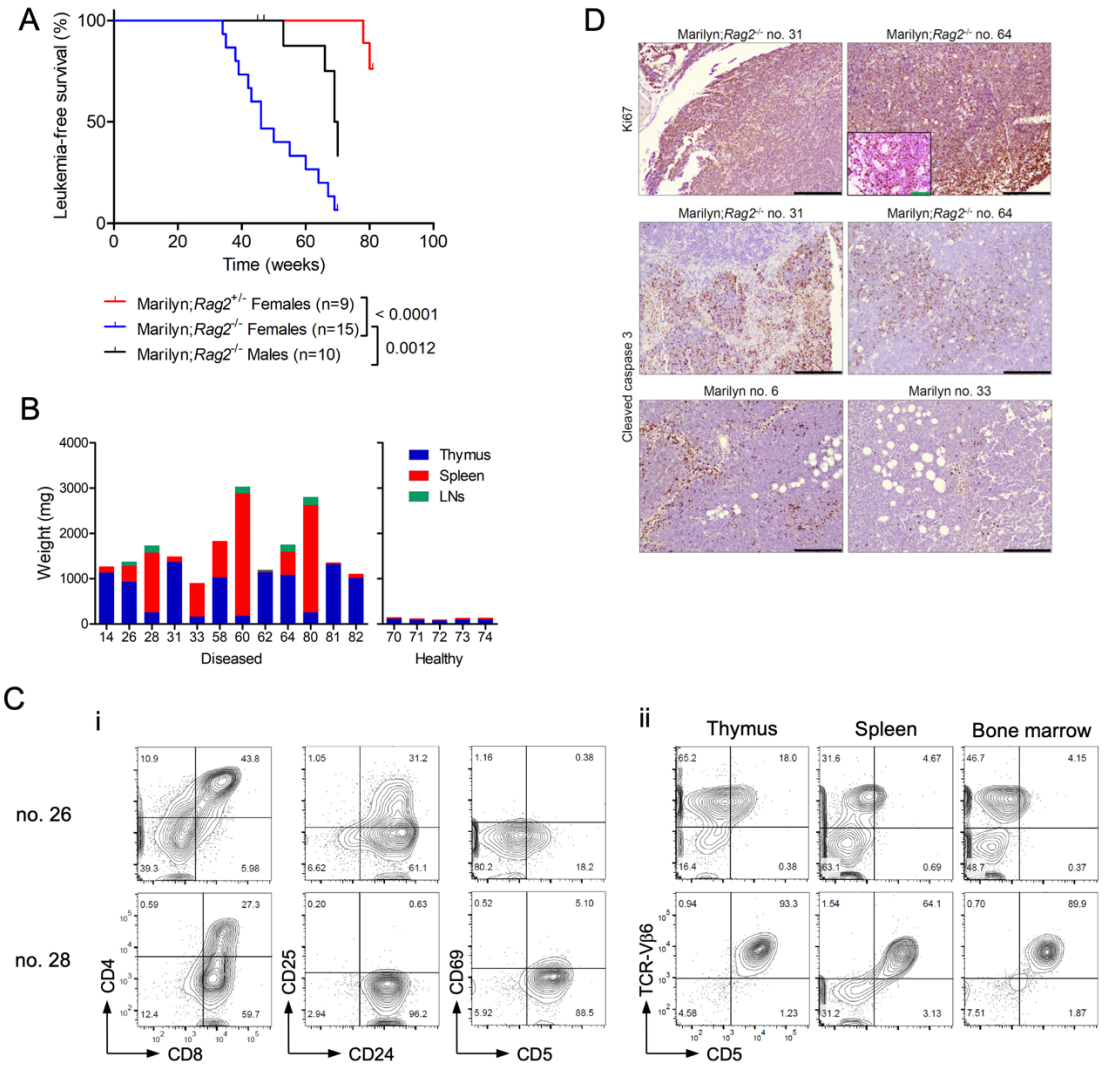


Figure 5. Transgenic HY-TCR expression in *Rag2*$^{-/-}$ mice drives development of T-ALL with thymic lymphomas. (A) Kaplan-Meier leukemia-free survival curve for the indicated genotypes. Log-rank test *P* values are indicated. (B) Representation of lymphoid organ weight (thymus, spleen and LNs) of Marilyn females with leukemia (left graph) and healthy (right graph). Each column represents a mouse. (C) Flow cytometry immunostaining of cells from the thymus (i) and indicated organs (ii) of two representative Marilyn;*Rag2*$^{-/-}$ leukemic mice with the indicated surface markers. (D) Immunohistochemical Ki67 and cleaved-caspase 3 staining of representative thymic lymphoma sections from Marilyn;*Rag2*$^{-/-}$ and Marilyn;*Rag2*$^{+/+}$ diseased mice. Black scale bars 500 μm, green scale bar 200 μm.

4. Mixed B and T lineage hematological disease in HY-TCR transgenic females is associated with microbial status

Marilyn and Marilyn;*Rag2*$^{+/-}$ cohorts had very distinct disease incidence, with more than 90% of Marilyn females at CBMR/Faro facility developing disease (Figure 3B), and only 22% of Marilyn;*Rag2*$^{+/-}$ females at i3S/Porto facility developing disease and at an older age (Figure 5B). Considering that the health status was not equal in both facilities (see Materials

and methods), we posit that the microbiota could have influenced leukemia development. Based on the finding that bacteria can be found in tumors (Nejman et al., 2020), we assessed Gram-negative bacterial lipopolysaccharide (LPS) in lymphomas obtained in both facilities. Indeed, Marilyn lymphomas (from CBMR/Faro facility) displayed more conspicuous LPS staining than Marilyn;*Rag2*$^{-/-}$ lymphomas (Supplementary Figure 7A), suggesting higher bacterial exposure could have driven the mixed B and T disease arising in Marilyn females. Interestingly, the pattern of LPS staining in Marilyn splenic lymphomas was associated with B-cell areas (B220 staining) (Supplementary Figure 7B). Although not conclusive, these data suggest microbiota could contribute to TCR-driven hematological malignancies.

5. Marilyn leukemias present *Notch1* PEST domain mutations and genetic copy number alterations

Notch1 mutations are frequent in human and mouse T-ALL and have been associated with T-ALL progression (Albertí-Servera et al., 2021). To further characterize Marilyn T-ALL cells, we performed sequencing of *Notch1* exon 34 (PEST domain), in which most *Notch1* mutations in murine T-ALL are found (Lin et al., 2006; O'Neil et al., 2006). Four out of 5 Marilyn T-ALL analyzed (Figure 6A) carried exon 34 frameshift mutations. Additionally, by low-coverage whole-genome sequencing, we found that T-ALL samples were largely diploid, with only a few copy number alterations detected: a chromosome 15 trisomy (mouse no. 6), a chromosome 10 trisomy (mouse no. 33) (Supplemental Figure 8A), and loss of the *Cdkn2a* locus (chromosome 4) in two out of five T-ALL leukemias analyzed (Figure 6B). The identification of these genetic alterations from bulk populations indicate that they are clonal in nature. In contrast, no *Notch1* mutations or consistent copy number alterations were found in 2 mixed B + T-cell leukemias assessed (Supplementary Figure 8A). Deletions of the *Tcra/d*, *Tcrb* and *Tcrg* loci were also found in Marilyn T-ALL (Supplementary Figure 8B), indicating that these loci underwent genetic rearrangements and that leukemia arose from post-β-selection thymocytes. As expected from T lineage leukemias, no clonal alterations were found in *Ighm* or *Igk* loci (Supplementary Figure 8B).

CDKN2A deletions are frequent in T-ALL (González-Gil et al., 2021). The *CDKN2A* locus encodes ARF and INK4a, two distinct tumor suppressor proteins. Considering that *Arf* or *Ink4a* genetic inactivation accelerated T-ALL development in various mouse models (Catarino et al., 2022; di Cello et al., 2013; Shank-Calvo et al., 2006; Treanor et al., 2011; Volanakis et al., 2009), we crossed HY-TCR mice with the *Cdkn2a* constitutive knock-out

allele, thus generating cohorts with *Cdkn2a* haploinsufficiency or normal copy number. Interestingly, Marilyn;*Cdkn2a*$^{+/-}$ females developed disease, characterized by increased organ weight (spleen, LNs and liver), faster than Marilyn;*Cdkn2a*$^{+/+}$ mice (Supplementary Figure 9A and B). Additionally, 1 of 3 cases showed loss of the *Cdkn2a* WT allele (Supplementary Figure 9C), consistent with the occurrence of *Cdkn2a* LOH in a different T-ALL model (Catarino et al., 2022). Immunophenotypically, these leukemias were composed of CD4 and CD8 single positive cells with TCR-Vβ6 expression (Supplementary Figure 9D).

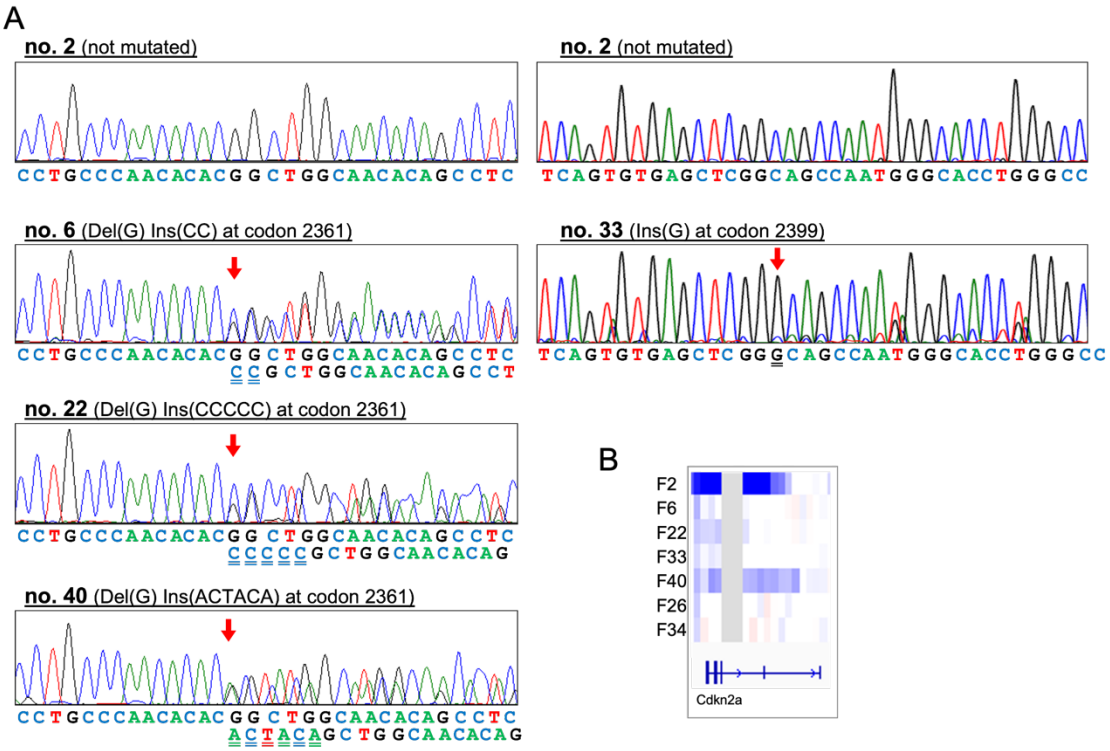


Figure 6. *Notch1* mutations and *Cdkn2a* copy number variations were detected in immature type HY-TCR leukemic cells. *Notch1* exon 34 mutations (disrupting the PEST domain) were detected in Marilyn T-ALL cells by Sanger sequencing. Thymic leukemic cells from mice nos. 6, 22, and 40 carried heterozygous frameshift mutations in codon 2361, as revealed by the presence of overlapping peaks, representing two parallel sequences. Thymic leukemic cells from mouse no. 33 carried a homozygous frameshift mutation in codon 2399, while those from mouse no. 2 had no PEST domain mutation. Mutation location is indicated by a red arrow. (B) DNA copy number variation analysis shows deletion of *Cdkn2a* locus in two cases of HY-TCR T-ALL. Blue represents loss and red represents gain.

6. The transgenic HY-TCR is functional in Marilyn T-ALL cells and its stimulation delays leukemogenesis

ETV6::JAK2;Marilyn T-ALL cells were previously reported to have a functional TCR and to undergo apoptosis following stimulation with cognate-antigen (Trinquand et al., 2016). To assess whether the HY-TCR in Marilyn T-ALL cells was functional, these cells were stimulated *in vitro* for 16 h with plate-bound CD3 antibody or PMA and ionomycin, as a positive control. Stimulated Marilyn T-ALL cells showed increased CD69 expression, accompanied by increased cell size (FSC-A) and increased CD5 expression (Figure 7) indicating that the transgenic TCR is functional in Marilyn T-ALL cells.

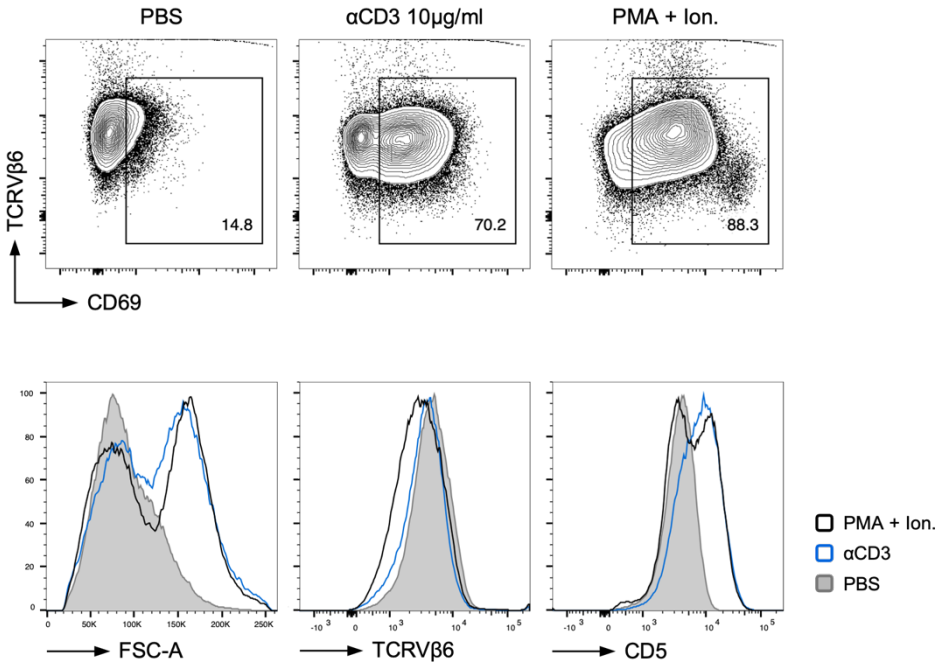


Figure 7. HY-TCR T-ALL cells are responsive to TCR signaling activation. Flow cytometry detection of TCR-Vβ6, CD5 and CD69 expression in TCR-HY T-ALL cells stimulated ex vivo with 10 μg/mL plate-bound anti-CD3 (αCD3) or 10 ng/mL PMA and 250 ng/mL ionomycin (Ion.) for 16 hours. Bottom histograms show forward side scatter (to gauge cell size) and expression of TCR-Vβ6 and CD5 in CD8+ stimulated HY-TCR T-ALL cells. Data representative of 3 independent experiments performed.

Since TCR stimulation *in vivo* has been reported to delay double transgenic ETV6::JAK2;Marilyn T-ALL leukemogenesis (Trinquand et al., 2016), we tested whether Marilyn T-ALL cells would also be susceptible to TCR stimulation. To this end, Marilyn T-ALL cells were injected in syngeneic C57BL/6 males and females and disease progression was followed by peripheral blood collection. CD45.1^{+} Marilyn T-ALL cells were found in the blood of 4 out of 5 females (HY-positive) as early as day 30 post-injection, but not in males

(HY-negative; Figure 8A). Most female recipients rapidly developed fatal T-ALL, as compared to male recipients, which failed to develop leukemia and therefore survived longer (Figure 8B). In parallel, a different group of males and females infused with Marilyn T-ALL was sacrificed 21 days post-injection to compare disease burden. Females presented significantly higher spleen and kidney size and weight (Figure 8C), and more extensive BM infiltration with CD45.1^{+} Marilyn T-ALL cells than males (Figure 8D). To address whether the leukemia-delaying effect of agonist-induced TCR stimulation in Marilyn T-ALL cells was MHC-II-dependent, Marilyn T-ALL cells were infused in syngeneic (C57BL/6) and allogenic (CBA) males and females, both under the immunodeficient Nude background to avoid graft rejection in CBA mice. As expected, T-ALL engraftment was significantly delayed in C57BL/6 males as compared to female counterparts (Figure 8E, left panel). However, no difference between males and females was observed in the context of non-compatible MHC-II molecules (CBA males and females; Figure 8E, right panel). These findings indicate that the T-ALL delaying effect of the male antigen was MHC-dependent (Figure 8E), and corroborated similar observations with ETV6::JAK2;Marilyn leukemic cells (Trinquand et al., 2016). To confirm that delayed Marilyn leukemogenesis in male mice was due to male antigen presentation, we next treated syngeneic immunodeficient females infused with Marilyn T-ALL with the DBY cognate peptide. Indeed, administration of DBY significantly delayed T-ALL development in females (Figure 8F). This indicates that a strong agonist peptide suppresses leukemogenesis.

Since the transgenic HY-TCR drove leukemogenesis, we asked whether TCR signaling was necessary for disease maintenance. Females infused with Marilyn T-ALL were treated with the tyrosine kinase inhibitor dasatinib, which inhibits Lck kinase, a key mediator of the TCR signaling pathway. Interestingly, dasatinib failed to delay T-ALL development, and all mice succumbed to disease (Figure 8F). Collectively these data show that Marilyn T-ALL cells have a functional TCR and are susceptible to TCR-mediated cell death, that the TCR-mediated delay in T-ALL leukemogenesis depends on interactions with agonist peptide presented by MHC, and that Marilyn T-ALL cell propagation is not dependent on continued TCR signaling.

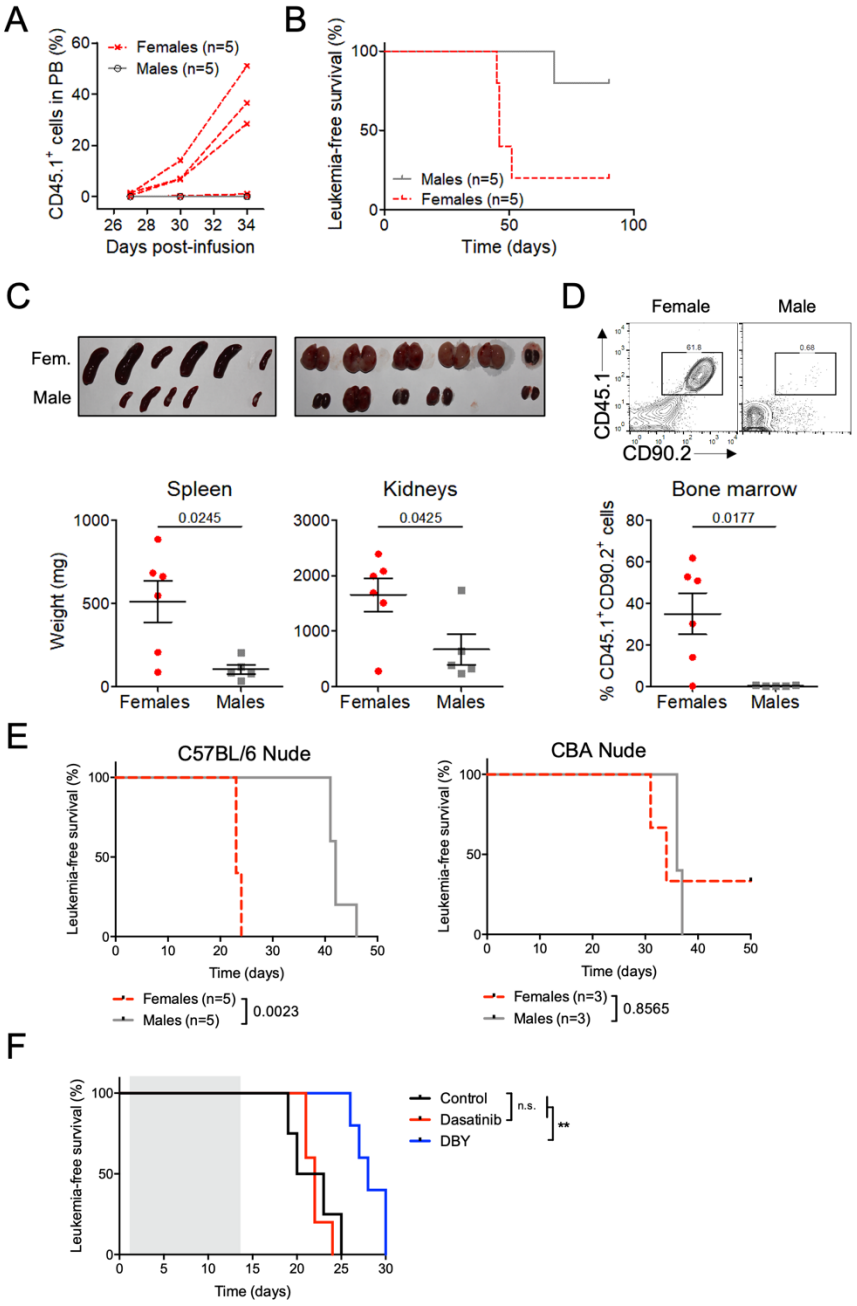


Figure 8. HY-TCR T-ALL antigenic stimulation in vivo hampers leukemia development. (A) Percentage of CD45.1^{+} T-ALL cells detected by flow cytometry in the peripheral blood of C57BL/6 males and females infused with HY-TCR T-ALL cells. *p*=0.0087, two-way ANOVA. (B) Kaplan-Meier leukemia-free survival curve for the same mice as in (A), representative of experiments performed with two different primary leukemic samples. *p*=0.033, log-rank test. (C) Spleens and kidneys (top panels) and respective organ weights (bottom panels) for male (n=5) and female (n=6) C57BL/6 mice 21 days after infusion with HY-TCR T-ALL cells. (D) Percentage of CD90.2 and CD45.2 double-positive HY-TCR T-ALL cells present in the bone marrow of same males and females as in (C). P values in (C) and (D) were obtained from unpaired t tests with Welch's correction. (E) Kaplan-Meier leukemia-free survival curves for syngeneic C57BL/6 nude or allogeneic CBA nude male and female mice injected with Marilyn T-ALL cells. Number of mice and P values are indicated in legends. (F) Kaplan-Meier leukemia-free survival curves for syngeneic *Rag2*$^{-/-}$ females infused on day 0 with Marilyn T-ALL cells and treated with dasatinib (10 mg/kg), 5 days/week (n=5), DBY peptide (0.01mg),

once a week (n=5), or Citrate buffer (n=4). Gray area represents treatment duration of 2 weeks. Control vs dasatinib p=0.7810, Control vs DBY p=0.0027, Dasatinib vs DBY p=0.0019, log-rank test.

7. Discussion

In this report we addressed whether TCR expression had a functional role in T-ALL, and showed that expression of the transgenic Marilyn TCR not only had a synergistic effect with JAK2 constitutive activation, accelerating T-ALL onset in ETV6::JAK2 mice, but also was sufficient to induce T-ALL development on its own. Leukemia development was observed in females but not in males, consistent with the hypothesis that tonic, self-peptide MHC-dependent TCR interactions in females (as opposed to strong, negative-selection-inducing, TCR interactions in male Marilyn thymocytes) underlie disease development. Marilyn females developed hematological disease with different presentations (e.g. mixed B and T cell leukemia and mature T cell leukemia) but immature T-ALL was the most common, characterized by thymic lymphomas, high CD24 and low CD44 expression levels, and was tendentially associated with earlier disease onset. Interestingly, T-ALL cells did not express the activation market CD69, while T cells in mixed B and T leukemias expressed high levels of TCR-Vβ6 and CD69, indicative of proliferative mature T cells and consistent with a non-malignant TCRβ$^+$/CD69$^+$/CD44$^+$ lymphoproliferative phenotype (Tivol et al., 1995).

Genetic alterations frequently found in human and mouse T-ALL were also observed in Marilyn-TCR T-ALL cells. *NOTCH1*-activating mutations, present in the majority of human T-ALL cases, are associated with disease progression (Albertí-Servera et al., 2021). Similarly, *Notch1* mutations, particularly exon 34 PEST domain mutations, are also common in mouse T-ALL models (Ghezzo et al., 2018; O'Neil et al., 2006). *Notch1*-activating mutations were also frequently detected in Marilyn T-ALL. Additionally, *CDKN2A* inactivation by deletion or epigenetic mechanisms occurs frequently in human T-ALL and has reported implications in patient survival (Sulong et al., 2009; H.-P. Wang et al., 2021). Two out of 5 Marilyn T-ALL samples presented deletions in chromosome 4 encompassing the *Cdkn2a* locus, replicating previous observations in pre-TCR-deficient ETV6::JAK2 mice (dos Santos et al., 2007) and ICN1-driven T-ALL mouse models (Volanakis et al., 2013). Interestingly, three Marilyn T-ALL showed a more subtle loss in *Cdkn2a* locus which might suggest deletion of a single copy of the gene or subclonal *Cdkn2a* deletion. Furthermore, *Cdkn2a* haploinsufficiency accelerated leukemia development in Marilyn females, with LOH found in 1 of 3 leukemias analyzed. *Cdkn2a* LOH was previously reported in different mouse T-ALL models (Catarino et al., 2022; di Cello et al., 2013; Shank-Calvo et al., 2006; Treanor et al., 2011; Volanakis et al., 2009). Together, these results indicate that transgenic HY-TCR expression drives the development of T-ALL, but secondary genetic alterations are probably required.

Although Rag recombinase-mediated mutagenic events contribute for leukemia development (Bianchi et al., 2019), *Rag2* recombinase activity was not necessary for Marilyn T-ALL development. In fact, leukemia developed with higher penetrance and much faster in Marilyn;*Rag2*$^{-/-}$ females than in Marilyn;*Rag2*$^{+/-}$ females generated in the same animal facility. As compared to *Rag2*-positive Marilyn females, diseased Marilyn;*Rag2*$^{-/-}$ females were characterized by frequent occurrence of thymic lymphomas, dissemination of leukemic cells to peripheral organs, and leukemic cells expressing markers of immature T-ALL. Furthermore, Marilyn;*Rag2*$^{-/-}$ leukemic cells presented high proliferative rate accompanied by high apoptotic rate, indicative of a high T-ALL cell turnover. The observed high *in vivo* apoptosis is consistent with the observation that Marilyn T-ALL cells die very rapidly in standard culture conditions (not shown).

Of note, Marilyn and Marilyn;*Rag2*$^{-/-}$ females were housed in different animal facilities, in CBMR/Faro and i3S/Porto, respectively, and Marilyn;*Rag2*$^{+/-}$ females hardly developed leukemia when housed at the i3S animal facility. This suggested that microbiota may influence transgenic TCR-induced leukemogenesis. These findings are reminiscent of the discovery that B-ALL was initiated in *Pax5* heterozygous mice only when they were exposed to common pathogens (Martín-Lorenzo et al., 2015). Microbiota plays a critical role in shaping the immune system, in particular T cell numbers and repertoire in the thymus (Cebula et al., 2013; Cheng et al., 2021) or in the periphery (Zegarra-Ruiz et al., 2021). In fact, we found higher levels of LPS staining in Marilyn T-ALL than Marilyn;*Rag2*$^{-/-}$ T-ALL, indicative of higher exposure to bacterial antigens and consistent with the hypothesis that different thresholds of TCR stimulation might have different outcomes in T cell function and different potential to drive leukemogenesis. We posit that the abundance of bacterial antigens increases the chance of TCR interactions with MHC molecules presenting non-cognate peptides that generate basal TCR stimuli, thus facilitating leukemogenesis. In contrast, lower bacterial antigen exposure conditions might generate less and/or lower intensity TCR-MHC interactions and decrease the probability of TCR-induced malignant transformation.

