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Cooperation between IL-7R signaling and HOXA9 in leukemogenesis

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Resumo

Leucemia linfoblástica aguda (LLA) é o cancro mais frequente em pacientes pediátricos sendo caracterizados por um crescimento anormal de linfócitos imaturos que se inicia na medula óssea e pode evoluir para outros órgãos. O subtipo LLA de linfócitos B é o mais comum, representando 85% dos casos em crianças e 75% em adultos. Têm sido feitos grandes desenvolvimentos no tratamento, no entanto, 20% dos pacientes pediátricos ainda têm recidivas e o tratamento ainda resulta em efeitos secundários importantes, existindo a necessidade de desenvolver novas abordagens terapêuticas.

A Interleucina-7 (IL-7) é uma citocina importante no desenvolvimento de células B e T. Quando a IL-7 se liga ao seu receptor (IL-7R), que é composto pela subunidade alfa e a cadeia gama comum, ativa vias de sinalização incluindo JAK/STAT, MEK/ERK e PI3K/Akt e que desempenham um papel fundamental na manutenção da viabilidade e progressão do ciclo celular. A via de sinalização IL-7/IL-7R tem sido descrita como sendo crucial na LLA, papel particularmente caracterizado em LLA subtipo de linfócitos T. O nosso laboratório descobriu uma mutação de ganho de função no exão 6 do gene *IL7R,* que codifica para a subunidade alfa do IL-7R, em pacientes com LLA do subtipo T. Tendo todas estas descobertas interessantes em mente, era relevante investigar a sinalização anormal da via IL-7/IL-7R em contexto de leucemia. Para isso, o nosso laboratório desenvolveu um modelo de ratinho com um *knock-in* condicional onde a mutação de ganho de função do IL-7R está no seu contexto genómico correto, e onde a expressão é mediada pela recombinação de Cre, que por sua vez é controlada pelo promotor CD2. Posto isto, as células hematopoiéticas irão expressar a forma mutada do receptor desde a fase de célula progenitora da linhagem linfóide até à sua maturação.

O primeiro objectivo desta tese que iremos abordar é a caracterização do nosso modelo de ratinho em que obtivemos grandes resultados pois notámos um impacto da mutação IL-7Rα nas células B, levando então ao desenvolvimento de leucemia linfoblástica aguda de células B. Após análises destes resultados, fomos mais além e através das análises do transcriptoma das células B leucémicas descobrimos o gene *Hoxa9* com baixa expressão, que nos levou ao segundo objectivo – explorar o papel do gene *HOXA9.* HOXA9 é amplamente caracterizado como sendo essencial na hematopoiese e apesar de ser geralmente encontrado como sobre-expresso em leucemia, alguns artigos mostram o oposto. Através da sobre-expressão deste gene nas células B leucémicas dos nossos ratinhos nós obtivemos resultados promissores

mostrando efeitos anti-proliferativos e apoptóticos revelando uma nova função de supressor de tumor.

Palavras-chave: leukemia linfoblástica aguda de células B (LLA-B); interleucina-7 (IL-7); receptor de IL-7; HOXA9; citometria de fluxo; transdução viral

Abstract

Acute lymphoblastic leukemia (ALL) is the most frequent cancer in pediatric patients being characterized by an abnormal proliferation of immature lymphoid cells which initiates in the bone marrow and can evolve to extramedullary sites. The subtype B-ALL is the most common, consisting in 85% of the children cases and 75% of the adults. There have been made great developments in the treatment field, however, 20% of pediatric patients still relapse and treatment still presents relevant secondary effects, therefore, there is an urge to develop novel therapeutic approaches.

Interleukin-7 (IL-7) is a cytokine with vital importance in B- and T-cell development. Upon IL-7 binding to its receptor (IL-7R), which itself is composed by alpha and common cytokine gamma chain subunit, it activates a signaling landscape comprising the JAK/STAT, MEK/ERK and PI3K/Akt pathways that play a key role in maintaining cell viability and cell cycle progression. IL-7/IL-7R signaling has been described as being crucial in ALL, particularly characterized in T-ALL. Our laboratory discovered a gain-of-function mutation in the exon 6 of the gene *IL7R*, which encodes for the alpha subunit of IL-7R, in T-ALL patients. Having all these interesting findings in mind, it was relevant to investigate the aberrant IL-7/IL-7R signaling in leukemia context. For that reason, our laboratory developed a conditional knock-in mouse model where *IL7Rα* gain-of-function mutation is in its correct genomic context, where expression is mediated upon Cre recombination, which in its turn is controlled by the promoter CD2. That being said, IL-7R expressing hematopoietic cells will express the mutated form of the receptor from the common lymphoid stage until maturation.

The first aim of this thesis is the characterization of our mouse model in which we achieved great results since we noticed an impact of IL-7Rα mutation on B cells eventually leading to the development of B cell acute lymphoblastic leukemia. Upon these findings we went further and through the transcriptome analysis of leukemic B cells we discovered *Hoxa9* downregulated, which led us to the second goal – explore the role of *HOXA9* gene. HOXA9 is widely characterized as being essential in hematopoiesis and although is generally found overexpressed in leukemia, a few reports indicate the opposite. Through overexpression of this gene in our mice B-leukemic cells we obtained promising results showing anti-proliferative and apoptotic effects revealing a novel tumor suppressor function.