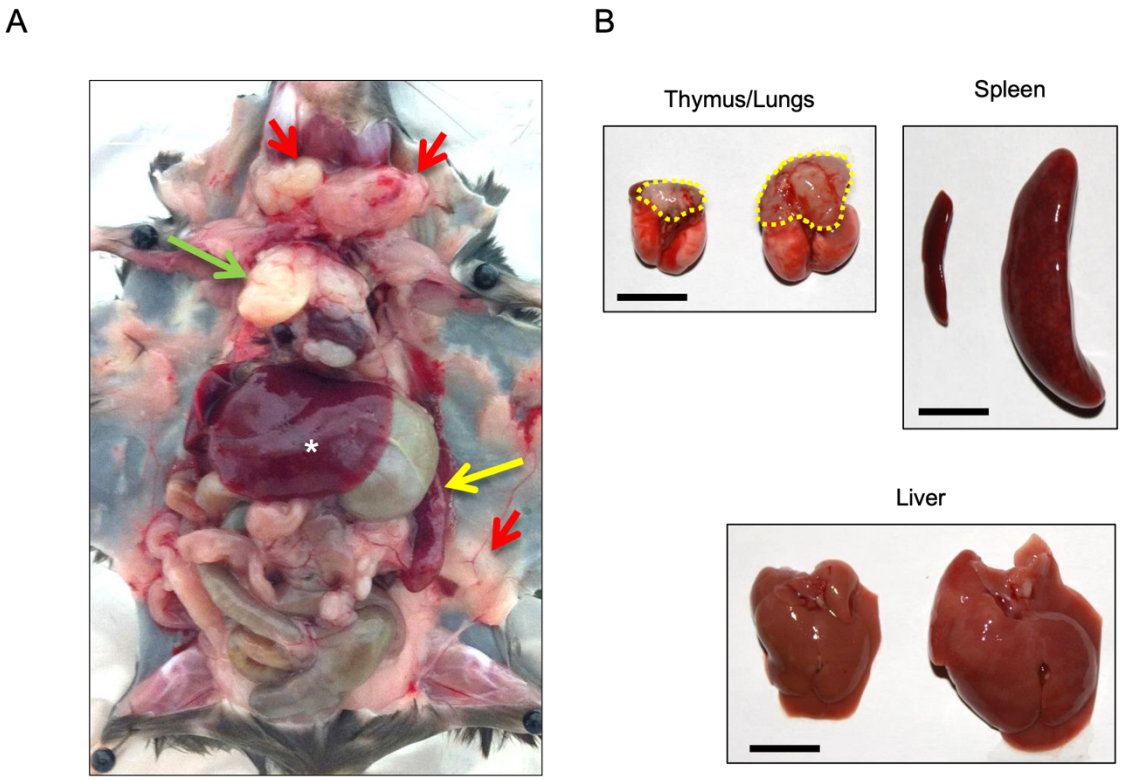
Transgenic HY-TCR expression had been previously reported to drive T-ALL development (Strzadala et al., 1997). Interestingly, the percentage of females developing leukemia was significantly lower than here reported (48% versus more than 90% at 80 weeks) and 38% of males developed leukemia while none did in our cohort. Although health status was not mentioned in this study, microbiota might have played a role in the differences of incidence observed. How exposure to microbial agents impacts positive or negative selection of thymocytes is not known. However, based on the differences in T-ALL development

between the two studies (Strzadala et al., 1997), we hypothesize that increased exposure to microbial antigens might explain the increase in T-ALL development in females, and might increase basal TCR signaling in males, consequently increasing negative selection efficacy. Additionally, we show here that transgenic TCR expression is important for leukemia development but TCR signaling is not required for Marilyn T-ALL maintenance as treatment with the tyrosine kinase inhibitor dasatinib had no effect in leukemia development. These results contrast with reports of TCR signaling being necessary for lymphoma cell growth in TCR-expressing reprogrammed T-cell model (Serwold et al., 2010). Of note, expression of a transgenic TCR recognizing a peptide from the murine peptide survivin protein was also shown to result in increased TCR signaling and to induce T-ALL (Cui et al., 2015). In this context, the specific (self) antigen was present, in contrast with the absence of the cognate peptide (DBY) of the HY-TCR in Marilyn females. Interestingly, Dubois and colleagues showed that mice expressing the OT-I TCR developed T-ALL but only when NK cells were absent (Dubois, Feigenbaum, et al., 2020). Furthermore, ovalbumin stimulation of OT-I TCR-expressing lymphoma cells resulted in lymphoma development in the presence of NK cells mediated by the secretion of NK cell inhibitory factors (Dubois, Waldmann, et al., 2020). Considering these results, we cannot discard the possibility that NK cell tumor suppressing activity might be impacted by microbiota composition and that NK cells might have played a role in preventing T-ALL in the Marilyn cohort maintained in the i3S/Porto facility.

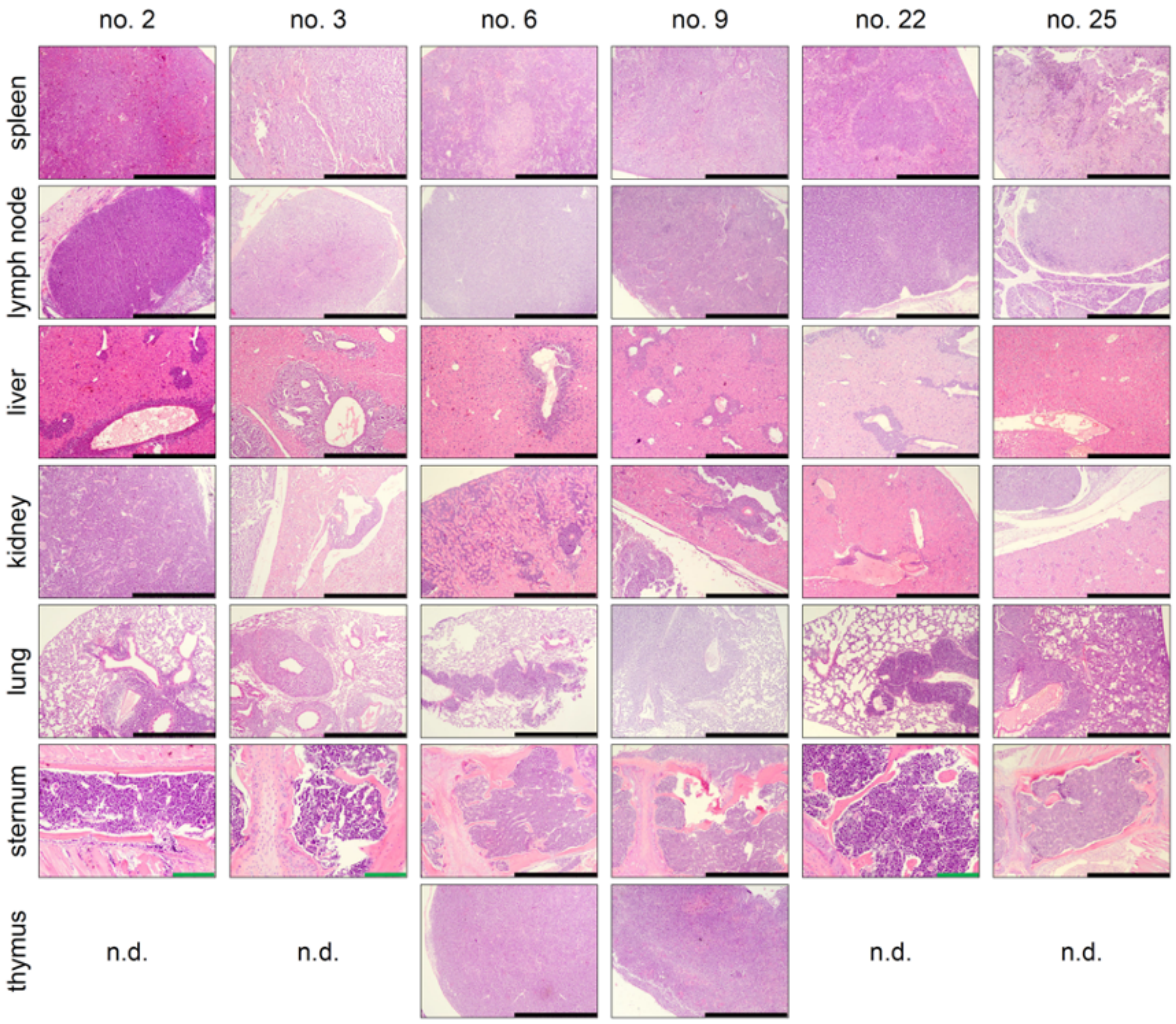
We showed that Marilyn T-ALL cells had a functional TCR and could be activated through TCR stimulation, leading to CD69 upregulation *in vitro*. Furthermore, TCR stimulation by cognate antigen, either in syngeneic males infused with Marilyn T-ALL or syngeneic females infused with Marilyn T-ALL and treated with DBY peptide, was associated with impaired Marilyn T-ALL leukemogenesis. These observations are in agreement with the reported murine ETV6::JAK2;Marilyn and human T-ALL apoptosis induced by TCR stimulation (Trinquand et al., 2016).

Together, our results suggest a dual role for TCR signaling in T-ALL: while basal TCR stimulation (Marilyn females) facilitates leukemia development, strong agonist-induced TCR engagement (Marilyn males) suppress Marilyn T-ALL.

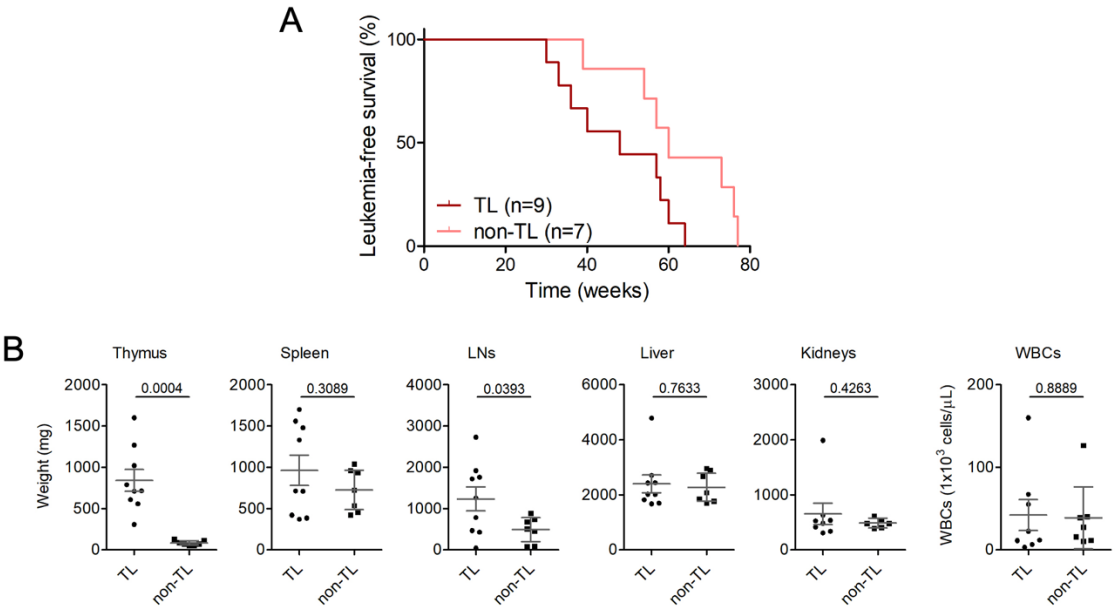
8. Supplementary Figures



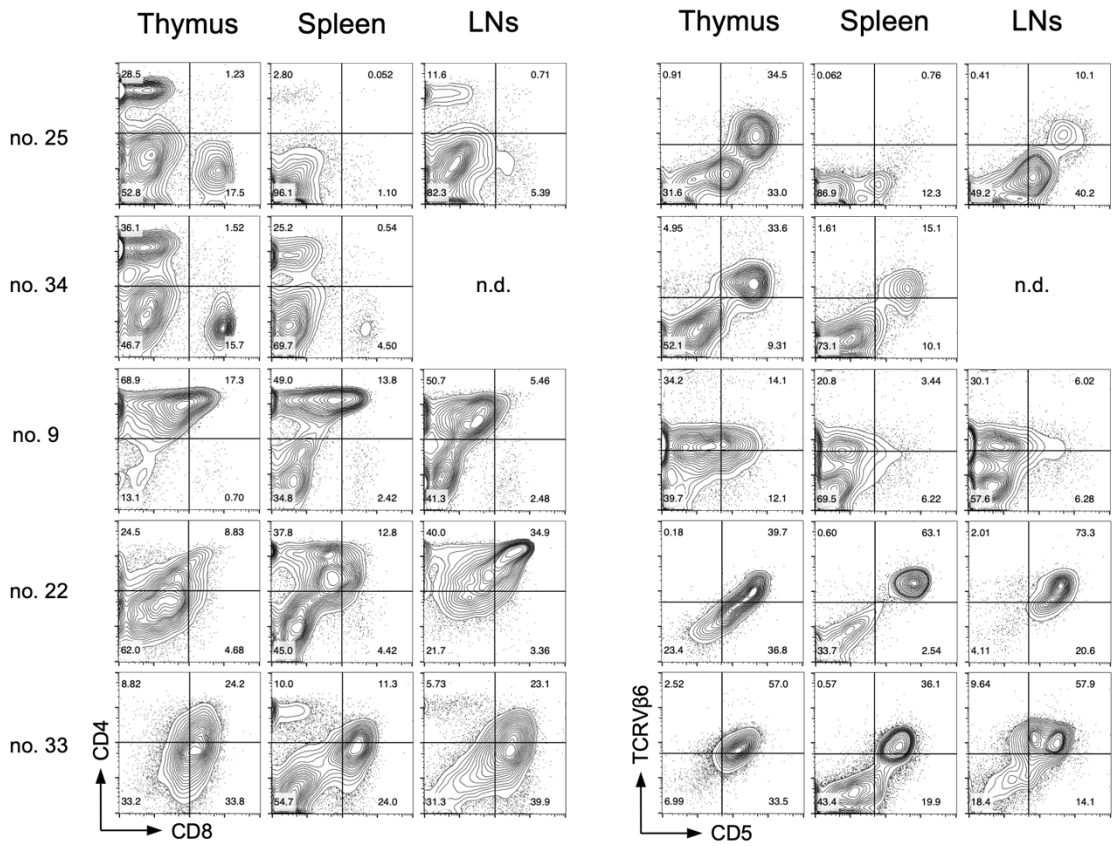
Supplementary Figure 1. Marilyn female mice developed hematological tumors. (A) Representative female Marilyn mouse (no. 34) presenting enlarged thymus (green arrow), LNs (red arrows) and spleen (yellow arrow). (B) Enlarged thymus, spleen and liver from a female Marilyn mouse (no. 3), compared to wild-type mouse organs. Yellow pointed line highlights the thymus. Scale bar: 1 cm.



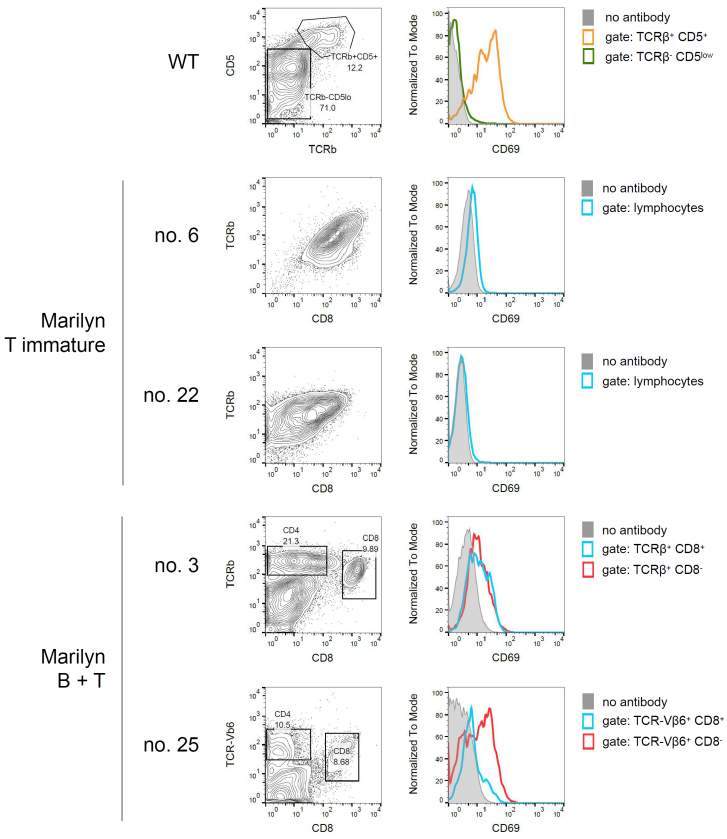
Supplementary Figure 2. Histological analysis of HY-TCR female mice with leukemia/lymphoma. Hematoxylin and eosin staining of sections of the indicated organs of TCR-HY female mice with leukemia. Black scale bars: 500 μm; green scale bars: 200 μm.



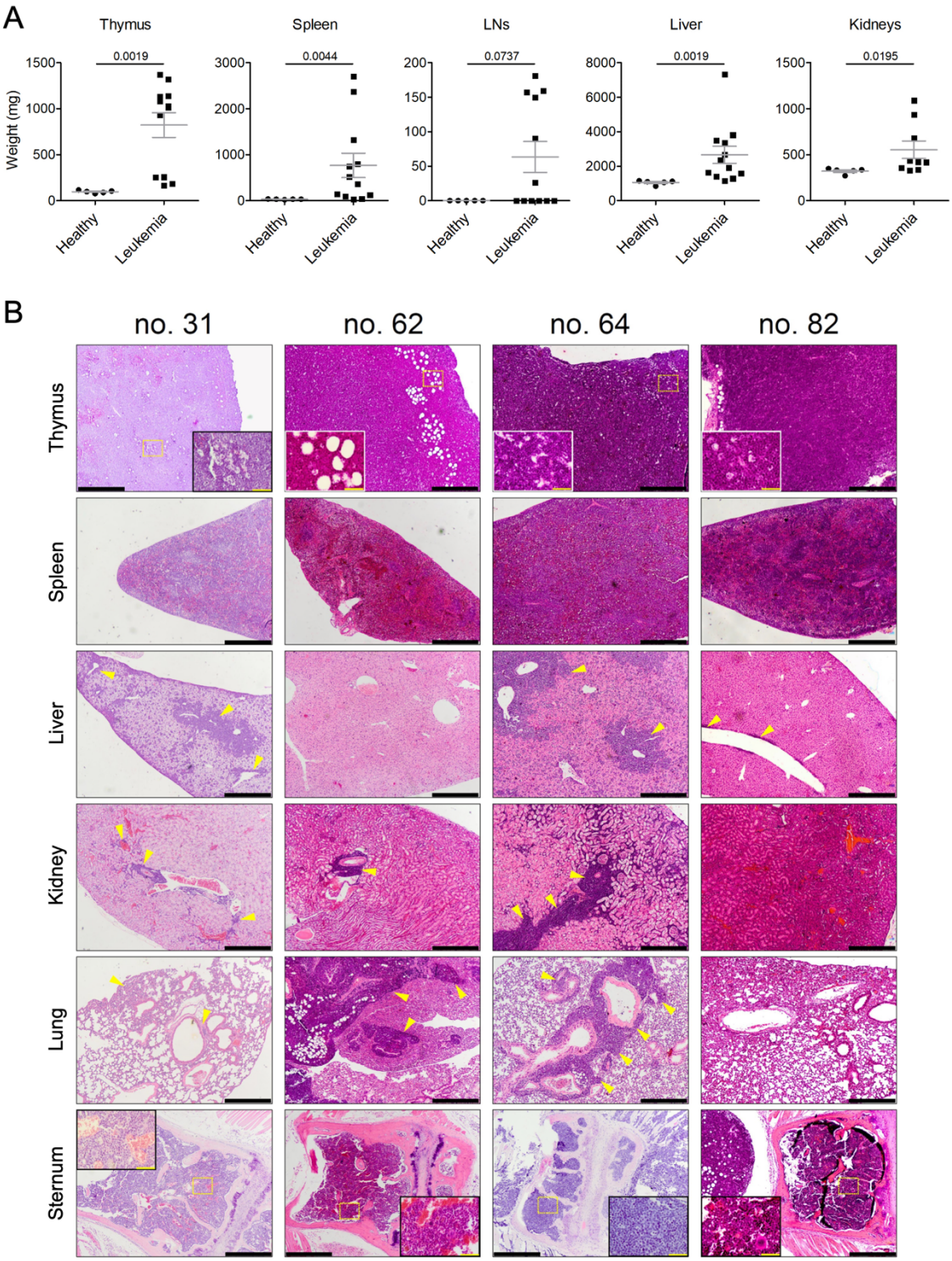
Supplementary Figure 3. Marilyn female mice with thymic lymphoma lived shorter and presented more severe lymphadenopathy than remaining diseased mice. (A) Kaplan-Meier survival curves for female with thymic lymphoma (TL) or developing lymphoma in organs other than thymus (non-TL). *p*=0.0509, log-rank test. (B) Organ weights and white blood cell counts (WBCs) of mice developing thymic lymphoma versus others. P values were obtained from unpaired t tests, with Welch's correction when variances were significantly different. Mean and SEM are shown.



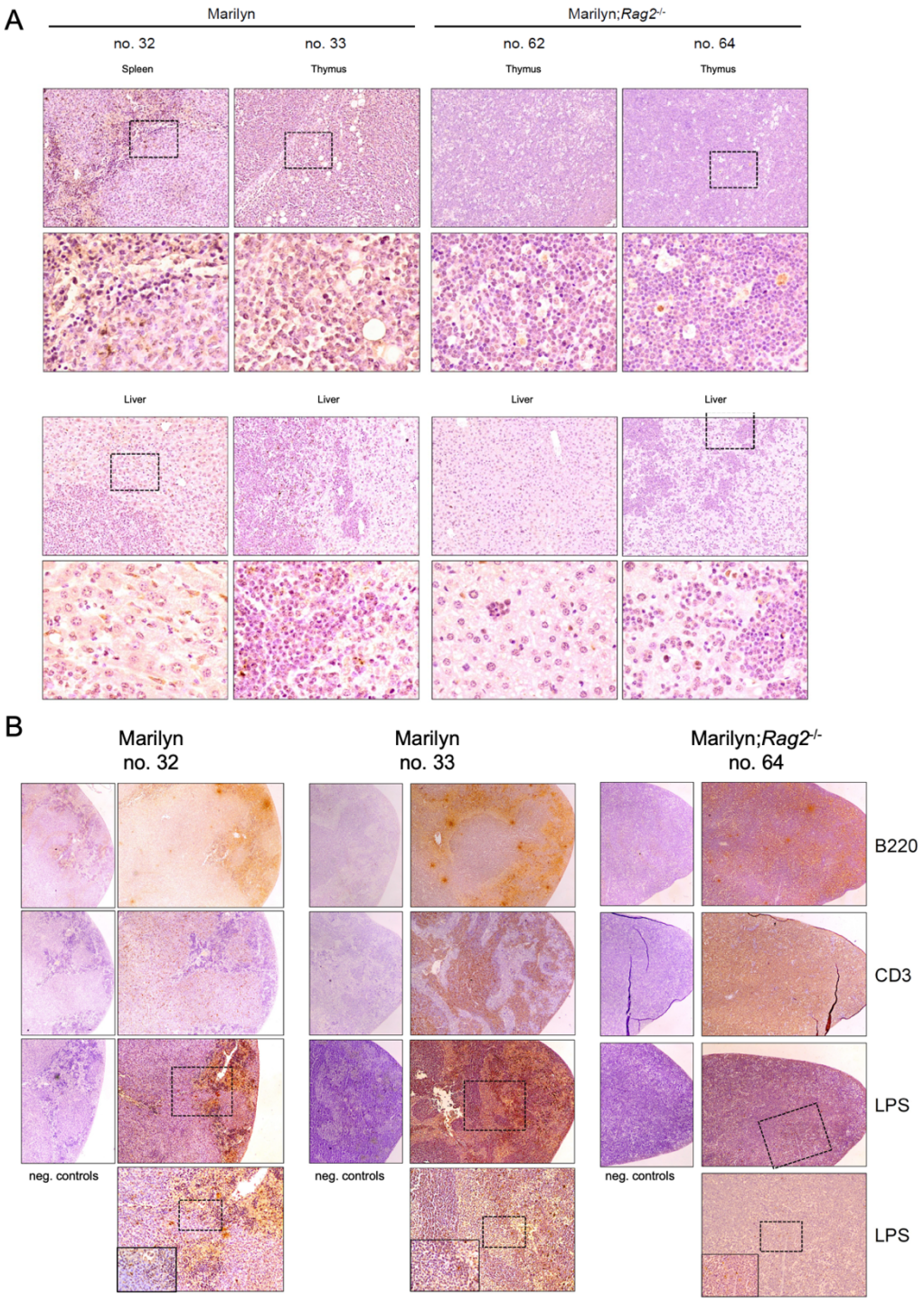
Supplementary Figure 4. The immunophenotype of pathological HY-TCR T cells is similar among different lymphoid organs. Flow cytometry detection of CD4, CD8, TCR-Vβ6 and CD5 surface expression in thymus, spleen and LNs from diseased Marilyn females.



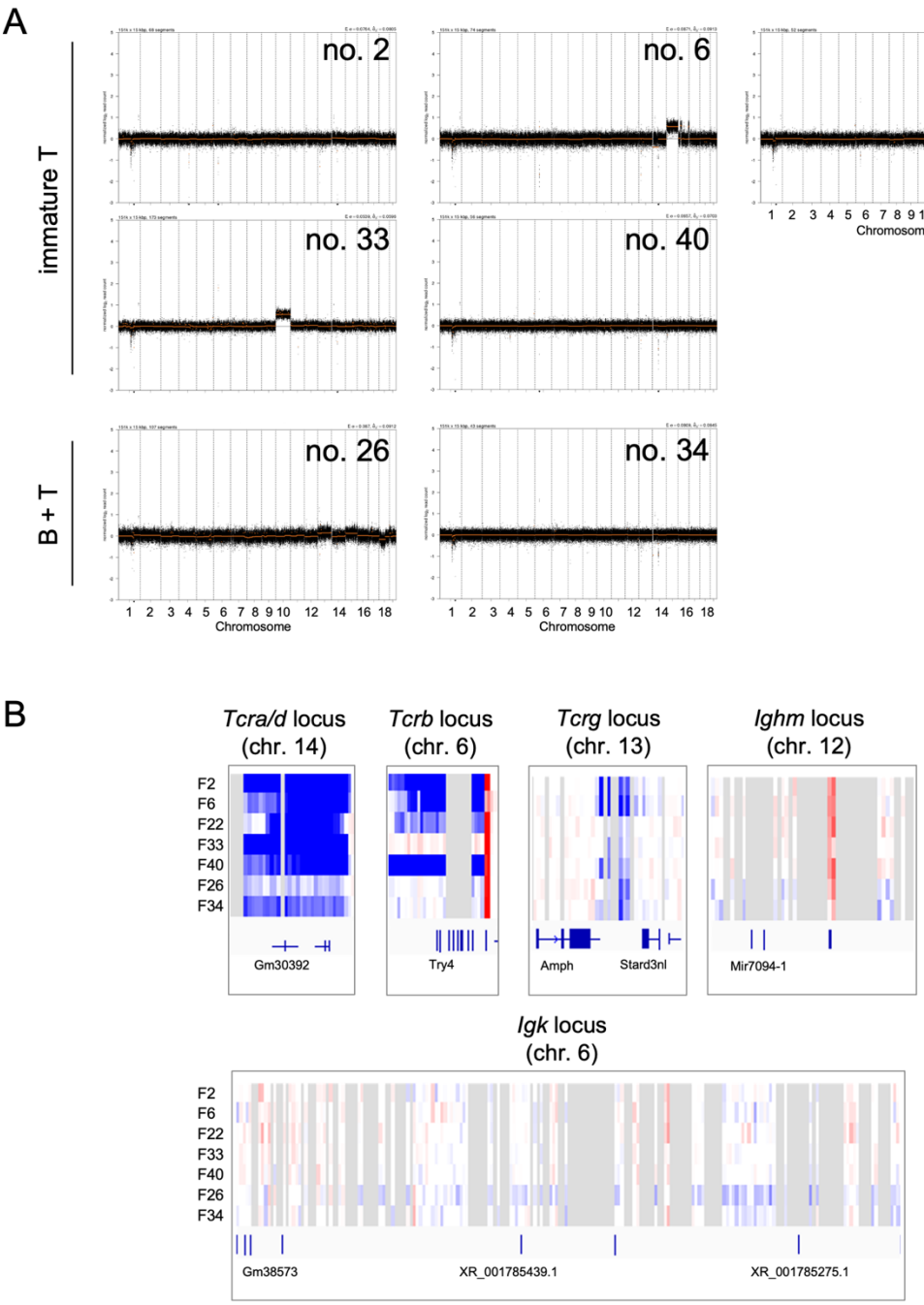
Supplementary Figure 5. CD69 expression in pathological HY-TCR T cells. Flow cytometry analysis of TCRβ, TCR-Vβ6, CD8 and CD69 expression showing TCR-positive cells in immature T-cell leukemias do not express CD69 while they do in mixed B- and T-cell counterparts. CD69 expression in positively selected ($TCR\beta^{+}CD5^{+}$) wild-type (WT) thymocytes was used as control.



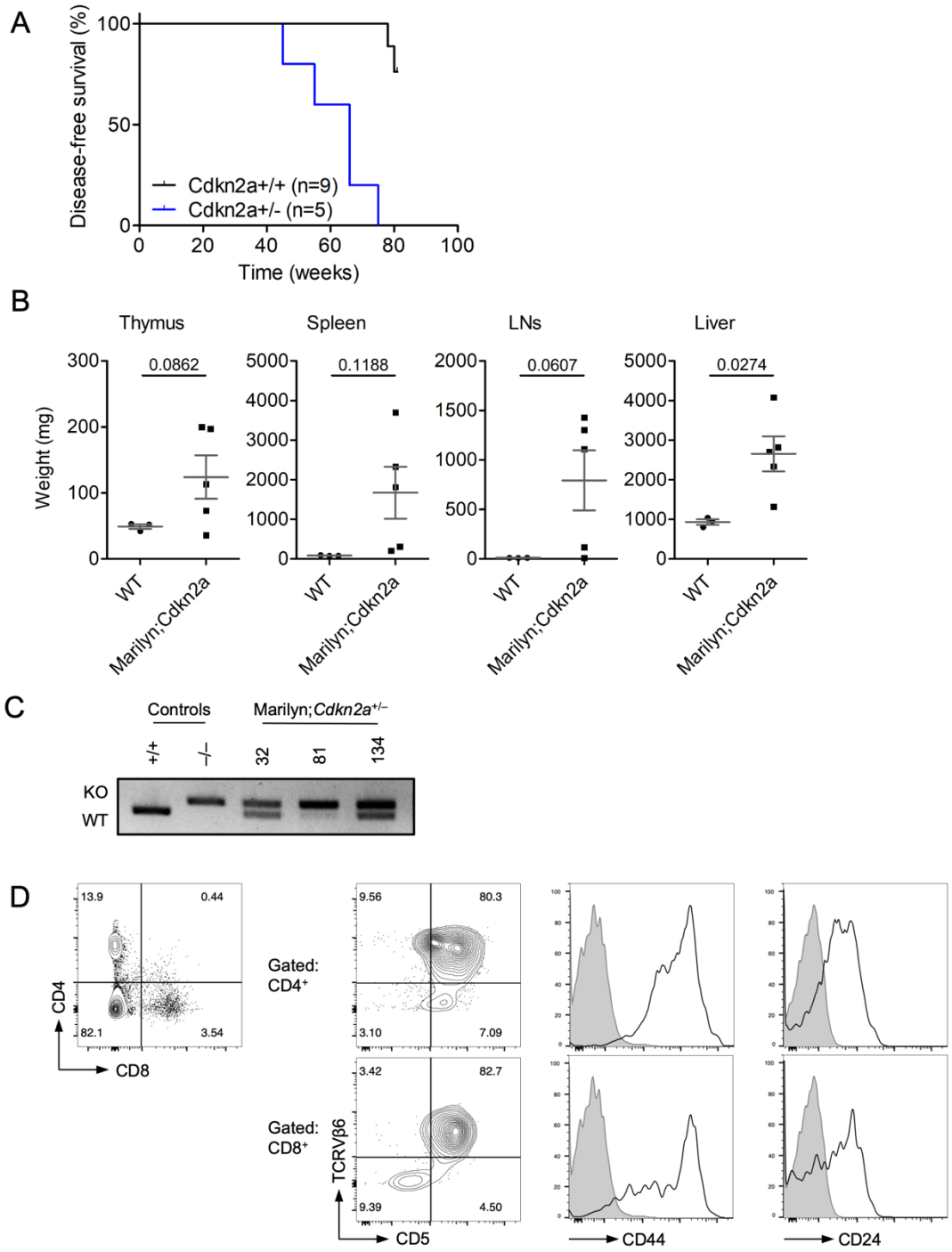
Supplementary Figure 6. Transgenic HY-TCR expression in *Rag2*$^{-/-}$ mice develop aggressive T-ALL disseminated to several organs. (A) Organ weights of Marilyn;*Rag2*$^{-/-}$ females with leukemia/lymphoma. Healthy females were used as control. Indicated *P* values were derived from unpaired *t* tests with Welch's correction. (B) Hematoxylin and eosin staining of sections of the indicated organs of TCR-HY;*Rag2*$^{-/-}$ females with T-ALL. Black scale bars: 500 μm; yellow scale bars: 200 μm.



Supplementary Figure 7. Stronger lipopolysacharide expression in organs from diseased Marilyn leukemic females than *Rag2*$^{-/-}$ counterparts raised in a different animal facility. (A) Anti-lipopolysacharide (LPS) immunohistochemistry for livers and thymus (or spleen for no. 32) of representative Marilyn and Marilyn;*Rag2*$^{-/-}$ mice. Top panels: 100x magnification; bottom panels: 400x magnification. (B) LPS, CD3 and B220 immunohistochemical detection in spleen sections of representative Marilyn or Marilyn;*Rag2*$^{-/-}$ females with splenomegaly. Staining with secondary reagent without primary antibody was performed as negative control (left panels for each mouse sample). Top 3 panels: 40x magnification; bottom panels: 100x magnification; inset: 400x magnification.



Supplementary Figure 8. DNA copy number alterations detected in Marilyn T-ALL cells. (A) DNA copy number alterations of representative HY-TCR leukemic cells assessed by low coverage whole-genome sequencing. Each graph represents DNA copy number distribution for each case. (B) DNA copy number variation analysis for *Tcra/d*, *Tcrb*, *Tcrg*, *Ighm* and *Igk* loci in cells from diseased Marilyn mice. In (B) blue represents copy number loss and red represents copy number gain relative to reference mouse genome (mm10).



Supplementary Figure 9. *Cdkn2a* loss accelerates HY-TCR T-ALL development. (A) Kaplan-Meier leukemia-free survival curve for Marilyn *Cdkn2a* heterozygous female mice and *Cdkn2a*$^{+/+}$ counterparts. p<0.0001, log-rank test. (B) Organ weights of Marilyn;*Cdkn2a*$^{+/-}$ females with leukemia/lymphoma. Indicated *P* values were obtained from unpaired *t* tests with Welch's correction. (C) PCR detection of wild-type (WT) and Cdkn2a knockout (KO) alleles in Marilyn;*Cdkn2a*$^{+/-}$ leukemic cells showing loss of the WT allele (loss of heterozygosity) in one (no. 81) of three cases analyzed. DNA from *Cdkn2a*$^{+/+}$ and *Cdkn2a*$^{-/-}$ mice was used as control. (D) CD44-positive mature immunophenotype of splenic cells from a representative diseased Marilyn;*Cdkn2a*$^{+/-}$ mouse.

Chapter 5. Qualitative differences in TCR stimulation drive distinct outcomes in leukemic cells

TCR transgenic expression in the absence of cognate peptide leads to the development of mouse T-ALL (Chapter 4) (Serwold et al., 2010; Strzadala et al., 1997). HY-TCR expression and potential stimulatory interactions with self-peptide presented by MHC-II molecules leads to T-ALL development in Marilyn female mice. In contrast, MHC-II presentation of cognate peptide to Marilyn T-ALL cells leads to strong TCR stimulation and impaired leukemogenesis. These findings suggests a dual role for TCR expression in leukemia: while TCR expression and positive selection-inducing signals from MHC-II molecules (basal TCR signals) might be sufficient for leukemogenesis, agonist-induced (strong) TCR signals suppress leukemia development. It remains to be deciphered the mechanism by which basal TCR signals favor leukemogenesis and strong TCR signals suppress leukemia. One possibility is that varying TCR signal strength leads to the expression of different gene sets. In this line, it was reported that TCR stimulation of mature T-cells with varying cognate peptide concentrations and for varying periods of incubation resulted in distinct transcriptional signatures (Elliot et al., 2021). Here, we aimed to dissect the impact of TCR agonist stimulation with peptides of varying affinity on T-ALL suppression.

1. OT-I TCR expression delays the development of T-ALL sensitive to TCR activation

To address whether TCR stimulation with peptides of different affinity would impact differently T-ALL cell activation and survival, we crossed the MHC-I-restricted, SIINFEKL ovalbumin (OVA) peptide-specific transgenic TCR (OT-I; TCR-Vα2/Vβ5) mice with the ETV6::JAK2 T-ALL mouse model. ETV6::JAK2;OT-I mice were generated on a *Rag2*$^{-/-}$ background to exclude expression of endogenous TCR chains. T-ALL onset was delayed in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ mice (median survival of 15 weeks) as compared to ETV6::JAK2;Marilyn;*Rag2*$^{-/-}$ females (median survival of 11 weeks; Figure 9A). Of note, although leukemogenesis was delayed in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ mice, leukemic cells expressed TCRVβ5 (Figure 15A, top panels). The difference in disease onset was not due to pre-disease differences in TCRβ expression, because this was similar in normal DP thymocytes from Marilyn females and OT-I mice (Figure 9B). Delayed leukemogenesis in OT-I mice could be due to the higher affinity of TCR for self-peptide MHC complexes than the Marilyn HY-TCR. Since CD5 surface expression is regulated by TCR signal intensity

during thymocyte development (Azzam et al., 1998), we assessed the CD5 levels in the two transgenic TCR strains. As hinted from previous reports (Fulton et al., 2015; Mandl et al., 2013), DP thymocytes from OT-I mice displayed higher CD5 surface expression levels than those from Marilyn mice (Figure 9B). This indicates that the OT-I TCR has stronger affinity to self-peptides presented by MHC than the Marilyn TCR.

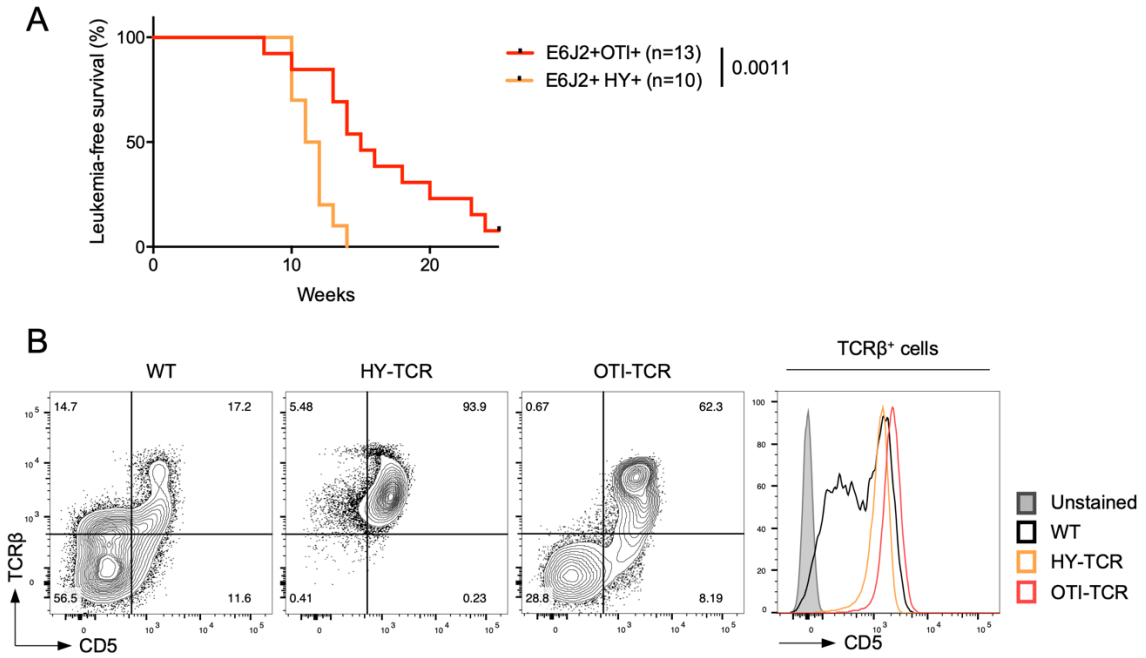


Figure 9. ETV6::JAK2-induced T-ALL onset was delayed in high-affinity transgenic OT-I TCR mice compared to lower affinity HY-TCR females. (A) Kaplan-Meier leukemia-free survival curves of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ mice and ETV6::JAK2;HY-TCR;*Rag2*$^{-/-}$ female mice. Log-rank test *P* value is shown in graph. (B) Flow cytometry analysis of TCRβ and CD5 expression in DP thymocytes of healthy wild type (WT), HY-TCR;*Rag2*$^{-/-}$ and OT-I;*Rag2*$^{-/-}$ mice. Histogram shows CD5 expression in TCRβ^{+} DP thymocytes.

Next, we set out to determine the impact of strong agonist-induced TCR stimulation on ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL leukemogenesis, first using a preventive approach. Thus, ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells were infused in *Rag2*$^{-/-}$ recipient mice and then treated the day after with OVA or PBS. Although OVA treatment failed to delay mouse survival (Figure 10A), it efficiently decreased T-ALL percentage in peripheral blood at day 17 (Figure 10B). To assess the impact of weaker agonist signaling on T-ALL leukemogenesis, we used the OVA mutant peptides SIITFEKL (T4) and SIIGFEKL (G4), which stimulate the OT-I TCR with progressively lower affinity (Salmond et al., 2014). ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL recipient mice were treated in parallel with OVA peptides on day 1 and sacrificed at day 20 post-infusion (Figure 10C). Although administration of lower affinity G4 and T4 peptides did

not lead to differences in T-ALL burden (measured by spleen and liver weight), as compared to PBS-treated counterparts, OVA treatment significantly decreased spleen weight (Figure 10D). Next, we tested whether OVA treatment would be deleterious to T-ALL leukemogenesis in a therapeutic approach. For this, mice infused with ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL were treated weekly with either OVA or PBS and sacrificed following the second treatment (day 15) (Figure 10E). Under this regimen, mice treated with OVA had a significant decrease in spleen but not liver weight (Figure 10F). IHC analysis of spleen sections from OVA-treated ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL recipient mice showed a striking reduction in CD3^{+} lymphoblasts, as compared to spleen sections of PBS-treated mice, where CD3^{+} cells were abundant (Figure 10G). Additionally, cleaved-caspase 3 staining was found in spleen histological sections of OVA-treated ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL recipient mice, but not in PBS-treated counterparts (Figure 10G). Collectively these data indicate that OT-I;*Rag2*$^{-/-}$ expression delays ETV6::JAK2-driven T-ALL development, as compared to HY-TCR expression, and that OVA-mediated OT-I TCR stimulation delays T-ALL leukemogenesis by inducing T-ALL apoptosis.

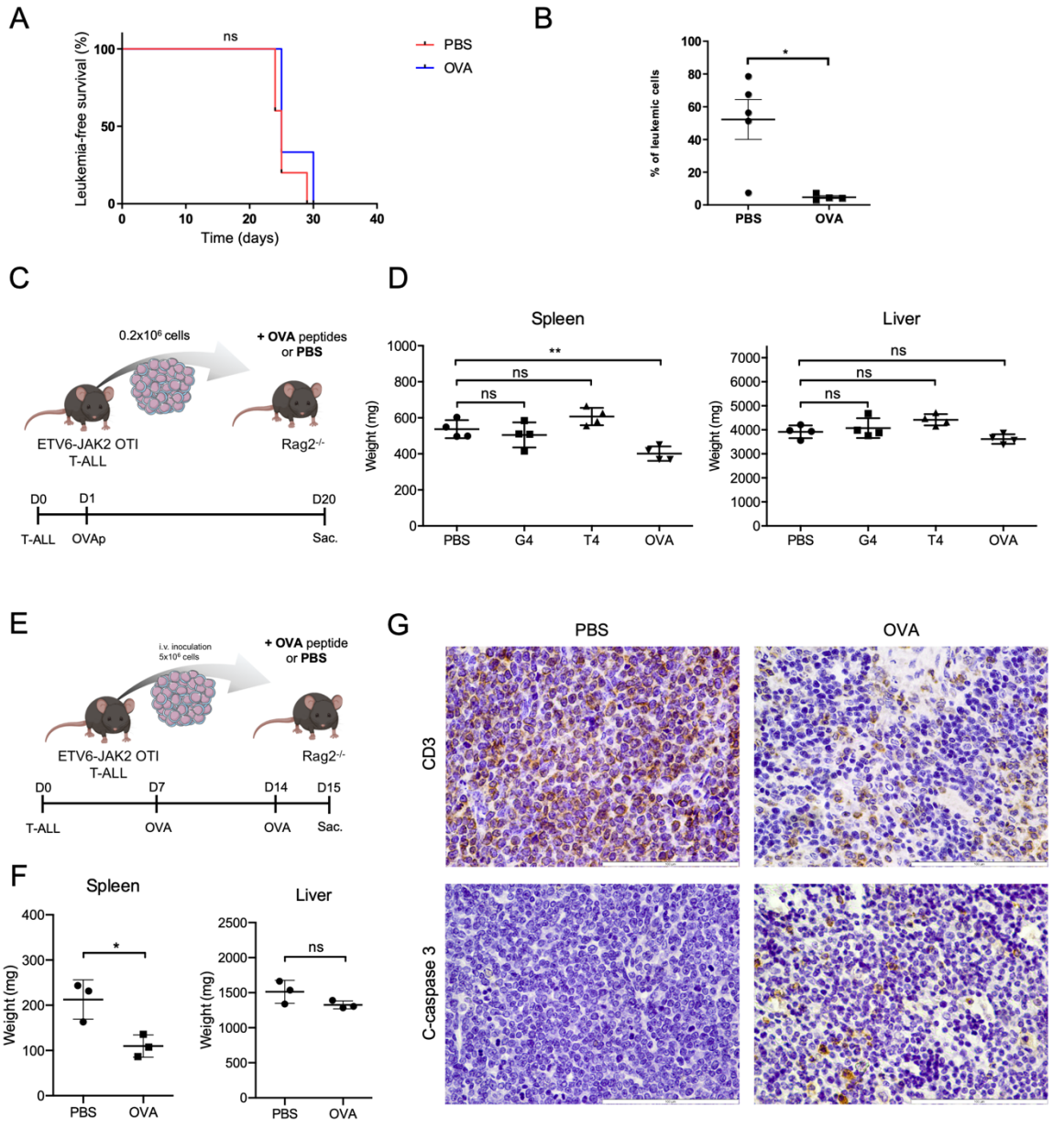


Figure 10. Strong agonist-induced OT-I TCR stimulation in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells is leukemia suppressive. (A) Kaplan-Meier leukemia-free survival curves for *Rag2*$^{-/-}$ mice infused with ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells and treated with PBS or OVA (0.01 mg) on day 1 post injection, euthanized at clinical endpoint. Differences are not statistically different (Log-rank test). (B) Percentage of CD90^{+} CD24^{+} ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells in the peripheral blood of mice from (A) at day 17 post injection. p=0.0171 (Unpaired t test with Welch's correction) (C) T-ALL cell infusion and treatment protocol scheme. *Rag2*$^{-/-}$ mice were infused with ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells and treated with PBS, OVA or OVA mutant peptides (0.01 mg) and sacrificed 20 days post infusion (n=4 for each group). (D) Spleen and liver weights of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL-infused mice in (C). One-way ANOVA test was used to compare differences between groups, multiple comparisons were made comparing each group to control. PBS vs OVA p<0.01. (E) T-ALL cell infusion and treatment protocol scheme. *Rag2*$^{-/-}$ mice were infused with 5x10^{6} ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells and treated with PBS or OVA (0.01 mg) once a week during 2 weeks and sacrificed 15 days post infusion (n=3 for each group). Spleen and liver weights of

ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL-infused mice in (E). Unpaired *t* test p=0.0235 for spleen weight. (F) Immunohistochemistry staining for CD3 and cleaved caspase-3 in representative histological sections of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL-infused mice in (E and F) treated with PBS or OVA.

2. OT-I TCR stimulation with increased affinity peptides correlates with ETV6::JAK2;OT-I T-ALL cell activation and apoptosis

To further dissect the role of OT-I TCR signal strength on leukemogenesis, we assessed the cellular effects of stimulation with peptides of different affinity. Normal OT-I thymocytes were cultured *in vitro* with OVA or the OVA mutant peptides G4 and T4. Stimulation of OT-I thymocytes with peptides with increasing OT-I TCR affinity (G4 < T4 < OVA) progressively increased the expression of the activation marker CD69 in both immature DP thymocytes and mature CD8$^+$ thymocytes (Figure 11A). Additionally, G4 stimulation induced an increase in DP thymocyte viability (measured by 7-AAD and Annexin V staining), while higher-affinity peptides resulted in increased cell death (Figure 11B-C). In contrast to DP thymocytes, TCR stimulation of CD8$^+$ thymocytes with high-affinity peptides (T4 and OVA) resulted in decreased in cell death (Figure 11B-C). These data suggest that immature thymocytes are more susceptible to being eliminated by strong TCR signals (consistent with negative selection), as previously reported (Khanom et al., 2019), while mature thymocytes survive and proliferate after strong TCR stimulation.

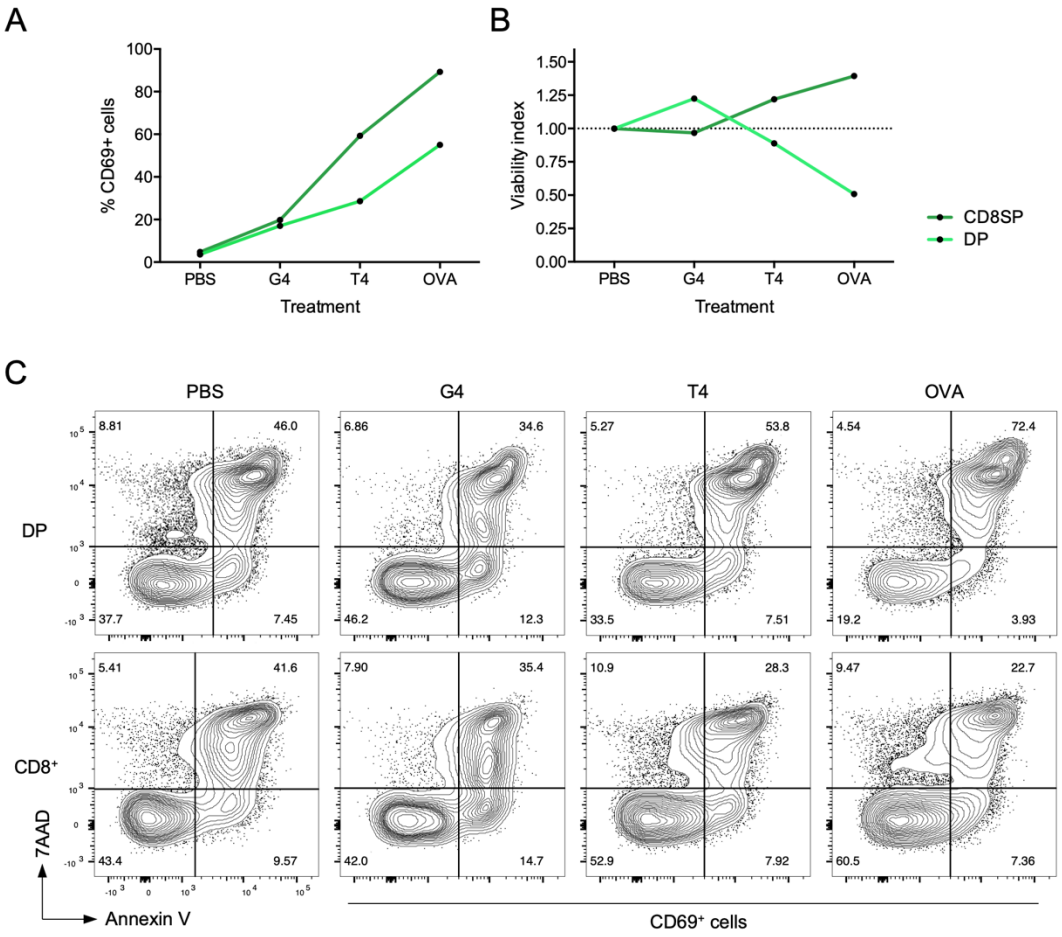


Figure 11. TCR activation of OT-I TCR thymocytes leads to apoptosis in a peptide affinity-dependent manner in DP immature thymocytes but not in mature CD8 SP cells. (A) Percentage of CD69$^+$ and (B) viability (Annexin V$^-$ and 7-AAD$^-$ cells) index of DP and CD8SP OT-I TCR normal thymocytes after *ex vivo* treatment with PBS or OVA peptides (10 μM) for 18 hours. In (B) viability was normalized to the PBS condition. (C) Flow cytometry analysis of 7AAD and Annexin V staining of OT-I TCR thymocytes treated with PBS or OVA peptides. In (B and C), for OVA peptides CD69$^+$ cells are shown.

Next, to evaluate the cellular effects of OT-I TCR stimulation with peptides of different affinity on ETV6::JAK2;OT-I T-ALL cells, these were cultured with OVA, T4 and G4 peptides. In general, treatment of T-ALL cells with all OVA peptides, even G4, the peptide with lowest affinity for OT-I, efficiently increased CD69 surface expression of TCR-Vβ5$^+$ T-ALL cells (Figure 12A and B). Concomitantly, TCR-Vβ5 expression decreased in CD69$^+$ activated cells, with stronger decrease after OVA treatment than T4 or G4 treatment. Interestingly, treatment with peptides with higher OT-I affinity was correlated with progressively increased percentage of apoptosis in CD69$^+$ activated T-ALL cells (Figure 12A and C). Different ETV6::JAK2;OT-I T-ALL samples showed different responses to OVA peptides. All ETV6::JAK2;OT-I leukemias were activated by OVA, T4 and G4 peptides, but the degree of CD69 induction after G4 treatment was highly variable (Figure 12B). Although

the T4 peptide not always induced T-ALL apoptosis, OVA consistently and significantly decreased T-ALL cell viability (Figure 12C). These data suggest that the apoptotic effect of TCR stimulation with high-affinity peptides in ETV6::JAK2;OT-I T-ALL cells more closely resembles that of normal OT-I DP thymocytes than normal CD8$^+$ counterparts, and that the ability for OVA peptides to induce CD69 surface expression is not strictly correlated with their ability to induce apoptosis in OT-I-expressing T-ALL cells.

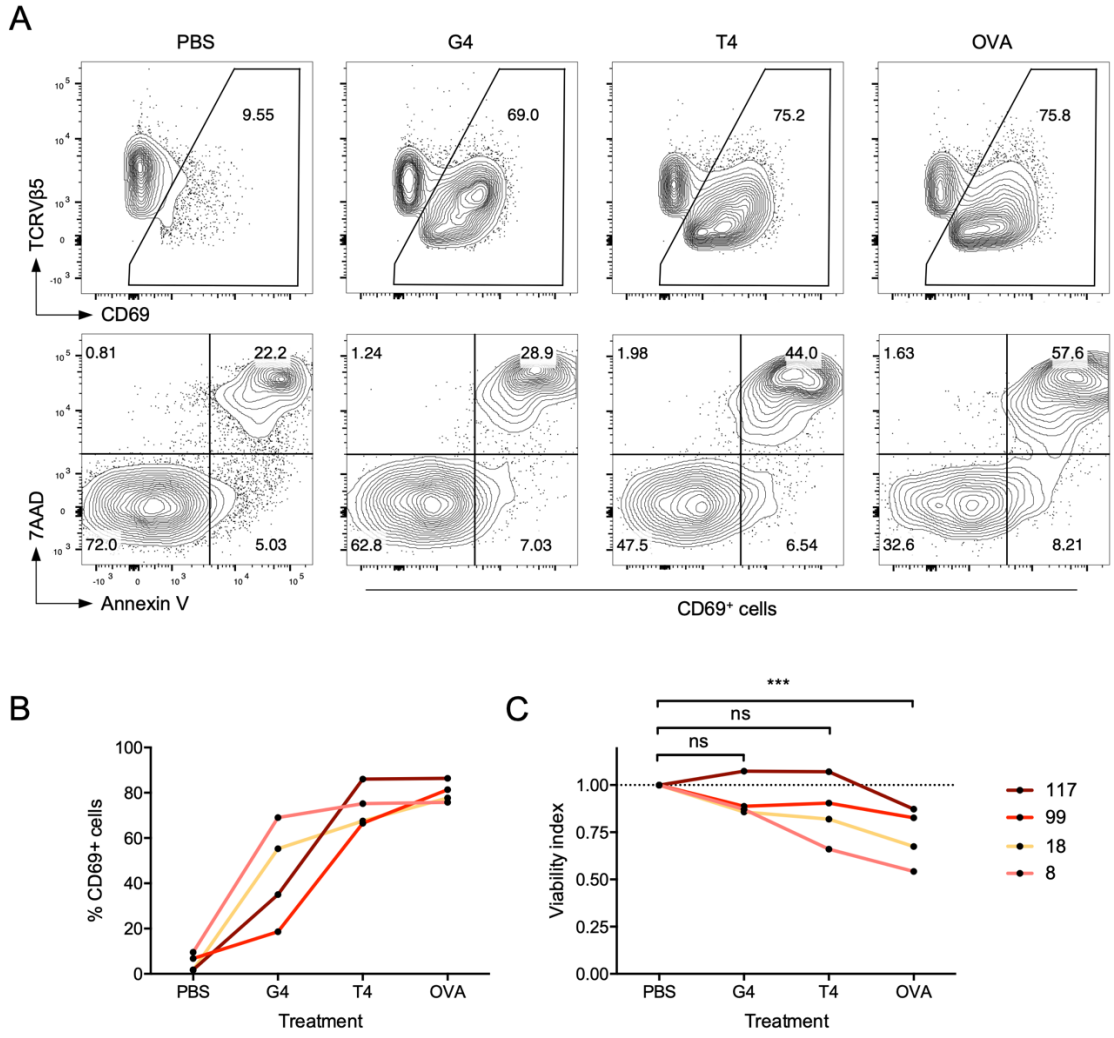


Figure 12. Agonist-induced activation of ETV6::JAK2;OT-I T-ALL cells results in apoptosis in a peptide affinity-dependent manner. (A) Representative plots for flow cytometry detection of *ex vivo* activation (TCRVβ5 and CD69 expression) and apoptosis (Annexin V and 7AAD) of ETV6::JAK2;OT-I leukemic cells following 18 h stimulation with OVA peptides or PBS. Top panels gated on CD90.2$^+$ lymphocytes and bottom panels gated on CD69$^+$ cells. (B) Percentage of CD69$^+$ cells obtained after 18 h stimulated with the indicated peptides for 4 independent leukemias. (C) Viability (Annexin V$^-$ and 7-AAD$^-$ cells) index of CD69+ cells, normalized to the PBS condition after 18 h *ex vivo* treatment of 4 independent ETV6::JAK2;OT-I leukemic samples with PBS or OVA peptides for 18 hours. One-way ANOVA test was used to compare differences between groups. Multiple comparisons were made comparing each group to PBS. ***, p<0.001.

Since lower-affinity OVA peptides (G4 and T4) were able to induce CD69 surface expression in OT-I^{+} T-ALL without triggering cell death, we next assessed the ability of OVA peptides to induce of other T cell activation markers. All OVA peptides induced high levels of CD69 and CD5 expression, whereas TCR-Vβ5 expression was concomitantly decreased with higher affinity peptide stimulation (Figure 13). Nur77 expression, an antigen-specific reporter for TCR signaling (Ashouri & Weiss, 2017), was more upregulated by OVA treatment as compared to G4 and T4, consistent with higher peptide affinity to the OT-I TCR (Figure 13). Interestingly, expression of immune checkpoint proteins PD1 and CTLA4 was induced differently by treatment with different OVA peptides. While all peptides similarly upregulated PD1 expression in CD69^{+} T-ALL cells, T4 was the most efficient peptide upregulating CTLA4 expression, while treatment with G4 and OVA resulted in modest and intermediate CTLA4 upregulation, respectively. These results indicate that TCR stimulation of T-ALL cells with peptides of varying affinity might result in the activation of different cellular programs that are not efficiently discriminated by CD69 expression.

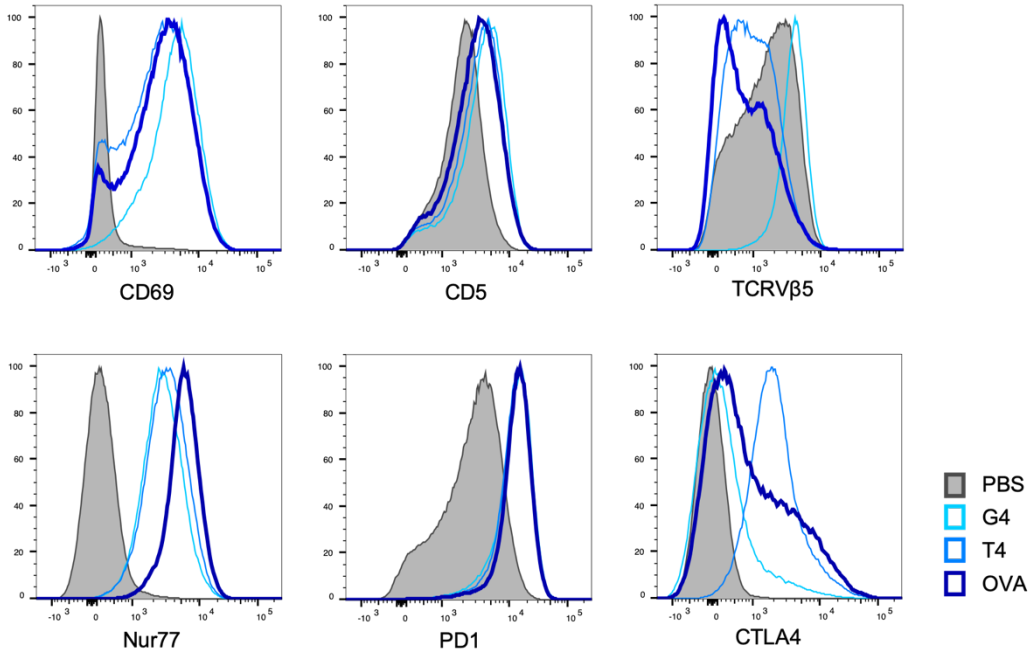


Figure 13. Strength of TCR stimulation in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL cells modulates the expression of T-cell activation markers. Flow cytometry analysis of T-cell activation markers including TCR co-inhibitory molecules PD1 and CTLA4 and TCR downregulation, in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ leukemic cells treated ex vivo with PBS or OVA peptides for 18 h. Representative of two independent experiments. CD69 expression is shown in CD8^{+} cells while expression of the markers CD5, TCRVβ5, Nur77, PD1 and CTLA4 is shown in CD69^{+} cells.

3. ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL development and TCR stimulation is not dependent on MHC-I

Because CD5 levels in developing thymocytes are established by TCR interactions with self-peptides presented by MHC and determine the TCR threshold for activation (Cho & Sprent, 2018), we next assessed whether the observed ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ delay in T-ALL onset, as compared to HY-TCR counterparts, was dependent on MHC-I. To this end, ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ mice were crossed with β-2 microglobulin (β2m) constitutive KO mice to generate ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ progeny with impaired MHC-I-mediated TCR interactions. The ETV6::JAK2;OT-I T-ALL onset on β2m haploinsufficiency or deficiency (median survival of 13 and 14 weeks, respectively) did not significantly differ from ETV6::JAK2;OT-I control mice (median survival of 15 weeks; Figure 14A). β2m-deficient mice lacked CD8^{+} thymocytes (Figure 14B), as expected from their dependency on MHC-I-mediated interactions. Confirming that TCR and CD5 surface expression levels depend on MHC interaction, TCR-Vβ5 and CD5 expression levels were decreased in β2m-deficient DP thymocytes in comparison with haploinsufficient or WT counterparts (Figure 14B).

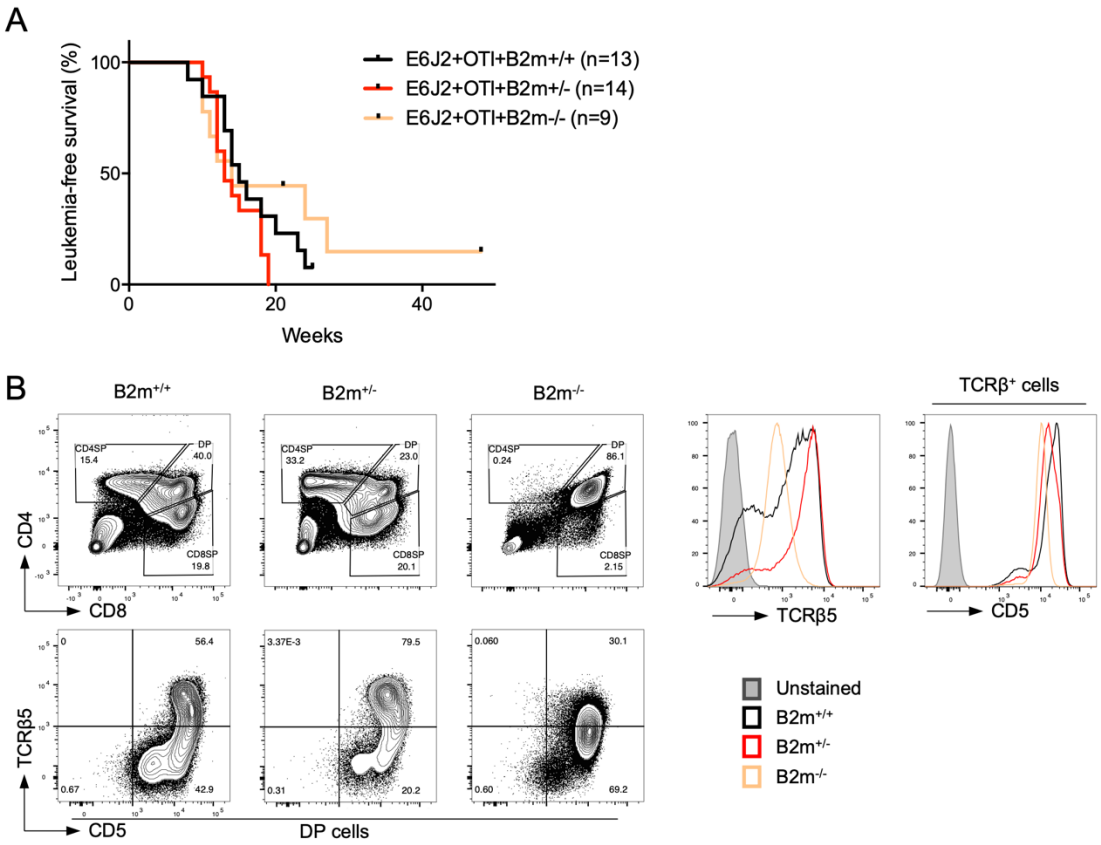


Figure 14. Leukemia development in ETV6::JAK2;OT-I mice is not MHC-dependent. (A) Kaplan-Meier leukemia-free survival curves of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ mice proficient, haploinsuficient and deficient for β2m. Differences are not statistically significant (*β2m*$^{+/+}$vs *β2m*$^{+/-}$ *p*=0.1219 and *β2m*$^{+/+}$vs *β2m*$^{-/-}$ *p*=0.4370 Log-rank test). (B) Flow cytometry analysis of TCRVβ5 and CD5 expression in normal *β2m*$^{+/+}$, *β2m*$^{+/-}$ and *β2m*$^{-/-}$ OT-I;*Rag2*$^{-/-}$ DP thymocytes. In histograms, CD5 levels were measured in TCRβ^{+} DP cells.

Although, β2m deficiency did not alter the ETV6::JAK2;OT-I CD4/CD8 immunophenotype and TCR-Vβ5 expression levels (Figure 15A), CD5 expression was significantly reduced in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ leukemias when compared to β2m WT counterparts (Figure 15A-B). Additionally, *in vitro* stimulation of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ T-ALL cells with OVA resulted in induction of CD69 expression, independently of the presence of β2m-proficient antigen-presenting cells from syngeneic (C57BL/6) or allogeneic (CBA) splenocytes (Figure 15C), indicating that activation was MHC-I-independent.

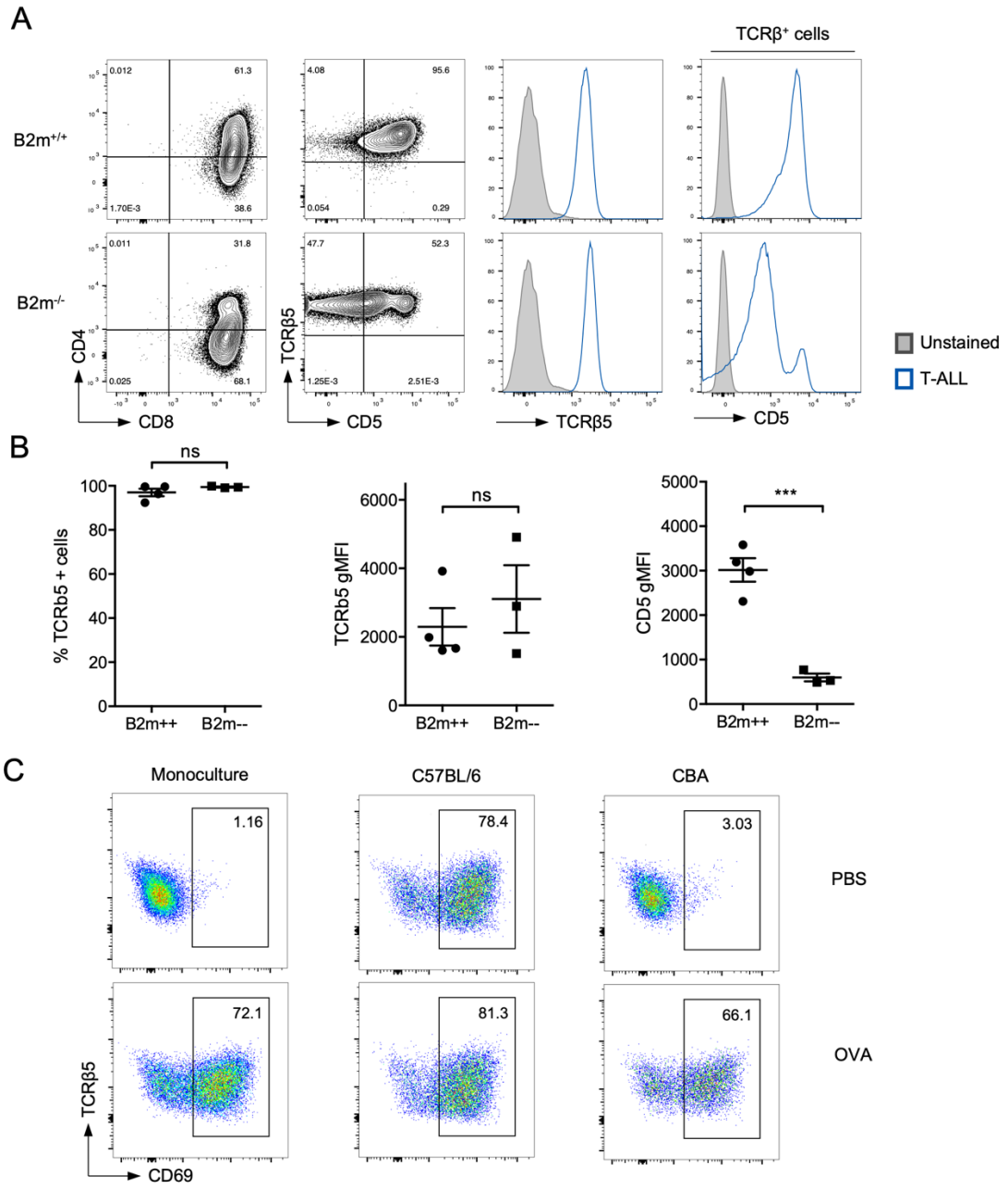


Figure 15. β2m-deficient ETV6::JAK2;OT-I T-ALL cells maintain TCR expression and can be activated by agonist-peptides in a MHC-independent manner. (A) Flow cytometry expression of TCRVβ5 and CD5 in *β2m*$^{+/+}$ and *β2m*$^{-/-}$ ET6::JAK2;OTI-TCR leukemic cells. CD5 histograms show TCRVβ5^{+} T-ALL cells. Representative plots are shown. (B) Quantification of flow cytometry percentage of TCRVβ5^{+} T-ALL cells and expression levels of TCRVβ5^{+} and CD5 in *β2m*$^{+/+}$ and *β2m*$^{-/-}$ ET6::JAK2;OT-I TCR leukemias. CD5 gMFI was measured in TCRVβ5^{+} cells. Unpaired t test. For CD5 gMFI p=0.0007. (C) Flow cytometry analysis of T-ALL expression levels of TCRVβ5 and CD69 in *β2m*$^{-/-}$ ET6::JAK2;OT-I;*Rag2*$^{-/-}$ leukemic cells after 18 hour treatment with PBS or OVA in monoculture or co-culture with MHC-I-expressing syngeneic (C57BL/6) or allogeneic (CBA) splenocytes. Representative of two independent experiments. Percentage of cells is indicated.

4. Discussion

TCRβ and/or CD3 expression is relatively frequent in human T-ALL (Asnafi et al., 2003), and because induction of agonist-induced TCR signaling was shown to suppress human and mouse T-ALL (Trinquand et al., 2016), it has been proposed as a potential target for T-ALL therapy (dos Santos et al., 2019). However, TCR signaling can have different roles in leukemogenesis and leukemia progression. While basal TCR signaling fostered T-ALL development in a TCR transgenic mouse model, strong agonist-induced TCR activation suppressed or significantly delayed leukemia progression (see Chapter 4). These observations suggest that the intensity of TCR signaling might be favorable for T-ALL up to a certain threshold when it becomes detrimental.

In this study we focused on dissecting the effect of varying the intensity of TCR stimulation, using peptides of variable affinity to a transgenic TCR expressed in a mouse model of T-ALL. We provide evidence that the intensity of TCR stimulation has different impacts on leukemogenesis and differential ability to suppress leukemia. First, we show that expression of different TCR transgenes impacts the development of ETV6::JAK2-driven T-ALL, in that expression of the MHC class I-restricted OT-I TCR significantly delayed leukemia onset in ETV6::JAK2 mice as compared to the MHC class II-restricted HY-TCR. This and the fact that OT-I TCR-expressing thymocytes showed higher levels of CD5 expression, a known regulator of TCR signaling (Azzam et al., 2001; Tarakhovsky et al., 1995) relatively to HY-TCR thymocytes, suggests that the lower CD5 expression levels may favor pro-leukemogenic basal TCR stimulation. Whether CD5 levels might have a direct impact on leukemogenesis in transgenic TCR T-ALL mouse models remains to be elucidated.

We earlier showed that DBY peptide administration delayed leukemogenesis in mice infused with Marilyn T-ALL (Chapter 4). Likewise, treatment of mice infused with OT-I^{+} T-ALL with OVA peptide, but not OVA mutant G4 or T4 peptides, significantly inhibited T-ALL infiltration in spleens and expansion in peripheral blood, and led to increased T-ALL cell apoptosis. However, these effects were not reflected in prolonged mouse survival. This discrepancy could be explained by limited elimination of leukemic cells, followed by rapid recovery of unaffected cells, or by the possibility that leukemic stem cells in specific niches were not eliminated and not twarted in their capacity to generate fatal disease by OVA peptide treatment. The possible existence of specific niches where ETV6::JAK2;OT-I;*Rag2*$^{-/-}$ T-ALL stem cells may reside and be able to resist OVA treatment is compatible with reports of leukemic stem cells present in adipose tissue accompanied by metabolic adaptation that contributes to increase T-ALL fitness (Ye et al., 2016). It is also possible that the

microenvironment of specific organs might contribute more significantly for T-ALL growth and survival than others, as has been reported for acute myeloid leukemia in the liver (Ye et al., 2021).

Interestingly, we showed that T-ALL cell TCR stimulation with peptides of increasing affinity resulted in increasing apoptosis, similarly to what is seen in immature thymocytes undergoing negative selection. Furthermore, treatment of OT-I T-ALL cells *ex vivo* with OVA peptides with increasing affinity resulted in a progressive increase in apoptosis, while not significantly affecting the percentage or levels of CD69 expression, indicating that TCR activation per se does not dictate the triggering of apoptosis and that only high-affinity interactions cross the thresholds for apoptosis induction. Interestingly, while all the peptides were comparably efficient in inducing surface CD69 in T-ALL cells, downregulation of TCRβ5 surface expression, characteristic of TCR signaling, was progressively more pronounced with treatment with increasing affinity peptides. In fact, peptides of different affinity led to differential induction of TCR activation markers: Nur77 expression was able to discriminate between strong TCR stimulation induced by OVA and weaker TCR stimulation by lower-affinity peptides (T4 and G4); PD1 was upregulated with TCR stimulation independently of peptide affinity; and CTLA4 was upregulated by treatment with the intermediate affinity peptide T4 but not as much with lower affinity G4 or high-affinity OVA. Our observations indicate that CD69, the commonly used marker of T cell activation, does not discriminate well between low- and high-affinity TCR stimulation of T-ALL cells, and that TCR stimulation with different affinity might result in the activation of specific molecular programs in T-ALL cells. Such molecular programs, for example differential expression of Nur77 and CTLA4, might determine T-ALL cell fate in response to TCR stimulation. Whereas Nur77 intermediate upregulation accompanied by CTLA4 expression in response to intermediate affinity TCR stimuli might promote T-ALL cell survival, high Nur77 upregulation and CTLA4 intermediate/low expression, in response to strong TCR stimulation, might promote apoptosis. This notion is consistent with the fact that Nur77 is a key protein in thymocyte negative selection (Calnan et al., 1995). We hypothesize that Nur77 is a master regulator of TCR-dependent T-ALL response to different stimuli and that CTLA4 is upregulated when a certain threshold of TCR signaling is achieved but not when TCR stimulation results in apoptosis. Recently, Elliot and colleagues reported that TCR stimulation induces different responses in splenic T-cells depending on time and peptide concentration. CTLA4 expression was consistently upregulated with high antigen availability and during continuous stimulation, together with other co-inhibitory molecules, such as Tigit and Lag3 (Elliot et al., 2021). Future studies are needed to elucidate the T-ALL response to stimulation with peptides of different affinity to TCR and which

transcriptional programs might be involved in defining the TCR signaling thresholds determining whether a T-ALL cell remains alive or enters apoptosis. It will also be interesting to assess whether T-ALL cells that survive strong TCR signaling are able to recalibrate their TCR signaling thresholds, as seen in splenocytes (Elliot et al., 2021).

A recent study showed that OT-I TCR transgenic mice spontaneously develop T-ALL (with approximately 10% of penetrance). T-ALL development in these mice was MHC-dependent, as demonstrated by its absence in OT-I;*β2m*$^{-/-}$ mice (Laffey et al., 2022). In accordance with these observations, transgenic OT-I;*Rag2*$^{-/-}$ mice also spontaneously developed T-ALL with similar frequency (not shown). Interestingly, *β2m* haploinsufficiency or deficiency had no impact on ETV6::JAK2 leukemia onset, although *β2m* inactivation caused a reduction in CD5 levels in normal thymocytes and ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ mice T-ALL cells, when compared to *β2m*-proficient counterparts. These results are in agreement with observations in a T-cell lymphoma model of TCR-expressing reprogramed T-cells, where TCR signaling was necessary for lymphomagenesis but interaction with MHC molecules was not required (Serwold et al., 2010). Considering these observations, we conclude that the presence of the oncogenic protein ETV6::JAK2 was sufficient to rescue MHC-dependency for T-ALL development in OT-I mice. Additionally, in ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ T-ALL cells, CD69 expression was induced *ex vivo* in response to agonist peptides in a MHC-independent manner. A few studies have reported MHC-independent αβTCR cells have been identified (Hanada et al., 2011; van Laethem et al., 2020). However, neither the ability of normal OT-I;*β2m*$^{-/-}$ cells to be stimulated by cognate peptides in a MHC-independent manner was not explored nor was the mechanism for MHC-independent activation of ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ cells. It is possible that in this context high-affinity peptides are able to stimulate ETV6::JAK2;OT-I;*Rag2*$^{-/-}$;*β2m*$^{-/-}$ leukemic cells independently of MHC interactions whereas lower affinity peptides are not, or that the process of malignant transformation in these cells is accompanied by a decreased dependency for MHC in TCR stimulation. These results highlight the fact that understanding TCR stimulation in T-ALL cells requires further elucidation and might provide useful insights for understanding the molecular basis of T-ALL.

This study highlights the heterogeneity of T-ALL response to TCR stimulation and provides evidence that TCR stimulation of T-ALL cells might induce distinct responses depending on TCR affinity with TCR. Further elucidation of the molecular programs induced by different TCR signals in T-ALL would provide useful insights for selection of patients who would benefit from TCR stimulation as a complement to standard therapy, while also aid in the

discovery of TCR signaling modulators that could be targeted to potentiate the response to TCR stimulation.

Chapter 6. *Cdkn2a* inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint

(Adapted from *Catarino et al. Exp Hematol (2022), 116,30-36.*)

Pre-T cell receptor (Pre-TCR) expression in $CD4^-/CD8^-$ double-negative (DN) thymocytes, following successful *Tcrb* locus rearrangements, is critical for the β-selection checkpoint. The pre-TCR is composed of a TCRβ chain paired with the invariant pTα protein and CD3 family proteins. Although pTα expression has been documented in a high proportion of human T-cell acute lymphoblastic leukemias (T-ALL) (Asnafi et al., 2003; Ivanyi et al., 2010), its role in leukemogenesis has been a matter of debate. In several T-ALL mouse models (Winandy et al., 1999; Allman et al., 2001), pTα or pre-TCR were shown to be essential for leukemogenesis. In turn, while not shown as essential, the pre-TCR favored leukemogenesis induced by intracellular Notch1 (ICN1) transduction (Campese et al., 2006), or transgenic expression of Notch3 (Bellavia et al., 2002), Myc (Smith et al., 2005) and ETV6::JAK2 fusion (dos Santos et al., 2007). Further indicating that pre-TCR expression can be a major driver of thymocyte malignant transformation, compensatory genetic alterations were observed in pre-TCR-deficient ETV6::JAK2-driven leukemias, including chromosome 15 gains and chromosome 4 microdeletions encompassing the *Cdkn2a* locus (dos Santos et al., 2007).

The *CDKN2A* locus encodes two distinct tumor suppressor proteins, ARF and INK4a, crucial to regulate cell survival and proliferation. *CDKN2A* deletions are common in solid and hematological malignancies, being frequent in T-ALL and specific subtypes of B-cell ALL (González-Gil et al., 2021). Additionally, *Cdkn2a* epigenetic silencing was found in the ICN1-driven T-ALL mouse model (Volanakis et al., 2013), and inactivation of *Arf* and/or *Ink4a* genes accelerated T-ALL development in mouse models driven by expression of intracellular Notch1, Tal1, Lmo2 or Hmga1a proteins (di Cello et al., 2013; Shank-Calvo et al., 2006; Treanor et al., 2011; Volanakis et al., 2009).

Arf expression was proposed to act as a fail-safe mechanism against aberrant pre-TCR-positive thymocytes by inducing cell death and impairing their maturation (Miyazaki et al., 2008). In this line, Volanakis and co-workers (Volanakis et al., 2013) reported that *Arf* prevents malignant transformation of thymocytes after β-selection, when thymocytes undergo massive proliferation after TCRβ rearrangements. However, since *CDKN2A* loss

has been detected across all maturation subtypes of human T-ALL (González-Gil et al., 2021; Hauer et al., 2011), it is likely that *Arf* and/or *Ink4a* act as tumor suppressors not only after but also before β-selection.

1. The ETV6::JAK2 fusion protein induces malignant transformation of *Rag2*-deficient thymocytes blocked at the DN3 stage

To determine whether *Cdkn2a* inactivation could act as a tumor suppressor in pre-β-selection thymocytes, we investigated a mouse model of T-ALL arising from thymocytes arrested at the DN stage. ETV6::JAK2;*Rag2*$^{-/-}$ mice develop late onset thymic lymphomas (median of 9 months) with malignant cells expressing mature markers such as CD4 and CD8.(dos Santos et al., 2007) Since *Rag2* deficiency imposes a strict arrest at the CD4^{-}/CD8^{-}/CD25^{+}/CD44^{-} DN stage 3 of thymocyte development, we assessed if ETV6::JAK2;*Rag2*$^{-/-}$ leukemia arises from thymocytes that bypass the DN3 arrest because of ETV6::JAK2 transgenic expression. Thymocyte cellularity of pre-leukemic ETV6::JAK2;*Rag2*$^{-/-}$ mice remained very low, similar to that of *Rag2*$^{-/-}$ mice (not shown). Eight of 9 mice retained the DN3 block (Figure 16A-B), indicating that the ETV6::JAK2 transgene did not systematically rescue the DN3 block caused by *Rag2* deficiency. One of 9 ETV6::JAK2;*Rag2*$^{-/-}$ thymuses presented DN4 cells (CD4^{-}/CD8^{-}/CD25^{-}/CD44^{-}), but these expressed aberrantly high levels of the immature thymocyte marker CD24 (not shown), indicating they were already transformed thymocytes. These data show that ETV6::JAK2 fusion protein does not bypass the β-selection block caused by *Rag2* deficiency and transforms malignantly cells at DN3 or earlier stage of thymocyte development.

2. *Cdkn2a* genetic inactivation accelerated T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice

Since chromosome 4 deletions encompassing the *Cdkn2a* locus were previously described in pre-TCR-deficient ETV6::JAK2 T-ALL (dos Santos et al., 2007), we performed *Cdkn2a* locus quantitative PCR (qPCR) in ETV6::JAK2;*Rag2*$^{-/-}$ (pre-TCR-deficient) T-ALL. *Cdkn2a* copy number loss, encompassing all exons, was found in 1 of 6 cases (Supplementary Figure 11). Compiling these results with reported array comparative genomic hybridization data,(dos Santos et al., 2007) we verified that *Cdkn2a* loss occurred only in pre-TCR-deficient and not in pre-TCR-proficient T-ALL (3/15 versus 0/11 cases analyzed; Figure 16C). Together, these data hint that *Cdkn2a* inactivation can cooperate with the ETV6::JAK2 fusion protein in the malignant transformation of pre-β-selection thymocytes.

Next, to determine the impact of germline *Cdkn2a* genetic inactivation on pre-TCR-deficient T-ALL, we bred ETV6::JAK2;*Rag2*$^{-/-}$ mice with the *Cdkn2a* constitutive knock-out allele to generate cohorts carrying normal or haploinsufficient *Cdkn2a* copy number. Although not statistically significant, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice developed lymphoma/leukemia tendentially faster and with higher frequency than ETV6::JAK2;*Rag2*$^{-/-}$ mice (median survival of 53 weeks versus 72 weeks, and survival proportions of 19.7% versus 49.2%, respectively; Figure 16D). ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented thymic lymphomas, reduced dissemination to the spleen and lymph nodes and immature immunophenotype, e.g. aberrant expression of CD4 and CD8, as well as high levels of CD24 and CD25, similar to that of ETV6::JAK2;*Rag2*$^{-/-}$ mice (Figure 16E-F). Of note, we could not investigate the impact of complete *Cdkn2a* deficiency on ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL development, because *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed rapid onset B-cell precursor leukemia (Chapter 7), a phenotype similar to that of *Rag2*$^{-/-}$;*Arf*$^{-/-}$ mice (Hauer et al., 2011).

We surmised the reduced latency of T-ALL in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice could be caused by a DN3 block bypass before leukemia onset. However, we found that *Cdkn2a* haploinsufficiency did not rescue the DN3 developmental block. Indeed, pre-leukemic ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented similar thymic atrophy, thymocyte hypocellularity and DN3 thymocyte developmental arrest as *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ or *Rag2*$^{-/-}$ mice (Supplementary Figure 12A-B).

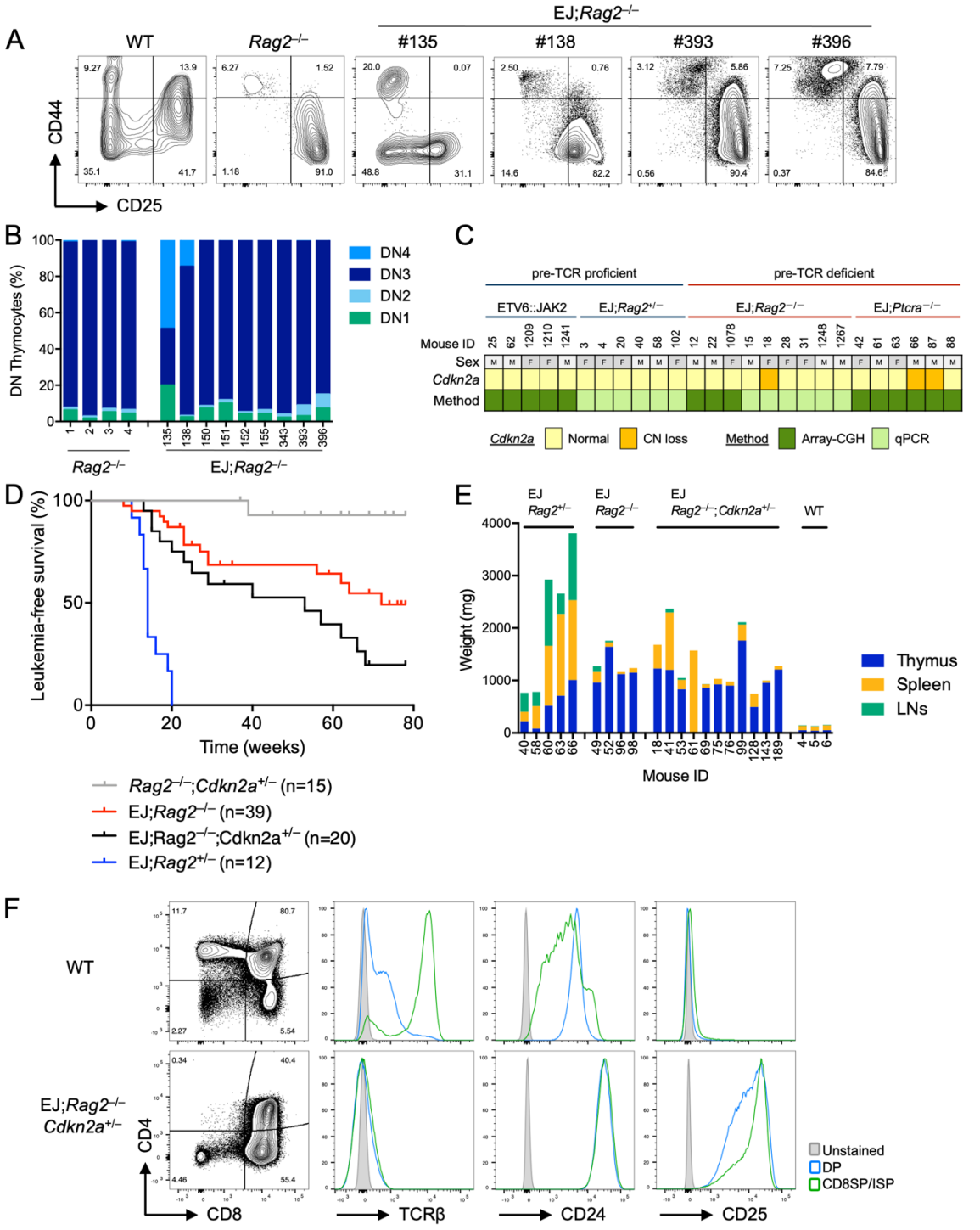


Figure 16. *Cdkn2a* genetic inactivation accelerates T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice. (A) Flow cytometry analysis of DN thymocytes (CD90.2^{+}CD4^{-}CD8) from representative 4-6 month-old *Rag2*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ preleukemic mice. Wild-type (WT) thymocytes were analyzed as a control. (B) Percentage of DN1-4 thymocytes, gated in CD90.2^{+}CD4^{-}CD8^{-} cells. Each column represents a mouse of the indicated genotype. (C) Summary of detected loss of *Cdkn2a* by quantitative PCR (Supplementary Figure 1) and array comparative genomic hybridization (Array-CGH) (dos Santos et al., 2007) in 3 of 15 pre-TCR-deficient and 0 of 11 pre-TCR-proficient T-ALL mice. (D) Kaplan-Meier leukemia-free survival curves for the indicated genotypes. Log-rank test, comparison of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ survival curves yielded p=0.0585. The hazard ratio was of 2.145, with 95% CI of 0.973 to 4.73. Mice euthanized due to

causes other than leukemia/lymphoma (e.g. solid tumors in old ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice) were censored in the analysis (tick marks). (E) Thymus, spleen and lymph node (LNs) weights of mice with leukemia/lymphoma. Each column represents a mouse. Mouse no. 61 developed B-cell leukemia and splenic lymphoma without involvement of other lymphoid organs. (F) Flow cytometry analysis of a representative ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia showing surface expression of CD4, CD8 and CD25, high levels of CD24, and absence of TCRβ expression. Expression of these markers in wild-type (WT) thymocyte subpopulations is shown as control. DP, double-positive; SP, single-positive; ISP, immature single-positive.

3. Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia

To determine whether leukemogenesis was associated with somatic inactivation of *Cdkn2a*, we assessed the *Cdkn2a* copy number status in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemic cells. Indeed, six of 10 cases showed loss of the WT allele (Figure 17A), indicating a selective advantage for complete *Cdkn2a* loss. Since ETV6::JAK2;*Rag2*$^{-/-}$ leukemias presented increased frequency of DNA copy number alterations,(dos Santos et al., 2007) we assessed the genome-wide copy number status of T-ALL from *Cdkn2a*-sufficient and -haploinsufficient mice by low-coverage whole-genome sequencing. We observed recurrent chromosomal numerical alterations in both ETV6::JAK2;*Rag2*$^{-/-}$ (occurring in 83% of cases, most frequently whole chromosome 15 gains) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL (found in 50% of cases, most frequently gains of chromosomes 4, 13 and 15; Figure 17B). These data suggest that complete *Cdkn2a* inactivation favors ETV6::JAK2-induced leukemogenesis, and that further genetic alterations are still required for malignant transformation of pre-TCR-deficient thymocytes.

4. *Cdkn2a* inactivation and Notch1-activating mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice are frequent, independent events

Since *Notch1* mutations occur frequently in human and mouse T-ALL, including ETV6::JAK2 mouse T-ALL (Ghezzo et al., 2018), and have been associated with disease progression (Albertí-Servera et al., 2021), we assessed whether the frequency of *Notch1* mutations was influenced by *Cdkn2a* allele loss. *Notch1* PEST domain (exon 34) mutations, which account for most *Notch1* mutations reported in murine T-ALL (Lin et al., 2006; O'Neil et al., 2006), were detected with similar frequency (~60%) among ETV6::JAK2, ETV6::JAK2;*Rag2*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemias, with no correlation with age of onset (Figure 17C). *Notch1* PEST domain mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL occurred independently of *Cdkn2a* copy number status, since four cases

carried both *Cdkn2a* LOH and *Notch1* mutation, two cases carried only *Cdkn2a* LOH, three cases carried only *Notch1* mutations, and one case neither alteration (Figure 17D and Supplementary Table 1). This indicates that *Cdkn2a* complete inactivation did not relieve the selective pressure for *Notch1* mutation acquisition and these two types of genetic alterations can complement each other in leukemogenesis.

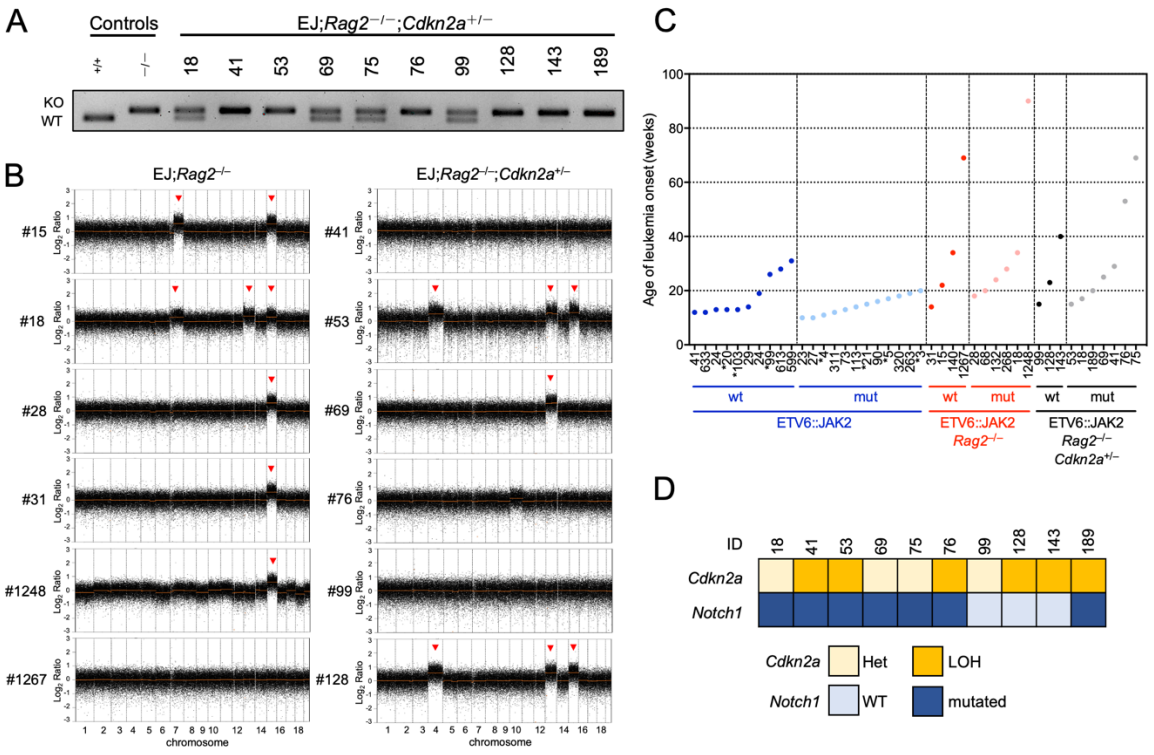


Figure 17. Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia. (A) PCR detection of wild-type (WT) and *Cdkn2a* knockout (KO) alleles in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells. Loss of the WT allele (loss of heterozygosity) was detected in 6 of 10 cases (numbers 41, 53, 76, 128, 143 and 189). (B) DNA copy number alterations assessed by low-coverage whole genome sequencing of T-ALL cells from *Cdkn2a*-sufficient and -haploinsufficient ETV6::JAK2;*Rag2*$^{-/-}$ mice. Recurrent copy number chromosomal alterations were found in both groups (chromosome 13 and 15 gains). Each graph represents DNA copy number distribution for each T-ALL case of the indicated genotype. Red arrowheads show chromosomal gains. (C) Schematic representation of age of T-ALL onset in mice of the indicated genotypes with or without *Notch1* exon 34 PEST domain mutation (mut and wt, respectively). Each dot represents a mouse. Asterisks mark mice of the ETV6::JAK2;*Rag2*$^{+/-}$ genotype (D) Schematic representation of detected *Cdkn2a* loss-of-heterozygosity (LOH) and *Notch1* mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases. *Het*=heterozygous

5. Discussion

In summary, using the ETV6::JAK2-driven T-ALL mouse model we show that *Cdkn2a* suppresses leukemogenesis in immature pre-β-selection thymocytes. This notion is supported by two main pieces of evidence. First, germline loss of one *Cdkn2a* copy aggravated leukemogenesis in ETV6::JAK2;*Rag2*$^{-/-}$ mice without bypassing the DN3 thymocyte developmental block. Second, *Cdkn2a* somatic allele loss was frequently observed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells, which suggests a selective advantage for total inactivation of *Cdkn2a*. Although *Cdkn2a* genetic inactivation facilitated thymocyte leukemogenesis, *Cdkn2a*-defective leukemic cells still presented other genetic alterations, such as *Notch1* mutations and chromosomal gains. This indicates that *Cdkn2a* deletion does not fully compensate for the absence of a pre-TCR signaling complex, and secondary alterations, such as *Notch1*-activating mutations, are required for leukemogenesis. The fact that 40% of cases retained the wild-type allele indicates that either *Cdkn2a* haploinsufficiency conferred a selective advantage for leukemic cells or *Cdkn2a* is inactivated through other mechanisms, such as epigenetic silencing, previously reported in Notch1-driven mouse T-ALL (Volanakis et al., 2013).

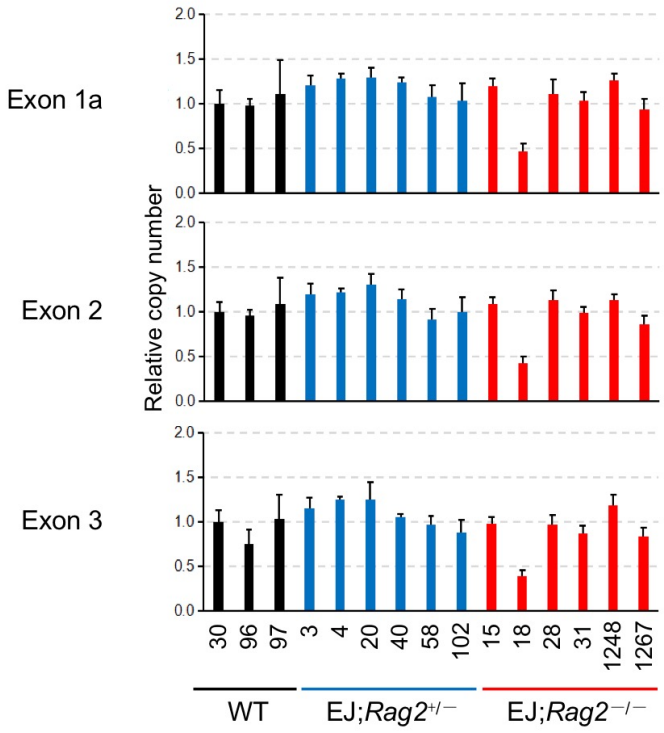
In two cases (nos. 53 and 128), *Cdkn2a* loss of heterozygosity (LOH) co-occurred with gain of chromosome 4, which harbors the *Cdkn2a* locus. *Cdkn2a* deletion in these cases with chromosome 4 gain was not detected by low-coverage sequencing, which suggests that the chromosome carrying the wild-type *Cdkn2a* allele was lost, resulting in LOH, and the chromosome carrying the mutant allele was triplicated. This conjecture is reminiscent of uniparental isodisomies of chromosome 9 segments harboring CDKN2A, which were detected in human T-ALL (Karrman et al., 2015).

Although *Cdkn2a* haploinsufficiency favored leukemogenesis, leukemia onset in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice occurred much later than in pre-TCR-proficient ETV6::JAK2 transgenic mice (median survival of 14 weeks; $P<0.0001$, log-rank test). The chance for acquisition of genetic alterations in *Rag2*-deficient pre-β-selection thymocytes is purportedly very low, due to absence of Rag-mediated mutagenic recombination events (Bianchi et al., 2019) and low rate of cell division. Although at this stage we cannot determine which events and in which order are required for leukemogenesis, we posit DN3 thymocytes expressing the ETV6::JAK2 fusion protein undergo malignant transformation upon acquisition of an odd oncogenic event that either inactivates completely *Cdkn2a* or cooperates with reduced INK4a/ARF tumor suppressive activity. Loss of Arf has been shown to promote leukemia self-renewal (Treanor et al., 2011), so this might be a crucial

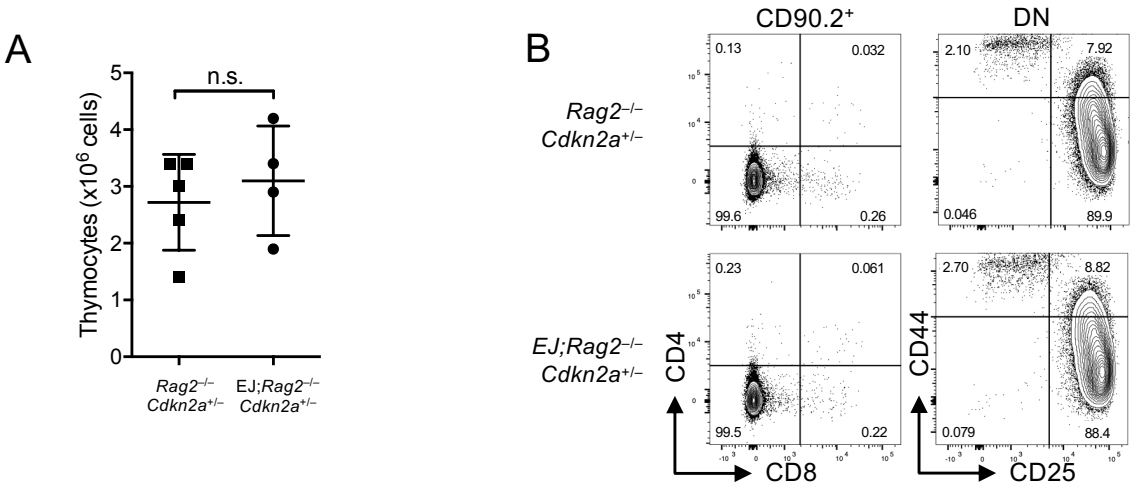
event in pre-β-selection thymocytes. *Notch1* mutations or other events (e.g. chromosome 13 or 15 trisomy) may bolster the self-renewal or malignant phenotype of leukemic cells with haploinsufficient or complete inactivation of *Cdkn2a*. *Notch1* mutations in our model are likely secondary events, paralleling human T-ALL, where *NOTCH1* mutations are often found in minor subclones (Albertí-Servera et al., 2021; Karrman et al., 2015).

Our results support the notion that *Cdkn2a* acts as a brake for leukemogenesis at early stages of thymocyte development before formation of the pre-TCR complex. Other reports indicated that *Cdkn2a* is an important tumor suppressor in post-β-selection thymocytes (Volanakis et al., 2009), which undergo massive proliferation and are prone to genetic alterations. These data and the occurrence of *CDKN2A* deletions in all subtypes of human T-ALL (Zhang et al., 2012) allow us to conclude that *Cdkn2a* acts as a gatekeeper for leukemogenesis from the most immature until the post-β-selection stages of thymocyte development.

6. Supplementary Figures



Supplementary Figure 10. *Cdkn2a* loss was found in *Rag2*-deficient T-ALL. Quantitative PCR of the indicated *Cdkn2a* exons in genomic DNA from leukemic cells of ETV6::JAK2;*Rag2*$^{+/-}$ (EJ;*Rag2*$^{+/-}$) and ETV6::JAK2;*Rag2*$^{-/-}$ (EJ;*Rag2*$^{-/-}$) mice and DNA from wild-type (WT) mice. One in 6 *Rag2*-deficient T-ALL cases showed loss of *Cdkn2a* (no. 18). Relative copy number was determined by normalization to *Vcpip1* gene (chromosome 1), but similar results were obtained upon normalization to *Spna2* gene (chromosome 2; not shown). Bars represent standard deviation.



Supplementary Figure 11. *Cdkn2a* haploinsufficiency does not rescue the DN3 block caused by *Rag2* deficiency. (A) Thymocyte cellularity of 11-week-old, pre-leukemic *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=5) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=4) mice (each symbol represents one mouse, mean and SD are shown). (B) Representative flow cytometry plots show CD90.2-positive thymocytes (left panels) and CD90.2-positive DN thymocytes (right panels) of the indicated genotype blocked at DN3 stage of differentiation.

Mouse ID	*Cdkn2a* copy number	*Notch1* exon 34	Chromosome alterations
ETV6::JAK2;*Rag2*$^{+/-}$			
3	wt	c.2351, Ins ATTA	n.d.
4	wt	c.2399, Ins CGGG	n.d.
20	wt	wt	n.d.
40	wt	n.d.	n.d.
58	wt	n.d.	n.d.
102	wt	n.d.	n.d.
ETV6::JAK2;*Rag2*$^{-/-}$			
15	wt	wt	+7 +15
18	Loss	c.280,Ins A	+7 +13 +15
28	wt	c.2398, Ins AGGGAT	+15
31	wt	wt	+15
1248	wt	c.2361, Del G, Ins CC	+15
1267	wt	wt	none
ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$			
18	het	c.2361, Del GG, Ins CCC; c.2364 Ins G	n.d.
41	LOH	c.2362, Ins GGG	none
53	LOH	c.2421, Ins GAC, Dup TGAGTGGGGAGCCCAGTCAGGCAGATGTACAAC	+4 +13 +15
69	het	Mut*	+13
75	het	c.2361, Ins CC	n.d.
76	LOH	c.2389, Del CC, Ins GAG	none
99	het	wt	none
128	LOH	wt	+4 +13 +15
143	LOH	wt	n.d.
189	LOH	c.2363, Ins GGCTCGG	n.d.

Supplementary table 1. Summary of genetic alterations found in leukemic cells of the indicated genotypes. *Notch1* mutations occurred concomitantly with *Cdkn2a* loss in one ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL case (no. 18) and with *Cdkn2a* loss of heterozygosity (LOH) in four ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases (numbers 41, 53, 76 and 189). *Mouse no. 69 presented a *Notch1* exon 34 frameshift mutation but the altered sequence was not identified. n.d., not determined.

Chapter 7. The ETV6::JAK2 fusion protein cooperates with *Cdkn2a* deletion to promote early onset precursor B-cell leukemia infiltrating the central nervous system

The *CDKN2A* tumor suppressor gene is commonly altered in B-cell ALL. In fact, *CDKN2A* deletion together with JAK2 kinase-activating mutations and *IKZF1* and *PAX5* genetic alterations, have been found in the Ph-like ALL subgroup of high-risk B-ALL. Ph-like ALL shares a gene expression signature with BCR::ABL1-positive ALL, despite lacking BCR::ABL1 fusions (Roberts, Li, et al., 2014). Ph-like B-ALL represents approximately 25% of all B-ALL cases in adults and is associated with frequent relapse, with particularly frequent central nervous system (CNS) involvement despite prophylactic intrathecal therapy (Frishman-Levy & Izraeli, 2017; Krishnan et al., 2010). Recurrent genetic alterations identified in Ph-like B-ALL (e.g. JAK2 alterations and deletions of *CDKN2A* and *IKZF1*) are associated with poor prognosis and resistance to therapy (Steeghs et al., 2019). However, these alterations are not currently used as molecular targets for therapy, creating the need for better understanding of disease biology and for the development of new therapeutic strategies.

1. ETV6::JAK2 transgene expression cooperates with *Cdkn2a* inactivation accelerating B-ALL development in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice

Since it has been reported that *Rag2*$^{-/-}$*;Arf*$^{-/-}$ mice develop B-cell hematological tumors (Hauer et al., 2011), the ETV6::JAK2 fusion protein can induce B-cell lymphomas in mice (dos Santos & Ghysdael, 2006) and *Cdkn2a* haploinsufficiency can accelerate and increase the frequency of T-ALL in *Rag2*$^{-/-}$*;Cdkn2a*$^{-/-}$ mice (Chapter 6), we sought to assess if ETV6::JAK2 expression could cooperate with complete *Cdkn2a* inactivation on leukemogenesis. The EμSRα-ETV6::JAK2 transgenic mice express this fusion protein in the lymphoid lineage, so it can drive both T and B leukemogenesis (Carron et al., 2000; dos Santos & Ghysdael, 2006). Interestingly, both *Rag2*$^{-/-}$*;Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$*;Cdkn2a*$^{-/-}$ mice developed fatal hematological pathology (splenomegaly, lymphadenopathy and high leukocyte counts; Figure 18A) with very frequent liver and lung infiltration by leukemic cells (not shown), but with significantly reduced latency for the latter (median latency of 28 and 15 weeks, respectively; Figure 18B). Although mice of both genotypes died relatively young with leukemia, solid tumors were occasionally detected in older animals, likely due to absence of *Cdkn2a* in all mouse tissues (not shown). Flow cytometry

immunophenotyping revealed that nearly all analyzed *Cdkn2a*-deficient mice with hematological disease developed B220-positive B-ALL, typically with pro-B immunophenotype ($CD43^+/CD24^+/CD127^+/CD25^+$, and BP-1 (Ly51) heterogeneous expression; Figure 18C; Supplementary Table 2). Of note, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells expressed significantly lower CD127 (IL-7Rα) levels than *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ counterparts and showed a trend for higher CD25 (IL-2Rα) expression levels (Figure 18D). Moreover, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells reinitiated disease in *Rag2*$^{-/-}$ recipient mice (Supplementary Table 2). Collectively these data indicate that *Cdkn2a* absence in immature B cells leads to B-cell precursor ALL, which is accelerated by ETV6::JAK2 expression.

Since *Rag1* or *Rag2* recombinase deficiency precludes immunoglobulin gene recombination and blocks B-cell differentiation at the pro-B (Hardy fraction C) stage in mice (Spanopoulou et al., 1994), we assessed whether *Cdkn2a* deficiency with or without ETV6::JAK2 expression could skew immature B-cell differentiation before overt disease development. However, no significant differences in B-cell stages from young non-diseased mice were observed (Supplementary Figure 13A-B), suggesting alterations in B-cell marker expression occurred after B-ALL development.

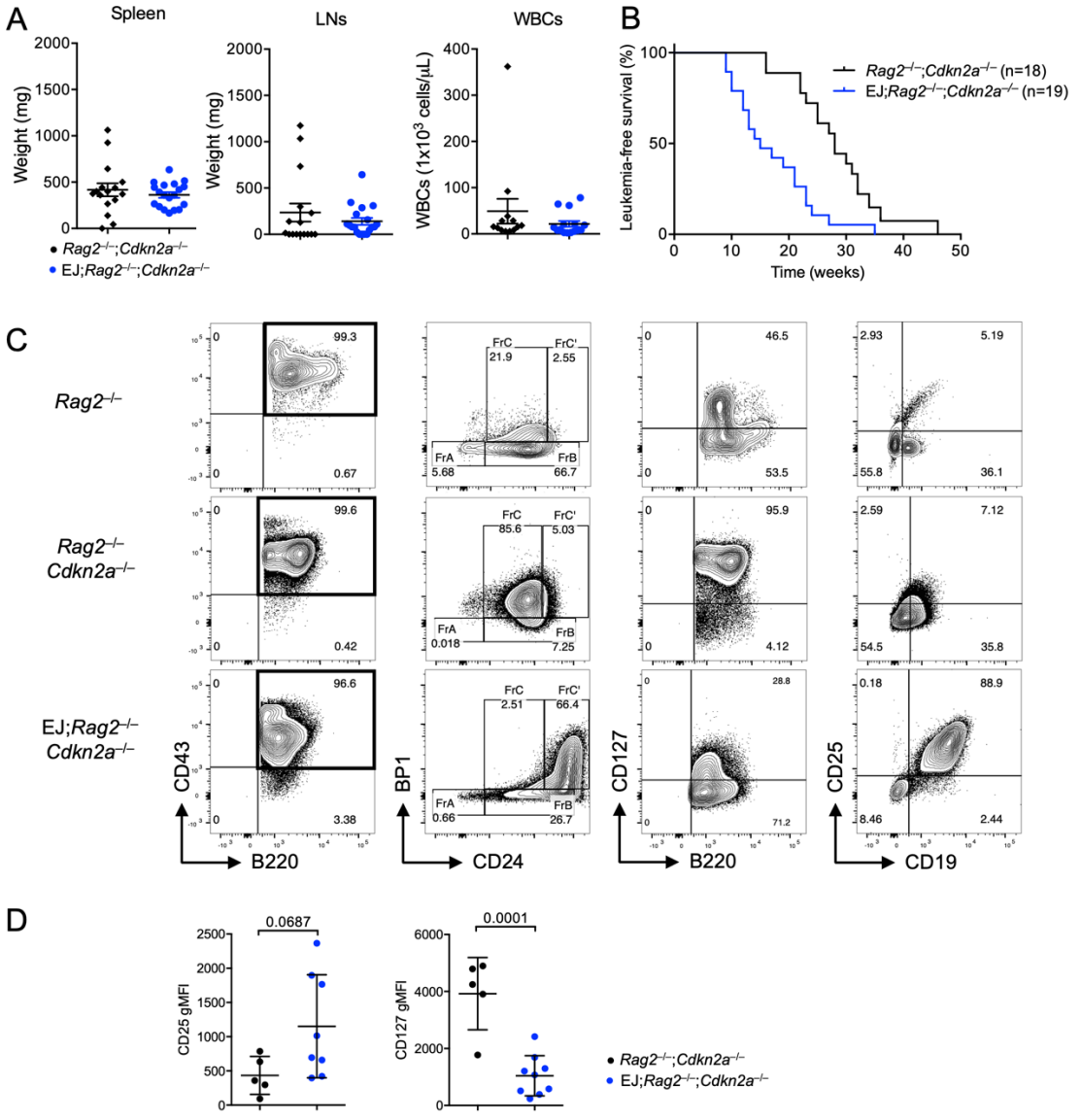


Figure 18. ETV6::JAK2 transgene expression accelerates B-ALL development in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice. (A) Kaplan-Meier leukemia-free survival curves for ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice. Log-rank test p=0.0002. (B) Organ weights (spleen, LNs) and WBC count of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=19 for organ weight and n=15 for WBC) and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=16 for organ weight and n=13 for WBCs) mice with leukemia. (C) Immunophenotypic analysis of representative ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ murine ALL splenic samples and *Rag2*$^{-/-}$ bone marrow control with the indicated surface markers. Expression of CD24, BP1, CD127 and CD25 were assessed on B220^{+}CD43^{+} cells, gated as shown in thick line rectangle. (D) CD25 and CD127 geometric mean fluorescence index (gMFI) in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=9 for CD127; n=8 for CD25 expression) and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=5) ALL cases gated in B220^{+};CD43^{high} cells. Each symbol represents a mouse, line and error bars represent mean and SD, and p-value (unpaired two-tailed t-test) is indicated in the graph.

2. B-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice was associated with extensive CNS infiltration and features of high-risk Ph-like ALL

Rag2$^{-/-}$;*Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ diseased mice often presented locomotor alterations (paraparesis or paraplegia), indicative of CNS infiltration, which were more frequent and severe in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice (Figure 19A). By histological analysis and B220 and PAX5 immunohistochemistry, we observed extensive infiltration of skull and vertebral leptomeninges in both ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice (Figure 19B and Supplementary Figure14). We questioned whether these leukemias expressed proteins involved in CNS infiltration by B-ALL cells, such as CD49f (integrin alpha-6 (Itgα6)) (Yao et al., 2018), CXCR4 and receptor activator of nuclear factor κB ligand (RANKL) (Rajakumar et al., 2021). While ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells expressed little to no RANKL and expressed CXCR4 at similar levels, Itgα6 expression was significantly higher in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias (Figure 19C and Supplementary Figure 15). Itgα6 is commonly found in human B-ALL (DiGiuseppe et al., 2009) and is thought to mediate CNS infiltration as well as survival of chemotherapy-treated B-ALL cells (Gang et al., 2020; Yao et al., 2018). These data further highlight the similarities of the ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mouse ALL model to human B-ALL and its potential as a pre-clinical model to identify molecular targets for CNS-infiltrating B-ALL.

To further characterize ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL, we performed transcriptomic analysis by bulk RNA-sequencing. Principal component analysis and unsupervised hierarchical clustering segregated ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL samples (Supplementary Figure 16A-B). Analysis of differential gene expression showed reduced expression of B-cell differentiation genes (*Rag1*, *Il7r*, *Ebf1*, *Myb*, *Lef1*, *Erg*, *Bach2*, *Lhx2*, *Cd93* and *Cd72*) in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells (Figure 19D). Canonical pathway analysis showed that ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias activated unfolded protein response (e.g. *Xbp1, Calr* and *Atf6*), and leukocyte extravasation and integrin signaling (*Itgam*, *Mmp8*, *Pik3r5*, and *Selplg*) pathways, as compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias (Figure 19E). Additionally, both ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias expressed several human Ph-like ALL signature genes (Chiaretti et al., 2018; Harvey et al., 2013), indicating both murine B-ALL types can be models for Ph-like ALL (Supplementary Figure 16C).

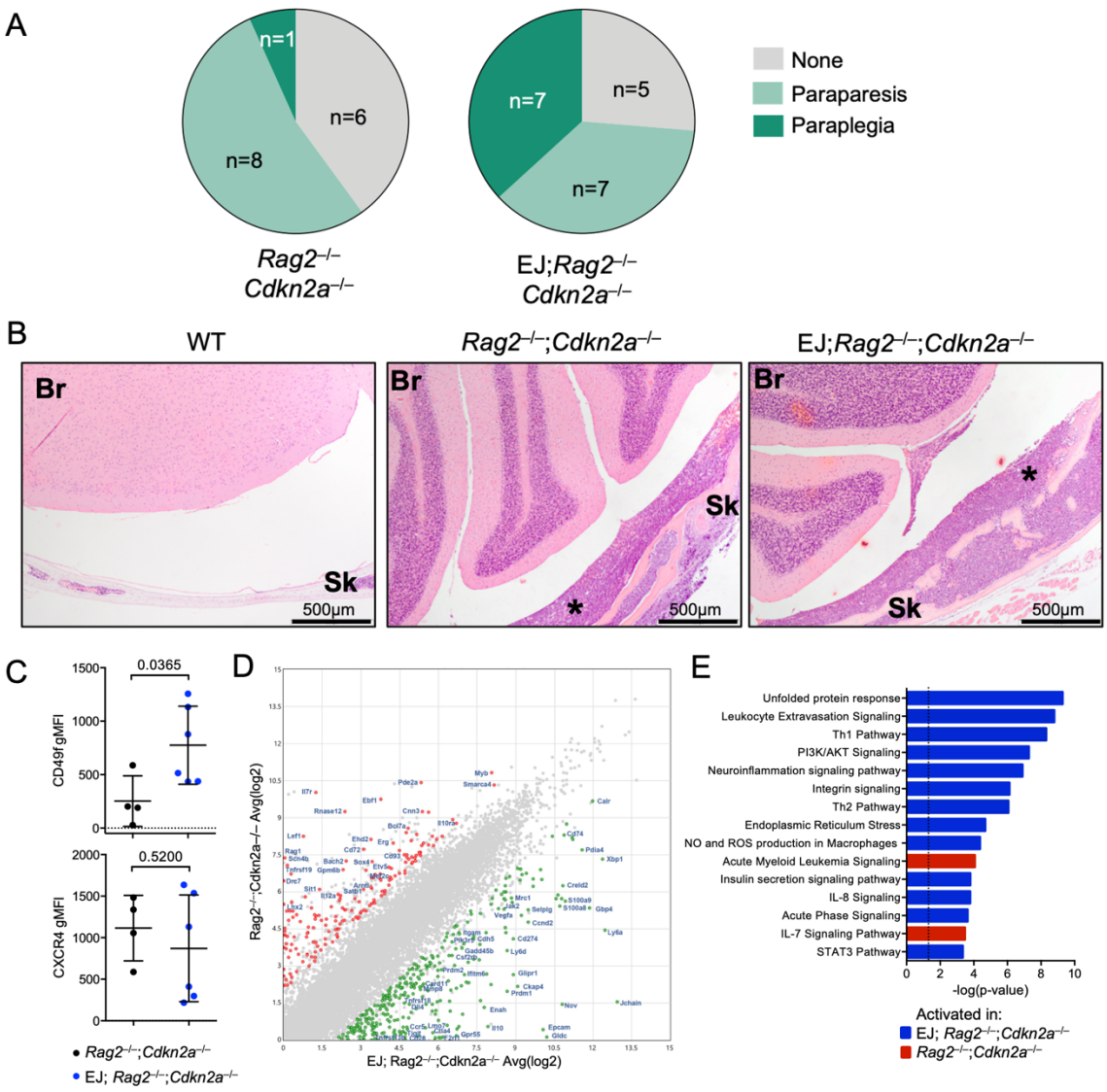


Figure 19. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed B-ALL with CNS infiltration and high-risk features. (A) Classification of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=19) and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=15) mice presenting no, mild (paraparesis) or severe (paraplegia) locomotor symptoms. Differences were not statistically significant. (B) Hematoxylin and eosin staining of representative wild-type (WT), ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mouse skull sections showing extensive leptomeningeal leukemia infiltration (marked by asterisk) of skull leptomeninges. Sk, skull bone; Br, brain. (C) CD49f and CXCR4 expression levels in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=4) and *ETV6::JAK2;Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=6 for CD49f and n=5 for CXCR4) ALL splenic leukemic cells gated in B220^{+};CD43^{high} cells. Each symbol represents a mouse, line and error bars represent mean and SD, and *p*-value (unpaired two-tailed *t*-test) is indicated in the graph. (D) Scatter plot representation of top 499 differentially expressed genes based on comparison of average expression in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=3) and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ (n=3) B-ALL cells (E) Representation of top 15 differentially expressed canonical pathways in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ counterparts based on -log(*p*-value). Threshold value is represented and corresponds to *p*=0.05.

3. Discussion

B-ALL detection in the CNS is associated with poor prognosis and the need for specific therapy. Additionally, B-ALL CNS infiltration is more frequent in relapse than at diagnosis. Prophylactic intrathecal chemotherapy frequently decreases CNS relapse in B-ALL patients however, it is associated with morbidity (Frishman-Levy & Izraeli, 2017; Krishnan et al., 2010). Although the mechanisms for B-ALL CNS infiltration are still largely unknown, JAK/STAT pathway activation has been reported to increase CNS colonization by B-ALL cells in mice (Williams et al., 2014).

Here we show that both *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed early onset B-ALL, accelerated in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice as compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ littermates. No overt alterations in the composition of immature B cell populations in the bone marrow was found in both models before detection of B-ALL cells. *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells showed similar immunophenotype, but among the differences found between the two models was a higher expression level of CD127 (IL-7Rα) in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and a tendency for higher CD25 (IL-2Rα) expression in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ counterparts. Interestingly, and activated IL-7Rα mutant was reported to be sufficient to drive B-ALL development in mice (Almeida et al., 2021; Thomas et al., 2022). Although not addressed here, it is possible that constitutively activated JAK2 and subsequent JAK/STAT signaling (Carron et al., 2000) in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells contributed for a lower dependency on IL-7 as compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ counterparts, potentially contributing to a better adaptation to environments where IL-7 is reduced. The functional consequence of high CD25 expression in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL is unclear. Of note, CD25 has been reported as a stem cell marker in acute myeloid leukemia (Saito et al., 2010). More recently, it was reported that CD25 expression was found more frequently in BCR::ABL1-positive than other types of B-ALL, and that CD25 expression in BCR::ABL1-negative patients was associated with residual desiease, and therefore worse prognosis (Aref et al., 2020; Chen et al., 2016). Additionally, Aref and colleagues reported that CD25 and CD123 coexpression in B-ALL patients was associated with CNS infiltration and poor outcome (Aref et al., 2020). These data are therefore consistent with our observations of development of an earlier onset and more severe CD25-positive B-ALL in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice.

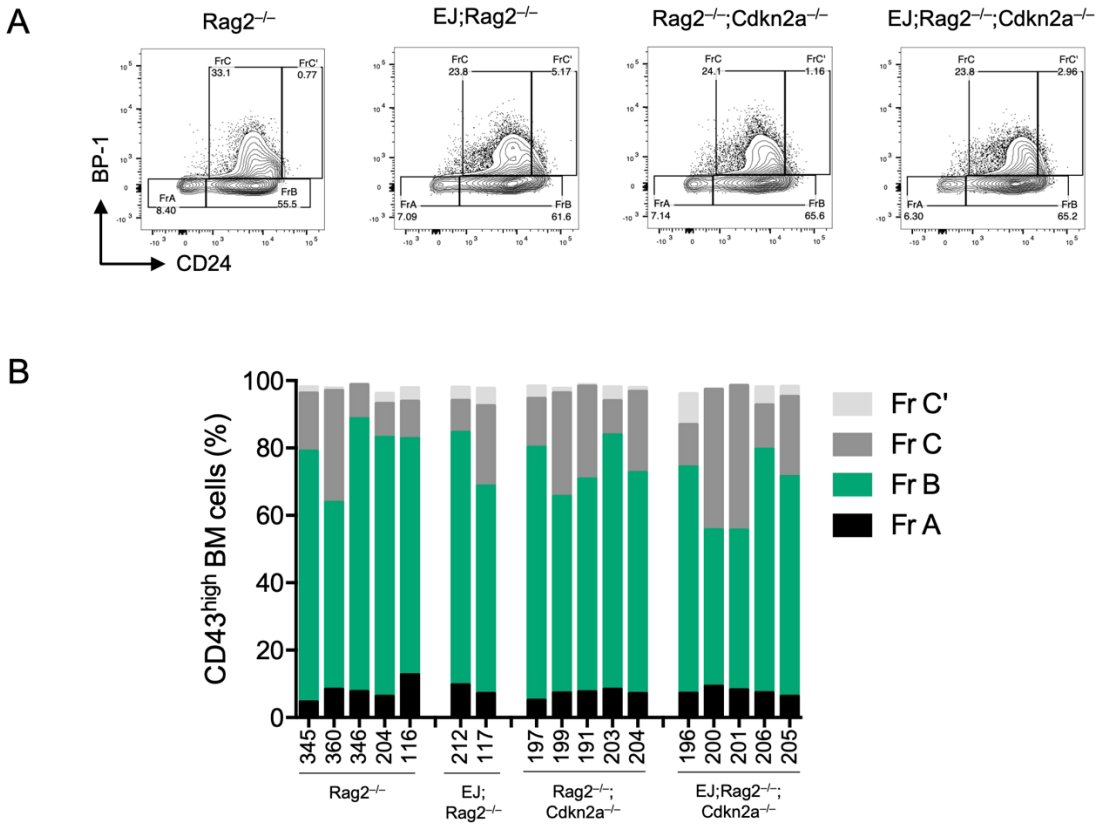
Both *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ presented signs of locomotor impairment (such as paraparesis and paraplegia), indicative of CNS infiltration, more

frequent and more severe in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice. These findings are consistent with other CNS-infiltrating B-ALL mouse models (Gladdy et al., 2003). Furthermore, extensive cranial leptomeningeal as well as spinal cord infiltration was detected in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice, although with no apparent difference in the extension of infiltration between the two models.

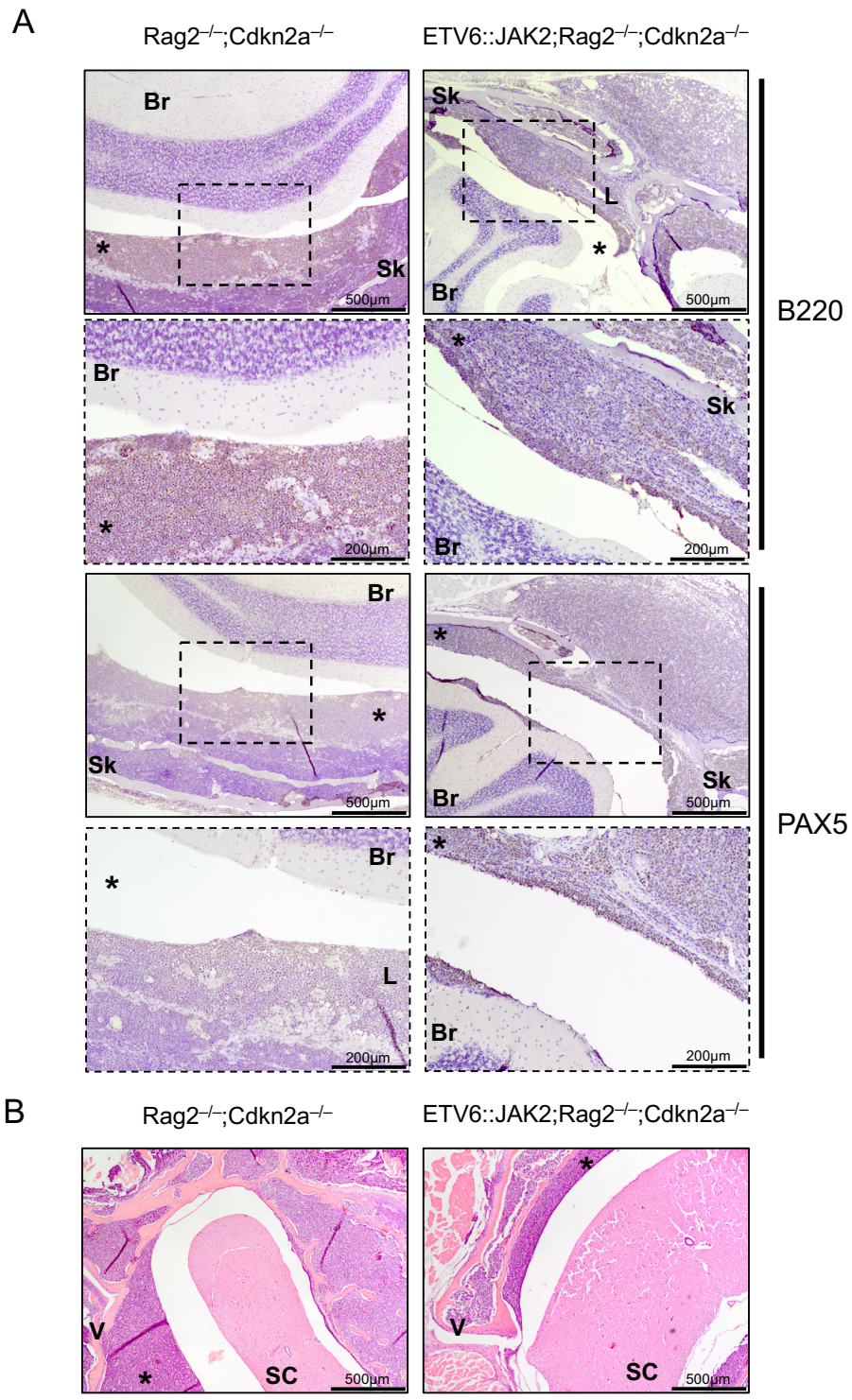
Interestingly, both *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells expressed similar levels of CXCR4, shown to mediate CNS colonization of mouse T-ALL and B-ALL (Jost et al., 2016; Rajakumar et al., 2021), but not RANKL, reported as a player in B-ALL CNS infiltration in a different mouse model (Rajakumar et al., 2020). Additionally, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells expressed significantly higher levels of Itgα6, thought to mediate B-ALL cell CNS colonization and resistance to therapy (Gang et al., 2020; Yao et al., 2018), which might explain the higher frequency of signs of neurological impairment such as paraparesis/paraplegia found in these animals.

Transcriptome analysis showed activation of leukocyte extravasation and integrin signaling in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells, consistent with observations of increased Itgα6 expression. Unfolded protein response, also significantly activated in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells as compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ counterparts, has been reported in B-ALL, particularly associated with poor outcome and activation of *Xbp1* mediated by STAT5 induced by BCR::ABL1 (Kharabi Masouleh et al., 2014). Interestingly, in melanoma preclinical models, Xbp1 expression was shown to favor tumor growth via cholesterol synthesis (Cousins et al., 2022; Yang et al., 2022). In turn, cholesterol synthesis in B-ALL was shown to be associated with CNS involvement. That, in addition to the expression of several genes characteristic of the Ph-like B-ALL signature in both models, suggest that ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice, particularly, but also *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice, can be murine models of high-risk, CNS-infiltrating, Ph-like B-ALL and might be a useful tool to identify the mechanisms of CNS infiltration and resistance to therapy.

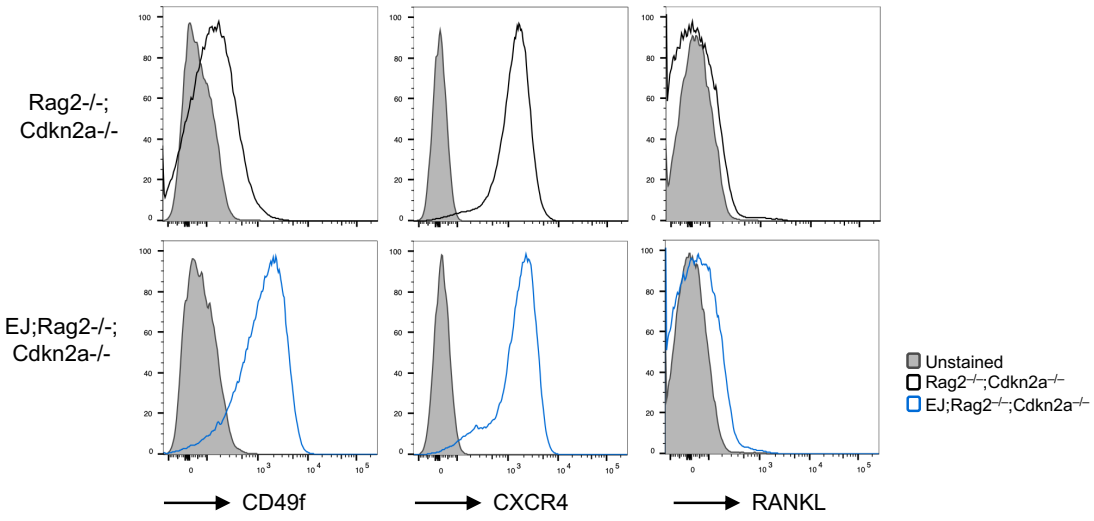
4. Supplementary Figures



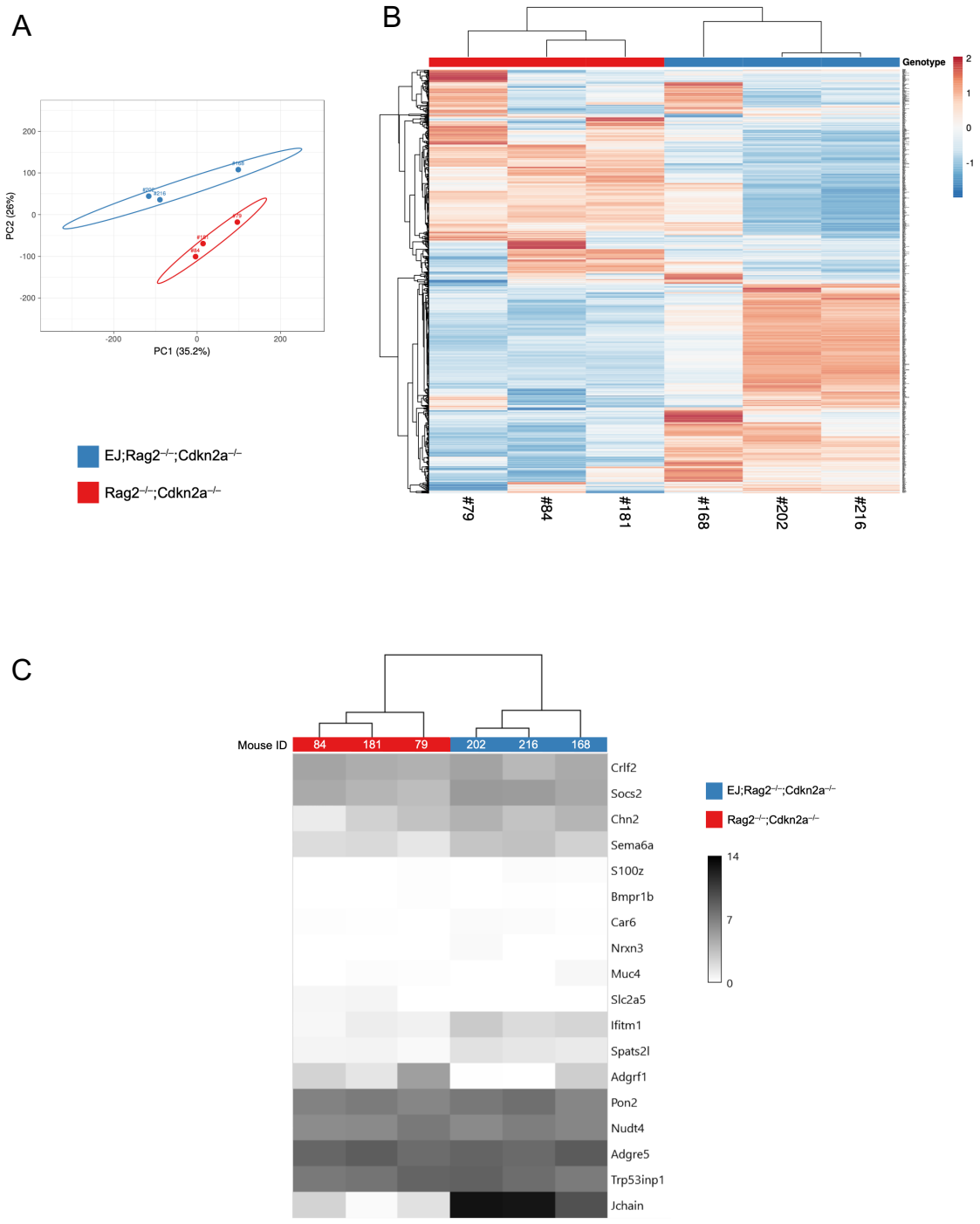
Supplementary Figure 12. ETV6::JAK2 expression and *Cdkn2a* deficiency did not rescue the B-cell developmental arrest induced by *Rag2* deficiency. (A) Representative flow cytometry plots showing B-cell developmental stages in bone marrow of 7-11 week-old *Rag2*$^{-/-}$ (n=5), *ETV6::JAK2;Rag2*$^{-/-}$ (n=2); *Rag2*$^{-/-}$*;Cdkn2a*$^{-/-}$ (n=5) and ETV6::JAK2;*Rag2*$^{-/-}$*;Cdkn2a*$^{-/-}$ (n=5) pre-leukemic (male and female) mice. (B) Percentage of Hardy fraction populations (A-C') found in the bone marrow of mice with the indicated genotypes. CD24^{-} BP-1^{+} outliers were excluded from total percentages. No statistically significant differences were found. Each column bar represents one mouse.



Supplementary Figure 13. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells infiltrate skull and vertebral leptomeninges. (A) Immunohistochemistry staining with B220 and PAX5 of representative skull sections from *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *ETV6::JAK2;Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ diseased mice confirming extensive B-ALL infiltration. (B) Extensive infiltration of vertebral leptomeninges was confirmed by hematoxylin and eosin staining of vertebral sections. Sk, skull bone; Br, brain; * leukemia infiltrate; V vertebra; SC spinal cord. Scale bars, 500 µm (for B220 and PAX5 top panels) and 200 µm (for B220 and PAX5 bottom panels).



Supplementary Figure 14. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells express CD49f and CXCR4 but not RANKL. Surface expression of CD49f, CXCR4 and RANKL proteins in representative ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cell cases gated in B220^{+};CD43^{high} cells.



Supplementary Figure 15. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemic cells segregate from each other but both express Ph-like ALL signature genes. (A) Principal component analysis of RNA-sequencing data from ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells (three cases each) generated with ClusVis web tool. Unit variance scaling was applied to rows. Singular value decomposition with imputation was used to calculate principal components. X and Y axis show principal component 1 and principal component 2, which explain 35.2% and 26% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. (B) Unsupervised hierarchical clustering of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells based on the 1200 genes with highest variance among the 6 samples. Rows are centered; unit variance scaling was

applied to rows. Both rows and columns were clustered using correlation distance and average linkage. (C) Heatmap clustering of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cases using a panel of 18 human Ph-like ALL signature genes, 15 from (Harvey et al., 2013) (*Adgrf1*, *Bmpr1b*, *Car6*, *Chn2*, *Crlf2*, *Ifitm1*, *Jchain*, *Muc4*, *Nrxn3*, *Pon2*, *S100z*, *Sema6a*, *Slc2a5*, *Spats2l*, and *Trp53inp1*) and 3 more from (Chiaretti et al., 2018) (*Adgre5*, *Nudt4*, and *Socs2*).

mouse ID	Sex	Age of death	Locomotion	Transplantability		CD90.2	B220	CD19	CD43	BP-1/Ly51	CD24	c-Kit	Hardy Fraction	CD127	CD127 gMFI	CD25	CD25 gMFI	CD49f	CD49f gMFI	CXCR4	CXCL4 gMFI	RANKL	RANKL gMFI
ETV6::JAK2;*Rag2-/-;Cdkn2a-/-*				*Rag2-/-*	*Rag2+*																		
70	M	24	paraparesia	4 in 4	nd	nd	low	pos	high	pos	high	neg	C'	pos	1299	pos	1897	pos	1256	pos	1636	nd	nd
83	F	23	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
87	M	35	nd	nd	nd	pos	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
101	F	15	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
102	F	13	normal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
104	M	10	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
105	F	9	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
106	F	17	normal	nd	nd	pos	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
107	F	10	normal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
109	F	21	paraparesia	0 in 2	nd	nd	pos	nd	high	pos	nd	nd	C-C'	low	1207	pos	1765	nd	nd	nd	nd	nd	nd
114	F	27	normal	1 in 5	nd	nd	low	nd	high	pos	nd	nd	C-C'	low	1070	neg	422	nd	nd	nd	nd	nd	nd
117	F	12	paraplegia	2 in 2	nd	nd	low	pos	high	pos	high	neg	C'	neg	510	pos	1013	pos	1321	pos	1138	nd	nd
168	M	14	paraplegia	nd	nd	nd	low	pos	high	pos	high	neg	B/C'	neg	385	pos	2365	pos	878	neg	218	neg	43
170	F	23	paraparesia	nd	nd	nd	low	pos	pos	pos	high	neg	C'	low	583	low	694	neg	437	neg	411	neg	36.3
188	F	20	paraparesia	nd	nd	nd	low	pos	pos	low	pos	neg	B-C	pos	2415	neg	397	neg	435	neg	297	neg	6.8
190	F	21	paraparesia	3 in 3	3 in 3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
202	F	12	paraparesia	nd	nd	nd	low	nd	high	neg	high	neg	B	neg	236	nd	nd	nd	nd	nd	nd	nd	nd
209	F	13	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
216	F	9	paraparesia	3 in 3	3 in 3	nd	low/pos	low	high	low	high	neg	B/C'	pos	1686	low	660	neg	515	pos	1535	neg	21.9
Rag2-/-;Cdkn2a-/-																							
79	M	23	normal	2 in 2	nd	nd	pos	neg	high	pos	pos	low	C	pos	1774	neg	92,7	neg	28.3	pos	1055	neg	55.2
80	F	27	normal	nd	nd	nd	pos	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
84	F	25	paraparesia	3 in 3	nd	nd	pos	neg	high	pos	pos	neg	C	pos	4792	neg	297	neg	203	pos	1482	neg	43.1
100	M	16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
108	F	25	paraparesia	2 in 2	nd	nd	pos	nd	high	pos	nd	nd	C-C'	pos	4246	neg	360	nd	nd	nd	nd	nd	nd
110	M	46	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
112	M	30	normal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
115	F	16	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
116	F	22	paraplegia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
118	F	32	paraparesia	2 in 2	nd	nd	low	neg	high	neg	high	neg	B	pos	3909	low	634	neg	191	pos	1338	neg	25.7
120	F	32	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
122	F	22	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
181	F	27	paraparesia	2 in 2	nd	nd	low	pos	pos	low	pos	neg	B-C	pos	4899	low	787	low	587	low	587	neg	37.9
182	F	34	normal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
191	M	32	paraparesia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
192	M	28	paraparesia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
193	M	31	paraparesia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
198	M	28	paraparesia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

gMFI, geometric mean fluorescence intensity; nd, not determined; pos, positive, neg, negative

Supplementary Table 2. Features and immunophenotype of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ and *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemias.

Chapter 8. Concluding remarks and future perspectives

Features frequently found in cancer have long been sought to develop therapeutic strategies. One such feature in T-ALL is surface expression of TCR, since it is found in the majority of cases. Moreover, genetic alterations such as loss of *CDKN2A* or activation of the JAK/STAT signaling pathway are frequently found in T-ALL and B-ALL, and are known to favor leukemia progression. That said, understanding how TCR expression and specific ALL mutations impact leukemia development and cell fate is crucial for the identification of new targets for the development of new and more efficacious treatment options.

In this thesis we showed that i) tonic TCR signaling in HY-TCR females was sufficient to drive development of T-ALL, characterized by acquisition of *Notch1* mutations and markers of positive selection (such as TCR-Vβ6 and CD5), ii) loss of *Cdkn2a* was common in HY-TCR female leukemias and Cdkn2a haploinsufficiency accelerated leukemia onset, and iii) strong TCR stimulation delayed HY-TCR T-ALL leukemia reinitiation. These findings highlight a dual role for TCR signaling in ALL: basal TCR signaling is leukemogenic (in HY-TCR females) while strong agonist-induced TCR signaling is leukemia-suppressive (in HY-TCR males and stimulation of female HY-TCR T-ALL cells).

In the context of ETV6::JAK2 transgenic protein expression, we showed that the high-affinity OT-I TCR delayed T-ALL development, when compared to lower-affinity HY-TCR expression, possibly associated with differential affinity for self-peptides presented by MHC and different CD5 levels of the two transgenic TCRs. T-ALL development in ETV6::JAK2;OT-I mice was not MHC-dependent, suggesting that the delayed leukemogenesis associated with OT-I expression was caused by relative strong, ligand-independent TCR signaling. Despite the demonstrated anti-leukemic effect of OT-I, all ETV6::JAK2;OT-I leukemias that developed expressed the transgenic TCR. Nevertheless, even stronger TCR stimulation of OT-I T-ALL cells with high-affinity agonist peptide resulted in apoptosis and decreased splenic infiltration by leukemic cells. The same was not observed with mutant lower-affinity peptides, suggesting that once ETV6::JAK2 leukemias develop, escaping the negative action of OT-I, only interaction with antigens of very strong affinity kill the malignant cells. TCR stimulation with peptides of variable affinity efficiently induced CD69 expression, all at similar levels, but resulted in the variable expression of different T-cell activation markers. In conclusion, basal signaling from TCRs with low affinity for self-peptides can promote T-ALL leukemogenesis, but once T-ALL has developed, TCR signaling induced by self-peptides or weak agonist peptides has no significant impact on

leukemogenesis. Strong agonist peptides trigger strong TCR signaling and induce apoptosis.

In the future, it would be interesting to investigate whether manipulating CD5 levels in T-ALL cells might impact leukemia development or the threshold for TCR activation and TCR-induced apoptosis. Subsequently, the ability of high versus low affinity stimuli to induce the expression of TCR regulators and cell death should be further characterized. The impact of CD5 expression levels on leukemia development in ETV6::JAK2;OT-I mice would also be interesting to dissect further, as differences in T-ALL onset observed between ETV6::JAK2 mice expressing HY-TCR or OT-I indicate that the strength of basal TCR signaling influences T-ALL development. Additionally, exploring if CD5 levels influence the activation of key mediators of the TCR signaling pathway without stimulation might shed light on the mechanism through which basal TCR signaling leads to T-ALL development.

Observations that TCR stimulation with varying affinity peptides results in distinct expression of T-cell activation markers and TCR regulators, while induction of apoptosis is more efficiently induced by high-affinity TCR agonists, suggest that TCR signaling intensity might induce the activation of distinct transcriptional programs in T-cells and in T-ALL. Transcriptomic analysis of T-ALL cells experiencing TCR stimulation with agonists of different affinity, using Nur77 as a surrogate marker for discrimination of T-ALL cells experiencing different levels of TCR activation, would be valuable to identify the transcriptional programs induced by low versus high affinity TCR stimulation. This would lead to a better understanding of how the decision between cell survival and death is regulated, and would allow the identification of critical mediators of TCR signaling-induced apoptosis, which may be used as therapeutic targets against T-ALL, regardless of surface TCR expression.

Regarding the impact of specific genetic alterations in leukemia development, we showed in this thesis that *Cdkn2a* haploinsufficiency in ETV6::JAK2;*Rag2*$^{-/-}$ mice accelerated T-ALL development. Because the β-selection developmental block imposed by Rag2 deficiency was not bypassed by either ETV6::JAK2 expression alone or in combination with *Cdkn2a* haploinsufficiency, we conclude that these two oncogenic events collaborate to malignantly transform immature thymocytes at the DN3 stage or earlier. T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice was frequently accompanied by total loss of *Cdkn2a* and additional mutations such as *Notch1*, indicating that Arf or Ink4a proteins prevent leukemogenesis of immature thymocytes prior to the β-selection checkpoint, but further genetic alterations might be necessary for leukemia development. Alternative mechanisms

for *Cdkn2a* inactivation were not explored here, but it would be interesting to address if T-ALL cases without *Cdkn2a* loss maintain Arf or Ink4a expression or *Cdkn2a* is inactivated by other mechanisms, for example epigenetic regulation, as previously described (Volanakis et al., 2013). Lastly, it would be interesting to investigate if and how *Cdkn2a* inactivation may favor the acquisition of further genetic alterations, such as Notch1 mutations.

Additionally, we showed in this thesis that constitutive JAK2 activation by expression of the transgenic ETV6::JAK2 protein cooperated with *Cdkn2a* genetic inactivation in precursor B-ALL development. Compared to *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL, leukemogenesis and CNS infiltration was accelerated in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice and the B-ALL cells expressed higher levels of proteins (e.g. integrin α6) and pathways (e.g. activation of the unfolded protein response, leukocyte extravasation and integrin signaling pathways) associated with CNS invasion. In addition, markers of human Ph-like B-ALL were more expressed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ than *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL. In future studies it would be interesting to explore if JAK2 activation (e.g. by ETV6::JAK2 transfection) in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL cells would impact leukemia development in recipient mice and CNS colonization, or if it would be sufficient to increase expression of integrin α6 and mediators of unfolded protein response and integrin signaling pathways to accelerate *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ leukemogenesis. Furthermore, the potential mechanisms through which JAK2 activation might regulate the expression of these proteins also merits investigation.

The fact that ETV6::JAK2 expression accelerated B-ALL development in *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice and was associated with activation of pathways previously associated with CNS infiltration (Cousins et al., 2022; Gang et al., 2020; Williams et al., 2014; Yao et al., 2018) opens the door for the investigation of the effect of JAK2, Xbp1 and integrin α6 inhibitors, alone or combined, on ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ B-ALL leukemogenesis and CNS colonization. Analysis and comparison of the transcriptome of B-ALL cells infiltrating various organs (spleen, BM or CNS) might provide useful insights into the molecular mechanisms used by B-ALL cells to infiltrate specific niches. Furthermore, expression analysis of the proteins and pathways here described and other candidate molecules upregulated in CNS-infiltrating lymphoblasts and their inhibition in PDX models of high-risk CNS-colonizing B-ALL would be crucial for the development and validation of new therapeutic targets.

Finally, although both leukemia models described here were generated in an immunodeficient background, B-ALL cells were able to grow and cause disease in

syngeneic immunocompetent mice, hence the models of B-ALL characterized in this thesis could be a useful tool to address how B-ALL cells evade the normal immune system.

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Annexes

Annex 1. Original research publication

Experimental Hematology

BRIEF COMMUNICATION

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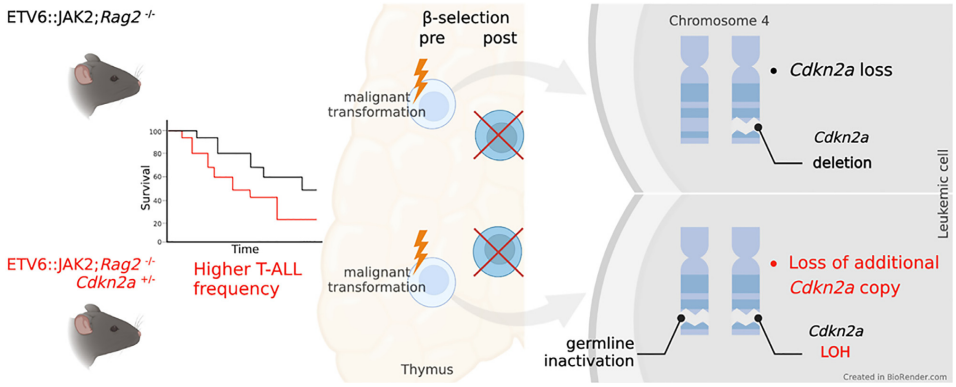
Cdkn2a inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint



BRIEF COMMUNICATION

Cdkn2a inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint

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HIGHLIGHTS

- *Cdkn2a* deletions occur in T-ALL driven by the ETV6::JAK2 fusion protein when the pre−TCR is absent.
- Germline *Cdkn2a* inactivation favors malignant transformation of DN3 thymocytes.
- Mouse T-ALL originating from *Cdkn2a*$^{+/-}$ DN3 thymocytes often presents *Cdkn2a* loss of heterozygosity.
- Further secondary events occur in T-ALL originating from *Cdkn2a*$^{+/-}$ DN3 thymocytes.

CDKN2A deletion is the most frequent genetic alteration in T-cell acute lymphoblastic leukemia (T-ALL), occurring across all molecular and immunophenotypic subtypes. *CDKN2A* encodes two functionally unrelated tumor suppressor proteins, ARF and INK4a, which are critical regulators of cell cycle and proliferation. *Arf* has been reported to suppress T-ALL development in post−β-selection thymocytes, but whether *CDKN2A* acts as a tumor suppressor gene in immature, pre−β-selection thymocytes remains to be elucidated. Resorting to a *Rag2*-deficient model of T-ALL, driven by the ETV6::JAK2 fusion, we report that *Cdkn2a* haploinsufficiency at early stages of T-cell development facilitates leukemia development. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL arose from pre−β-selection thymocytes because the thymocyte differentiation arrest caused by *Rag2* deficiency was maintained in preleukemic mice. Furthermore, somatic loss of the *Cdkn2a* wild-type allele was frequently observed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells, suggesting a selective advantage for total inactivation of *Cdkn2a*. Both *Cdkn2a*-sufficient and *Cdkn2a*-defective T-ALL presented additional genetic alterations, such as *Notch1* mutations and gains of chromosomes 13 and 15. These data indicate that *Cdkn2a* acts as a gatekeeper for leukemogenesis from the most immature stages of thymocyte development.

Pre−T-cell receptor (pre−TCR) expression in CD4$^-$/CD8$^-$ double-negative (DN) thymocytes, following successful *Tcrb* locus rearrangements, is critical for the β-selection checkpoint. The pre−TCR is composed of a TCRβ chain paired with the invariant pTα protein and CD3 family proteins. Although pTα expression has been documented in a high proportion of human T-cell acute lymphoblastic leukemia (T-ALL) [1,2], its role in leukemogenesis has been a matter of debate. In several T-ALL mouse models [3,4], pTα or pre−TCR was found to be essential for leukemogenesis. In turn, although not found as essential, pre−TCR favored leukemogenesis induced by intracellular Notch1 (ICN1) transduction [5] or transgenic expression of Notch3 [6], Myc [7], and ETV6::JAK2 fusion [8]. Further indicating that pre−TCR expression can be a major driver of thymocyte malignant transformation, compensatory genetic alterations were observed in pre−TCR-deficient ETV6::JAK2-driven leukemias, including chromosome 15 gains and chromosome 4 microdeletions encompassing the *Cdkn2a* locus [8].

The *CDKN2A* locus encodes two distinct tumor suppressor proteins, ARF and INK4a, which are crucial to regulating cell survival and proliferation. *CDKN2A* deletions are common in solid and hematological malignancies, being frequent in T-ALL and specific subtypes of B-cell ALL [9]. Additionally, *Cdkn2a* epigenetic silencing was found in the ICN1-driven T-ALL mouse model [10], and inactivation of *Arf* and/or *Ink4a* genes accelerated T-ALL development in mouse models driven by expression of intracellular Notch1, Tal1, Lmo2, or Hmga1a proteins [11−14].

Arf expression was proposed to act as a fail-safe mechanism against aberrant pre−TCR-positive thymocytes by inducing cell death and impairing their maturation [15]. Similarly, Volanakis et al. [10] reported that *Arf* prevents the malignant transformation of thymocytes after β-selection when thymocytes undergo massive proliferation after TCRβ rearrangements. However, because *CDKN2A* loss has been detected across all maturation subtypes of human T-ALL [9,16], it is likely that *Arf* and/or *Ink4a* act as tumor suppressors not only after but also before β-selection.

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METHODS

Mice

EμSRα-ETV6::JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) transgenic mice [8] were bred with *Rag2* (B6.129S6-Rag2tm1Fwa) and *Cdkn2a* (B6.129-Cdkn2a^{tm1Rdp}/NCI) knockout mice on the C57BL/6 background. The ETV6::JAK2 transgene was kept in hemizygosity. The *Cdkn2a* knockout mice [17] were obtained from the National Cancer Institute Mouse Repository (strain number 01XB1). Mice were bred and maintained at the i3S barrier animal facility (high efficiency particulate air filtration of incoming air, differential pressure, and disinfection or sterilization of room equipment and supplies) under 12:12-hour light:dark cycles and with food (2014S diet; Envigo) and water ad libitum. All experimental procedures were approved by the i3S ethics committee and Portuguese authorities (*Direção-Geral de Agricultura e Veterinária*) and followed recommendations from the European Commission (Directive 2010/63/UE) and the local Portuguese authorities (*Decreto-Lei* n°113/2013). Both female and male mice were used for all experiments. Mice were monitored for signs of disease (i.e., dyspnea, lethargy, enlarged lymph nodes, enlarged abdomen, and paraplegia) and euthanized by CO_2 inhalation upon reaching predefined experimental end points. Mice of different genotypes from the same litter were kept together in the same cages, and monitoring for signs of disease was performed blindly. Adult mice that were euthanized without leukemia were censored in Kaplan–Meier survival curves.

Low-coverage Whole Genome Sequencing

For low-coverage whole genome sequencing, the Ion Xpress Plus Fragment Library kit (ThermoFisher Scientific) was used for library preparation, and sequencing was performed using the Ion S5XL system (ThermoFisher Scientific). For copy number analysis, sequencing data were aligned with the mouse reference genome (Genome Reference Consortium Mouse Build 38 or mm10 reference genome) and further analyzed using QDNAseq (RRID:SCR_003174) package, R software. A bin size of 30 kb was used.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 software (RRID:SCR_002798). Unpaired Student *t* test was used for comparisons between the two groups. Log-rank test was used to compare the survival of different groups. Sample numbers are indicated in the figure legends. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To determine whether *Cdkn2a* inactivation could act as a tumor suppressor in pre–β-selection thymocytes, we investigated a mouse model of T-ALL arising from thymocytes arrested at the DN stage. ETV6::JAK2;*Rag2*$^{-/-}$ mice develop late-onset thymic lymphomas (median of 9 months) with malignant cells expressing CD4 and CD8 [8]. Because *Rag2* deficiency imposes a strict arrest at the CD4$^-$/CD8$^-$/CD25$^+$/CD44$^-$ DN stage 3 of thymocyte development, we assessed if the arrest could be overcome by ETV6::JAK2 transgenic expression. Thymocyte cellularity of preleukemic ETV6::JAK2 and *Rag2*$^{-/-}$ mice remained very low, similar to that of *Rag2*$^{-/-}$ mice. Eight of nine mice retained the DN3 block (Figure 1A,B), indicating that the ETV6::JAK2 transgene did not systematically rescue the DN3 block caused by *Rag2* deficiency. One of nine ETV6::JAK2;*Rag2*$^{-/-}$ thymuses presented DN4 cells (CD4$^-$/CD8$^-$/CD25$^-$/CD44$^-$), but these expressed aberrantly high levels of the immature thymocyte marker CD24, indicating they were already transformed thymocytes. These data show that ETV6::JAK2 fusion protein does not bypass the β-selection block caused by *Rag2* deficiency and transforms malignant cells at DN3 or earlier stages of thymocyte development.

Because chromosome 4 deletions encompassing the *Cdkn2a* locus were previously described in pre–TCR-deficient ETV6::JAK2 T-ALL [8], we performed *Cdkn2a* locus quantitative PCR in ETV6::JAK2;*Rag2*$^{-/-}$ (pre–TCR-deficient) T-ALL. *Cdkn2a* copy number loss, encompassing all exons, was found in one of six cases (Supplementary Figure E1). Compiling these results with reported array comparative genomic hybridization data [8], we verified that *Cdkn2a* loss occurred only in pre–TCR-deficient and not in pre–TCR-proficient T-ALL (3/15 vs. 0/11 cases analyzed; Figure 1C). Together, these data hint that *Cdkn2a* inactivation can cooperate with the ETV6::JAK2 fusion protein in the malignant transformation of pre–β-selection thymocytes.

Next, to determine the impact of germline *Cdkn2a* genetic inactivation on pre–TCR-deficient T-ALL, we bred ETV6::JAK2;*Rag2*$^{-/-}$ mice with the *Cdkn2a* constitutive knockout allele to generate cohorts carrying normal or haploinsufficient *Cdkn2a* copy number. Although not statistically significant, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice developed lymphoma/leukemia tendentially faster and with higher frequency than ETV6::JAK2;*Rag2*$^{-/-}$ mice (median survival of 53 weeks vs. 72 weeks and survival proportions of 19.7% vs. 49.2%, respectively; Figure 1D). ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented thymic lymphomas, reduced dissemination to the spleen and lymph nodes, and immature immunophenotype, that is, aberrant expression of CD4 and CD8, as well as high levels of CD24 and CD25, similar to that of ETV6::JAK2;*Rag2*$^{-/-}$ mice (Figure 1E, F). Of note, we could not investigate the impact of complete *Cdkn2a* deficiency on ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL development, because *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed rapid B-cell precursor leukemia, a phenotype similar to that of *Rag2*$^{-/-}$;*Arf*$^{-/-}$ mice [16].

We surmised that the reduced latency of T-ALL in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice could be caused by a DN3 block bypass before leukemia onset. However, we found that *Cdkn2a* haploinsufficiency did not rescue the DN3 developmental block. Indeed, pre-leukemic ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented similar thymic atrophy, thymocyte hypocellularity, and DN3 thymocyte developmental arrest as *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ or *Rag2*$^{-/-}$ mice (Supplementary Figure E2A,B).

To determine whether leukemogenesis was associated with somatic inactivation of *Cdkn2a*, we assessed the *Cdkn2a* copy number status in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemic cells. Indeed, six of 10 cases showed loss of the wild-type (WT) allele (Figure 2A), indicating a selective advantage for complete *Cdkn2a* loss. Because ETV6::JAK2;*Rag2*$^{-/-}$ leukemias presented increased frequency of DNA copy number alterations [8], we assessed the genome-wide copy number status of T-ALL from *Cdkn2a*-sufficient and -haploinsufficient mice by low-coverage whole genome sequencing. We observed recurrent chromosomal numerical alterations in both ETV6::JAK2;*Rag2*$^{-/-}$ (occurring in 83% of cases, most frequently chromosome 15 gains) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL (found in 50% of cases, most frequently chromosome 4, 13, and

Experimental Hematology
Volume 116

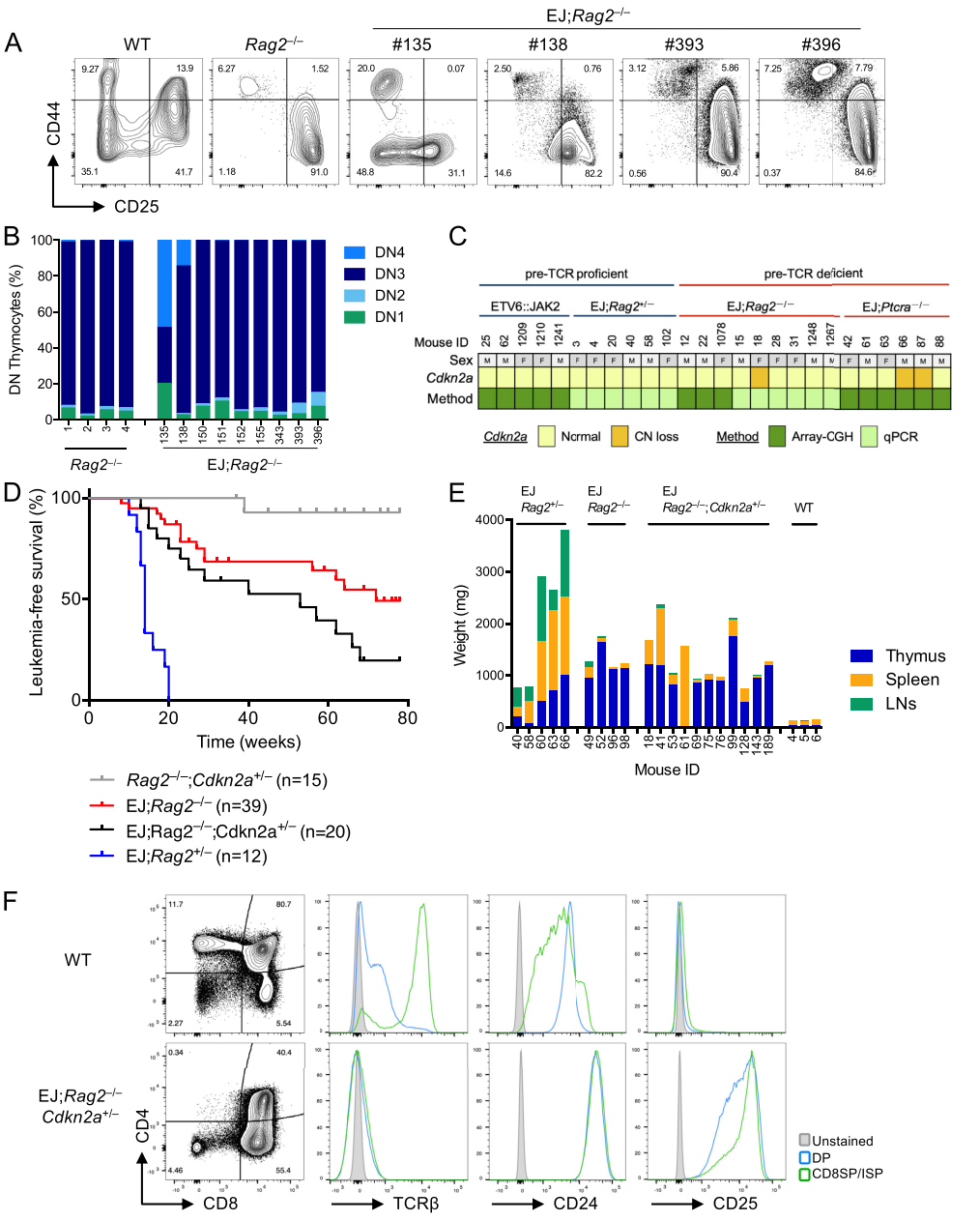

Figure 1 *Cdkn2a* genetic inactivation accelerates T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice. **(A)** Flow cytometry analysis of DN thymocytes (CD90.2^{+}CD4^{-}CD8^{-}) from representative 4- to 6-month-old *Rag2*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ preleukemic mice.

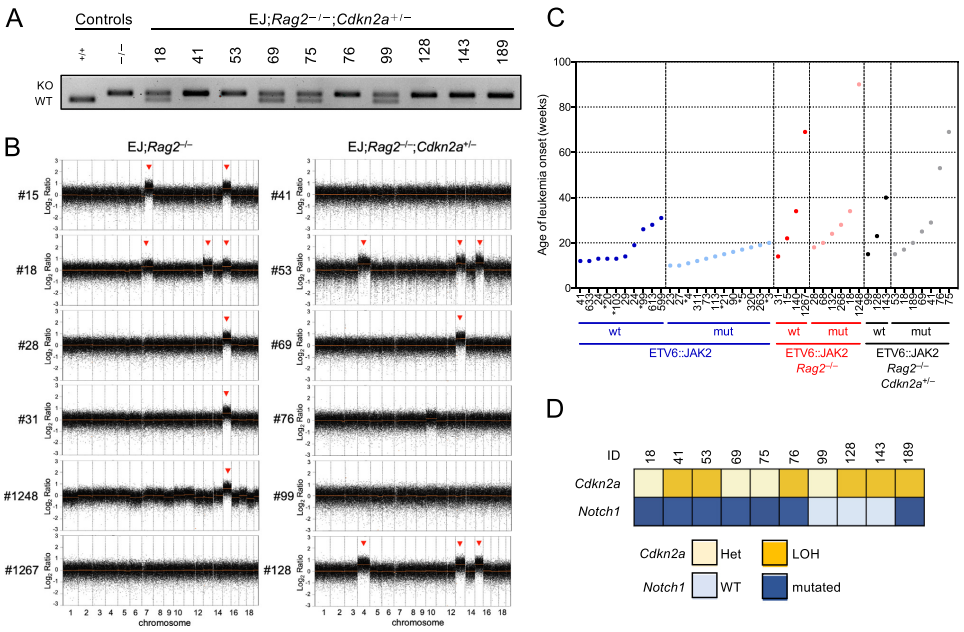

Figure 2 Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia. **(A)** PCR detection of wild-type (WT) and *Cdkn2a* knockout (KO) alleles in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells. Loss of the WT allele (loss of heterozygosity) was detected in 6 of 10 cases (numbers 41, 53, 76, 128, 143, and 189). **(B)** DNA copy number alterations assessed by low-coverage whole genome sequencing of T-ALL cells from *Cdkn2a*-sufficient and -haploinsufficient ETV6::JAK2;*Rag2*$^{-/-}$ mice. Recurrent copy number chromosomal alterations were found in both groups (chromosome 13 and 15 gains). Each graph represents DNA copy number distribution for each T-ALL case of the indicated genotype. Red arrowheads show chromosomal gains. **(C)** Schematic representation of the age of T-ALL onset in mice of the indicated genotypes with or without *Notch1* exon 34 PEST domain mutation (mut and wt, respectively). Each dot represents a mouse. Asterisks mark mice of ETV6::JAK2;*Rag2*$^{+/-}$ genotype **(D)** Schematic representation of detected *Cdkn2a* loss-of-heterozygosity (LOH) and *Notch1* mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases. *Het*=heterozygous.

15 gains; Figure 2B). In two cases (nos. 53 and 128), *Cdkn2a* loss of heterozygosity (LOH) co-occurred with the gain of chromosome 4, which harbors the *Cdkn2a* locus. From the low-coverage sequencing, there was no indication of *Cdkn2a* deletion in these cases with chromosome 4 gain. These findings suggest that the chromosome carrying the WT *Cdkn2a* allele was lost, resulting in LOH, and the chromosome carrying the mutant allele was triplicated. This conjecture is reminiscent of uniparental isodisomies of chromosome 9 segments harboring *CDKN2A* detected in human T-ALL [18]. These data suggest that complete *Cdkn2a* inactivation favors ETV6::JAK2-induced leukemogenesis and that further genetic alterations are still required for the malignant transformation of pre–TCR-deficient thymocytes.

Wild-type (WT) thymocytes were analyzed as a control. **(B)** Percentage of DN1–DN4 thymocytes, gated in CD90.2^{+}CD4^{-}CD8^{-} cells. Each column represents a mouse of the indicated genotype. **(C)** Summary of detected loss of *Cdkn2a* by quantitative PCR (Supplementary Figure E1) and array comparative genomic hybridization (Array-CGH) [8] in 3 of 15 pre–TCR-deficient and 0 of 11 pre–TCR-proficient T-ALL mice. **(D)** Kaplan–Meier leukemia-free survival curves for the indicated genotypes. Survival curve comparison of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ mice yielded $p = 0.0585$ (log-rank test). The hazard ratio was 2.15, with a 95% CI of 0.97–4.73. Mice euthanized because of causes other than leukemia/lymphoma (e.g., solid tumors in old ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice) were censored in the analysis (tick marks). **(E)** Thymus, spleen, and lymph node (LNs) weights of mice with leukemia/lymphoma. Each column represents a mouse. Mouse no. 61 developed B-cell leukemia and splenic lymphoma without the involvement of other lymphoid organs. **(F)** Flow cytometry analysis of a representative ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia showing surface expression of CD4, CD8, and CD25, high levels of CD24, and absence of TCR-β expression. Expression of these markers in wild-type (WT) thymocyte subpopulations is shown as the control. *DP*=Double-positive; *SP*=single-positive; *ISP*=immature single-positive.

Because *Notch1* mutations occur frequently in human and mouse T-ALL, including ETV6::JAK2 mouse T-ALL [19], and have been associated with progression [20], we assessed whether the frequency of *Notch1* mutations was influenced by *Cdkn2a* allele loss. *Notch1* PEST domain (exon 34) mutations, which account for most *Notch1* mutations reported in murine T-ALL [21,22], were detected with similar frequency (~60%) among ETV6::JAK2, ETV6::JAK2;*Rag2*$^{-/-}$, and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemias, with no correlation with age of onset (Figure 2C). *Notch1* PEST domain mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL occurred independently of *Cdkn2a* copy number status because four cases carried both *Cdkn2a* LOH and *Notch1* mutation, two cases carried only *Cdkn2a* LOH, three cases carried only *Notch1* mutations, and one case neither alteration (Figure 2D and Supplementary Table E1). This indicates that *Cdkn2a* complete inactivation did not relieve the selective pressure for *Notch1* mutation acquisition and these two types of genetic alterations can complement each other in leukemogenesis.

In summary, using the ETV6::JAK2-driven T-ALL mouse model, we show that *Cdkn2a* suppresses leukemogenesis in immature pre−β-selection thymocytes. This notion is supported by two main pieces of evidence: (1) germline loss of 1 *Cdkn2a* copy aggravated leukemogenesis in ETV6::JAK2;*Rag2*$^{-/-}$ mice without bypassing the DN3 thymocyte developmental block; and (2) *Cdkn2a* somatic allele loss was frequently observed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells, which suggests a selective advantage for total inactivation of *Cdkn2a*. The fact that 40% of cases retained the WT allele indicates that either *Cdkn2a* haploinsufficiency conferred a selective advantage for leukemic cells or *Cdkn2a* is inactivated through other mechanisms, such as epigenetic silencing, previously reported in Notch1-driven mouse T-ALL [10]. Although *Cdkn2a* genetic inactivation facilitated thymocyte leukemogenesis, *Cdkn2a*-defective leukemic cells still presented other genetic alterations, such as *Notch1* mutations and chromosomal gains. This indicates that *Cdkn2a* deletion does not fully compensate for the absence of a pre-TCR signaling complex, and secondary alterations, such as *Notch1*-activating mutations, are required for leukemogenesis.

Although *Cdkn2a* haploinsufficiency favored leukemogenesis, leukemia onset in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice occurred much later than in pre−TCR-proficient ETV6::JAK2 transgenic mice (median survival of 14 weeks; $p < 0.0001$, log-rank test). The chance for the acquisition of genetic alterations in *Rag2*-deficient pre−β-selection thymocytes is purportedly very low, due to the absence of Rag-mediated mutagenic recombination events [23] and a low rate of cell division. Although at this stage we cannot determine which events in which order are required for leukemogenesis, we posit DN3 thymocytes expressing the ETV6::JAK2 fusion protein undergo malignant transformation upon acquisition of an odd oncogenic event that either inactivates *Cdkn2a* completely or cooperates with reduced INK4a/ARF tumor suppressive activity. Loss of Arf has been shown to promote leukemia self-renewal [13], so this might be a crucial event in pre−β-selection thymocytes. *Notch1* mutations or other events (e.g., chromosome 13 or 15 trisomy) may bolster the self-renewal or malignant phenotype of leukemic cells with haploinsufficient or complete inactivation of *Cdkn2a*. *Notch1* mutations in our model are likely secondary events, paralleling human T-ALL, where *NOTCH1* mutations are often found in minor subclones [18,20].

Our results support the notion that *Cdkn2a* acts as a brake for leukemogenesis at the early stages of thymocyte development before the formation of the pre-TCR complex. Other reports indicated that *Cdkn2a* is an important tumor suppressor in post−β-selection thymocytes [11], which undergo massive proliferation and are prone to genetic alterations. These data and the occurrence of *CDKN2A* deletions in all subtypes of human T-ALL [24] allow us to conclude that *Cdkn2a* acts as a gatekeeper for leukemogenesis from the most immature until the post−β-selection stages of thymocyte development.

Conflict of Interest Disclosures

N.R.S. has received research funding from Gilead Sciences, Portugal, and *Fundação AstraZeneca*, Portugal, after applying in competitive calls for projects. The other authors do not have any conflicts of interest to declare in relation to this work.

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Author Contributions

T.A.C. designed, performed, and analyzed experiments and created the figures and wrote the manuscript; I.P.-L., F.A.-D., M.N.G., M. T.F., and T.C. performed experiments; and N.R.S. designed the study, performed experiments, and wrote the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request. Raw unaligned sequencing reads (fastq-format) that support the findings of this study have been deposited in the Sequence Read Archive under the accession number PRJNA843967.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.10.001.

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SUPPLEMENTARY INFORMATION TO:

***Cdkn2a* inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint**

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Supplementary Methods

Mice Genotyping

Rag2 knock-out mice were genotyped using the following primers: 5'-TGTCCCTGCAGATGGTAACA-3' and 5'-CCTTTGTATGAGCAAGTAGC-3' for WT allele and 5'-CTATTCGGCTATGACTGGG-3' and 5'-AAGGCGATAGAAGGCGATG-3' for the mutated allele. *Cdkn2a* knock-out mice were genotyped using 5'-GTGATCCCTCTACTTTTTCTTCTGACTT-3' primer in combination with 5'-CGGAACGCAAATATCGCAC-3' or 5'-GAGACTAGTGAGACGTGCTACTTCCA-3' for the detection of the WT or mutated alleles, respectively.

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs using cell strainers, washed with FACS buffer (phosphate-buffered saline (PBS) with 3% fetal bovine serum (FBS) and 10 mM NaN_3) and stained for 30-45 min with fluorochrome-labeled antibodies in FACS buffer. The following Biolegend antibodies were used: CD25-fluoresceine isothiocyanate (FITC) (clone PC61), TCRβ-phycoerithrin (PE) (clone H57-597), CD44-PE/Cyanine5 (clone IM7), CD90.2-PE-Cyanine7 (clone 30-H12), CD24-allophycocyanate (APC) (clone M1/69), CD4-APC/Cyanine7 (clone GK1.5), CD8α-Pacific Blue (clone 53-6.7). Immunostained cells were washed twice with FACS buffer and incubated in PBS with 10 mM NaN_3. Cell viability was determined using the Zombie Aqua Fixable Viability Kit (Biolegend). Samples were acquired using BD FACS CANTO II and analyzed using FlowJo software.

Quantitative PCR

Genomic DNA was isolated from $2x10^6$ thymic cell pellet using GeneJet genomic DNA purification kit (Thermo Fisher Scientific) according to manufacturer's instructions. Quantitative PCR was performed using the SsoFast Evagreen Supermix (BioRad) under

the following conditions: denaturation and polymerase activation at 95°C for 3 minutes and 40 cycles of denaturation at 95°C for 1 min, annealing and elongation at 60°C for 30 sec. All samples were tested in triplicate and the results expressed as the mean plus SD. Quantitative PCR results were analyzed with BioRad CFX Manager Software, version 1.6, using the comparative CT method (2-ΔΔC (T) method). DNA copy number alterations were detected relative to the amplification of DNA from the *Vcpip* and *Spna2* loci (chromosomes 1 and 2, respectively) which were used as a normalization control. The following primers were used for each locus.

Vcpip1:

5'-AGTGACATGGGCCTTCAAAC-3'

5'-GGCCCAGATCCAGAATAACA-3';

Spna2:

5'-AGGAGGAGGCTTGGATCAAT-3'

5'-CGAGGGTACTGCCACTGTCT-3'

Cdkn2a exon 1a:

5'-ATCTGGAGCAGCATGGAGTC-3'

5'-CTCCTTGCCTACCTGAATCG-3'

Cdkn2a exon 2:

5'-TCAACTACGGTGCAGATTCG-3'

5'-TCGCACGATGTCTTGATGTC-3'

Cdkn2a exon 3:

5'-GGTGGCTAACGGGTATCAGA-3'

5'-CTGAGGCCGGATTTAGCTCT-3'

Notch1 mutation detection

Genomic DNA from mouse leukemia samples were used for PCR amplification of two segments of *Notch1* exon 34: primer pair 5'-GCTCCCTCATGTACCTCCTG-3' and 5'-TAGTGGCCCCATCATGCTAT-3', generating a predicted amplicon of 904 bp, and

primer pair 5'-ATAGCATGATGGGGCCACTA-3' and 5'-CTTCACCCTGACCAGGAAAA-3', generating a predicted amplicon of 893 bp. PCR products were Sanger sequenced using the same primers at i3S Genomics (Porto, Portugal) or CCMAR *Serviços de Biologia Molecular* (Faro, Portugal).

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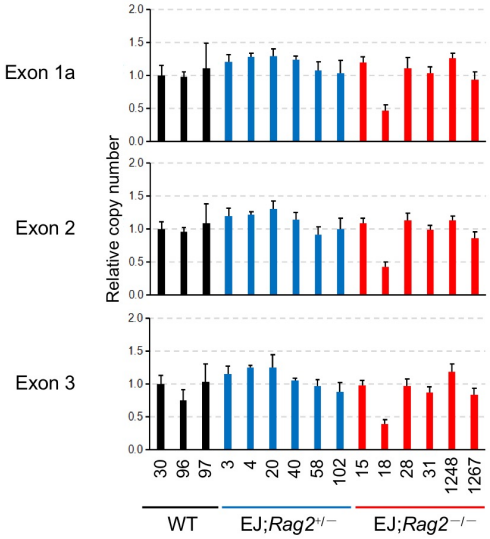
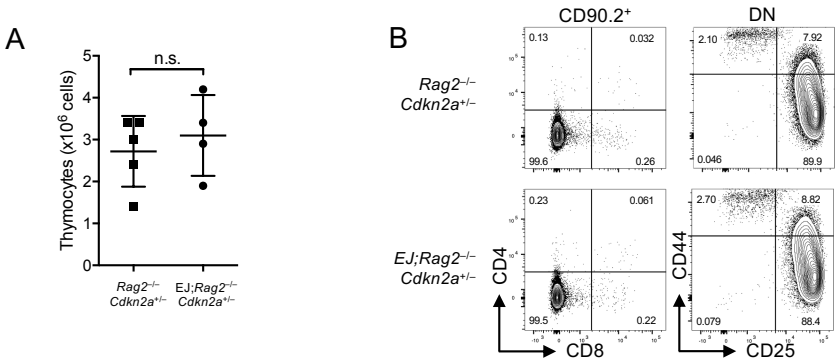


Supplementary Figure E1. *Cdkn2a* loss was found in *Rag2*-deficient T-ALL. Quantitative PCR of the indicated *Cdkn2a* exons in genomic DNA from leukemic cells of ETV6::JAK2;*Rag2*$^{+/-}$ (EJ;*Rag2*$^{+/-}$) and ETV6::JAK2;*Rag2*$^{-/-}$ (EJ;*Rag2*$^{-/-}$) mice and DNA from wild-type (WT) mice. One in 6 *Rag2*-deficient T-ALL cases showed loss of *Cdkn2a* (nº18). Relative copy number was determined by normalization to *Vcpip1* gene (chromosome 1), but similar results were obtained upon normalization to *Spna2* gene (chromosome 2; not shown). Bars represent standard deviation.

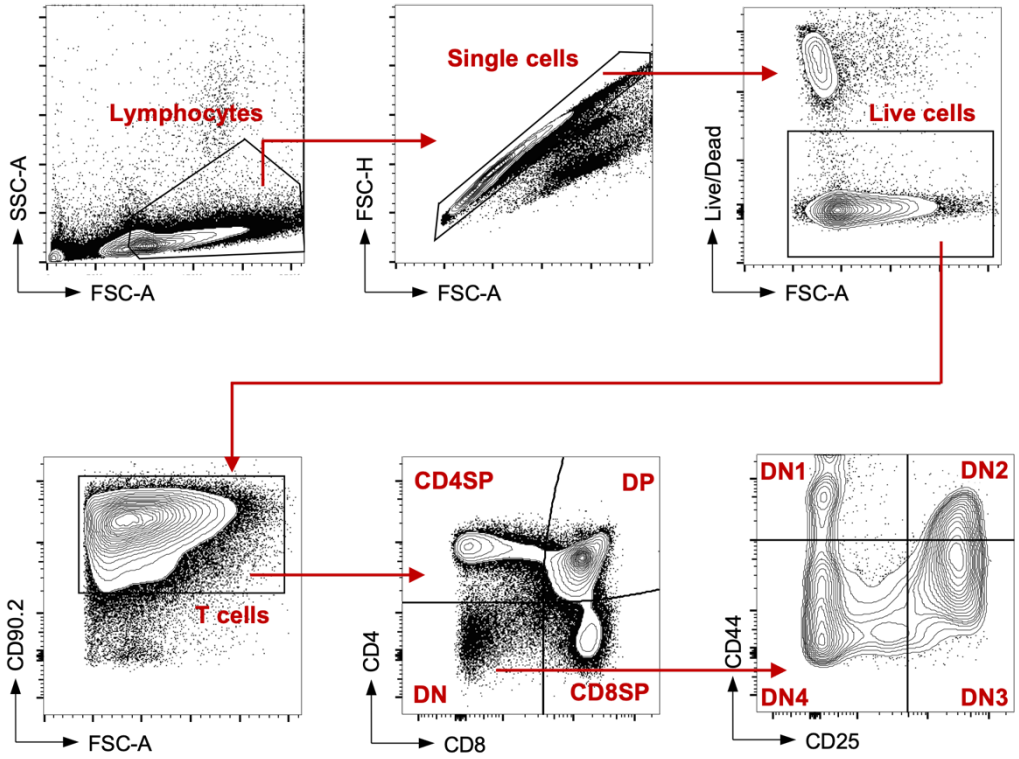


Supplementary Figure E2. *Cdkn2a* haploinsufficiency does not rescue the DN3 block caused by *Rag2* deficiency. (A) Thymocyte cellularity of 11-week-old, pre-leukemic *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=5) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=4) mice (each symbol represents one mouse, mean and SD are shown). (B) Representative flow cytometry plots show CD90.2-positive thymocytes (left panels) and CD90.2-positive DN thymocytes (right panels) of the indicated genotype blocked at DN3 stage of differentiation.

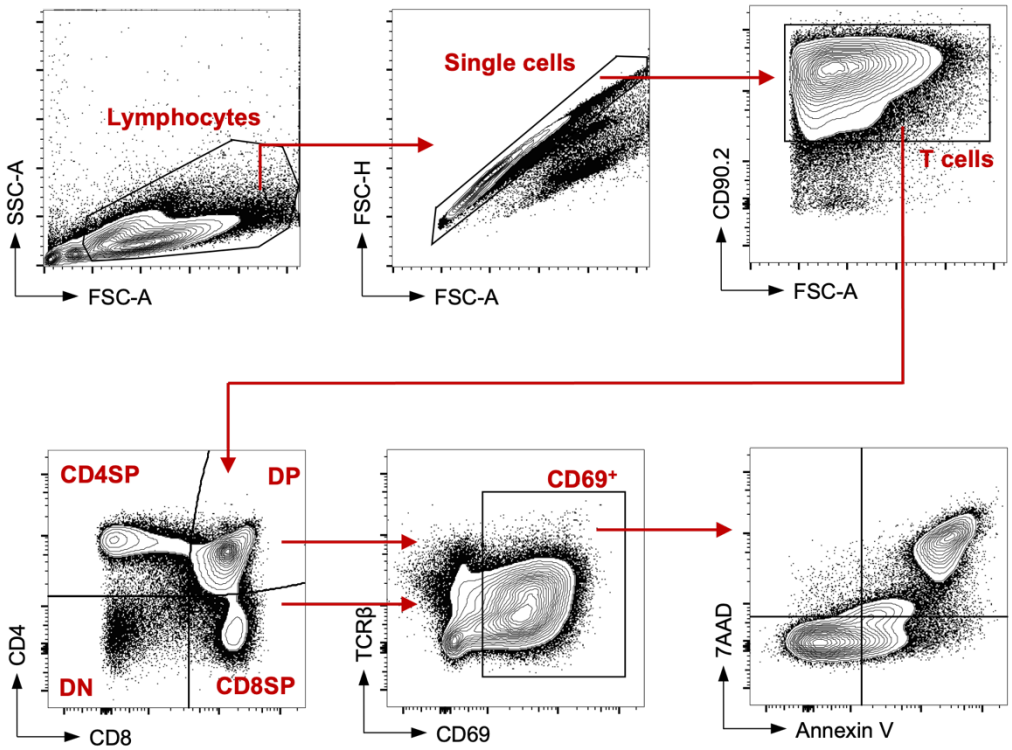
Mouse ID	*Cdkn2a* copy number	*Notch1* exon 34	Chromosome alterations
ETV6::JAK2;*Rag2*$^{+/-}$			
3	wt	c.2351, Ins ATTA	n.d.
4	wt	c.2399, Ins CGGG	n.d.
20	wt	wt	n.d.
40	wt	n.d.	n.d.
58	wt	n.d.	n.d.
102	wt	n.d.	n.d.
ETV6::JAK2;*Rag2*$^{-/-}$			
15	wt	wt	+7 +15
18	Loss	c.280,Ins A	+7 +13 +15
28	wt	c.2398, Ins AGGGAT	+15
31	wt	wt	+15
1248	wt	c.2361, Del G, Ins CC	+15
1267	wt	wt	none
ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$			
18	het	c.2361, Del GG, Ins CCC; c.2364 Ins G	n.d.
41	LOH	c.2362, Ins GGG	none
53	LOH	c.2421, Ins GAC, Dup TGAGTGGGGAGCCCAGTCAGGCAGATGTACAAC	+4 +13 +15
69	het	Mut*	+13
75	het	c.2361, Ins CC	n.d.
76	LOH	c.2389, Del CC, Ins GAG	none
99	het	wt	none
128	LOH	wt	+4 +13 +15
143	LOH	wt	n.d.
189	LOH	c.2363, Ins GGCTCGG	n.d.

Supplementary Table E1. Summary of genetic alterations found in leukemic cells of the indicated genotypes. *Notch1* mutations occurred concomitantly with *Cdkn2a* loss in one ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL case (no. 18) and with *Cdkn2a* loss of heterozygosity (LOH) in four ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases (nos. 41, 53, 76 and 189). *Mouse no. 69 presented a *Notch1* exon 34 frameshift mutation but the altered sequence was not identified. n.d., not determined.

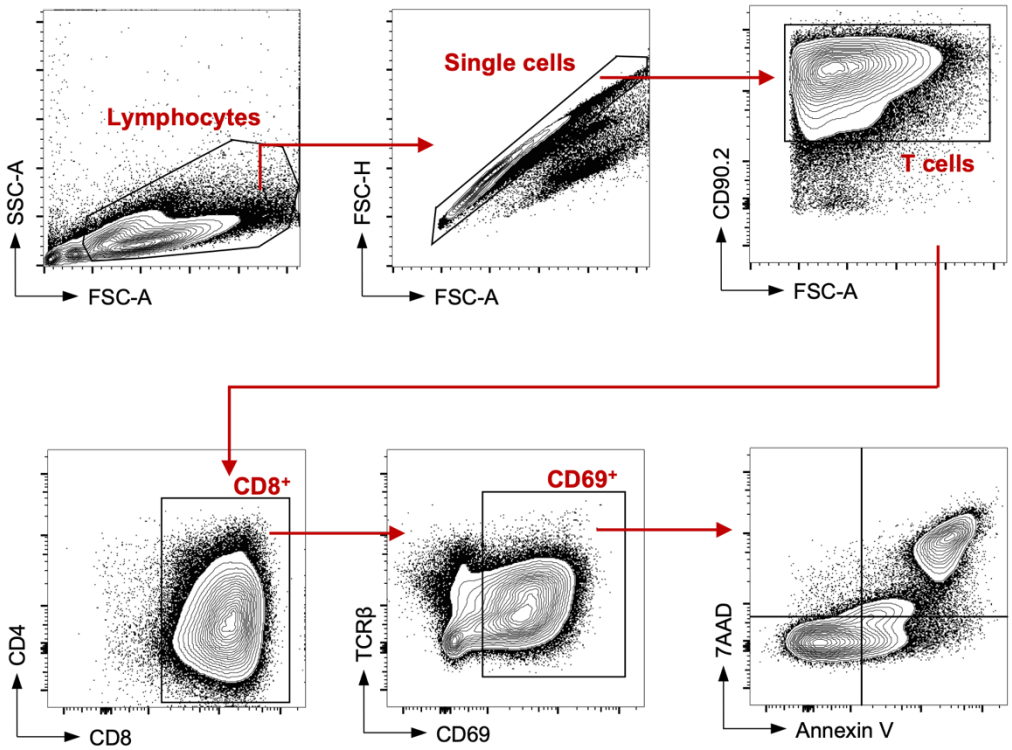
Annex 2. Gating strategies for flow cytometry analysis



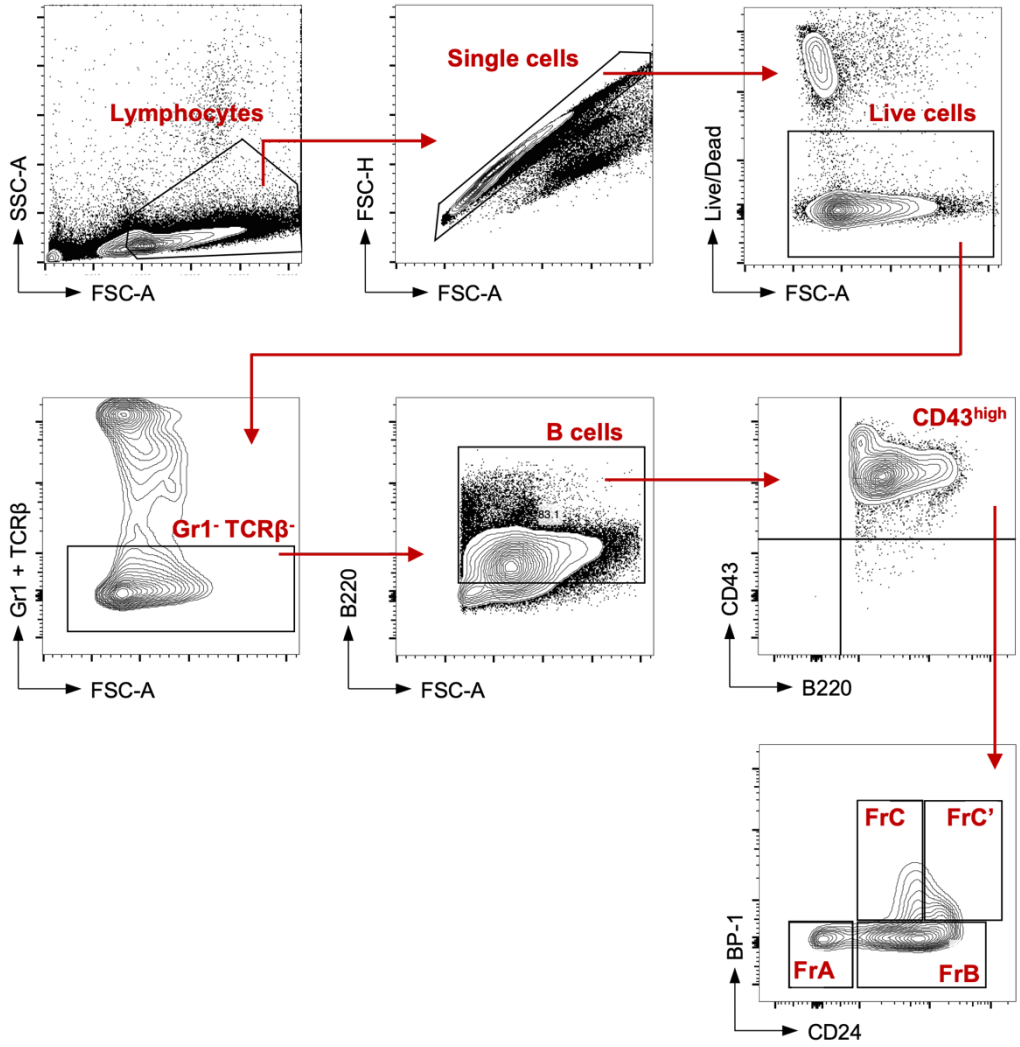
Gating strategy for flow cytometry analysis of normal thymocytes.



Gating strategy for flow cytometry analysis of cell activation and apoptosis of *ex vivo* stimulated normal thymocytes.



Gating strategy for flow cytometry analysis of cell activation and apoptosis of *ex vivo* stimulated T-ALL cells.



Gating strategy for flow cytometry analysis of normal B cells from BM.

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