Keywords: B cell acute lymphoblastic leukemia (B-ALL); interleukin-7 (IL-7); IL-7 receptor; HOXA9; flow cytometry; viral transduction

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Abbreviations

γc	Common gamma chain
7-AAD	7-Aminoactinomycin
Akt/PKB	v-akt murine thymoma viral oncogene homolog/Protein kinase B
ALL	Acute lymphoblastic leukemia
Allo	Allogeneic
AML	Acute Myeloid Leukemia
APC	Allophycocyanin
APC-CY7	Allophycocyanin Cy-7 Tandem
BAD	BCL2-associated agonist of cell death
B-ALL	B cell Acute lymphoblastic leukemia
Bcl-2	B cell CLL/Lymphoma 2
BCR	B cell receptor
BCR- ABL1	Breakpoint cluster region - Abelson murine leukemia viral oncogene homolog 1
BM	Bone marrow
BTG	B cell translocational gene
BV	Brilliant violet
CD	Cluster of differentiation
CDKNB2A/2B	Cyclin-dependent kinase inhibitor 2A/2B
cDNA	coding Deoxyribonucleic acid
CEBPA	CCAAT Enhancer Binding Protein Alpha
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
c-Myc	Myelocytomatosis viral oncogene
CNS	Central nervous system

Col1a1	Collagen Type I Alpha 1 Chain
CREB	cAMP response element binding
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2A	Immunoglobulin enhancer-binding factor
EBF	Early B cell factor
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
ETV6	ETS variant 6
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
FLT3	Fms-like tyrosine kinase 3
FOXO	Forkhead box class O
FSC-A	Forward scatter area
GFP	Green fluorescent protein
GSK3	Glycogen synthase-3
HEK 293T	Human embryonic kidney 293T cell line
HSC	Hematopoietic stem cells
iAMP21	Intrachromosomal amplification of chromosome 21
Ig	Immunoglobulin
IKZF1	Ikaros zinc finger family protein 1
IL	Interleukin
IL-7R	Interleukin-7 receptor
IL-7Rα	Alpha subunit of interleukin-7 receptor
ILF2	Interleukin Enhancer Binding Factor 2
IRES	Internal ribosome entry site

ITGAM	Integrin Subunit Alpha M
JAK	Janus kinase
KLF4	Kruppel-like factor 4
KMT2A	Histone-lysine N-methyltransferase 2A
MAPK	Mitogen activated protein kinase
MEF2C/2D	Myocyte-specific enhancer factor2c/2d
MEIS1	Myeloid Ecotropic Viral Integration Site 1 Homolog
MLL	Mixed lineage leukemia
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
NK	Natural killer cells
PAX5	Paired box protein 5
PBS	Phosphate buffered saline
PBX	Pre-B-Cell Leukemia Homeobox
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PE	Phycoerythrin
PE-CY7	Phycoerythrin Cy-7 Tandem
PerCP	Peridinin-chlorophyll-protein
Ph	Philadelphia chromosome
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
Pre-B	Precursor B cells
Pro-B	Progenitor B cells
RAG	Recombination-activating gene
RBC	Red blood cell

RNA	Ribonucleic acid
RSK	p90 ribosomal kinase
RUNX1	Runt-related transcription factor 1
SCT	Stem cell transplantation
SDS	Sodium dodecyl sulfate
SSC-A	Side scatter area
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
T-ALL	T cell acute lymphoblastic leukemia
TCRβ	T cell antigen receptor beta chain
WT	Wild-type

Introduction

B cell development

Hematopoiesis is a highly regulated process by which blood cells are formed, developed and differentiated from pluripotent hematopoietic stem cells (pHSCs)[1]. During embryonic development, hematopoiesis occurs in the fetal liver, while after birth the major hematopoietic site is the bone marrow (BM)[2], with B cells able to complete their development within the BM, in contrast to T cells, that require the thymus in order to develop. Functional maturation for both subsets occurs mainly in secondary lymphoid tissues such as the spleen and lymph nodes[3].

While pHSCs cells have the ability to self-renew, thus providing lifelong hematopoietic and regenerative potential, it is their differentiation that will provide the immediate precursors of lymphoid and myeloid lineages, namely the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). The latter is thus the cell stage where B-lineage development starts[4].

B cell development is a process in which a wide repertoire of cell-intrinsic and extrinsic signaling components such as cytokines, receptors and transcription factors collaborate in the maturation and functional diversity of B lymphocytes[5]. This implies an important degree of regulation in both the expression of molecules and receptors for environmental clues and in internal machinery dynamics, such as transcription factor expression and chromatin accessibility[6]. B cell lymphopoiesis can be characterized by the stage and maturation of the B cell and can be identified by fractions (from A to F) based on Richard Hardy's classification on the early 1990s, from pre-pro to mature B cells[7](Figure 1).

Early stages of B cell development are mainly regulated by signaling pathways of three important receptors: c-kit and fms-like tyrosine kinase-3 (FLT3) receptors and the interleukin-7 receptor[8], which are controlled by the transcription factors Ikaros and PU.1[4]. The first phases of B-lineage commitment are characterized by the critical process of rearrangement of the immunoglobulin (Ig, encoded by *IG* genes) heavy and light chain gene segments (variable V, diversity D, joining J)[9], although in the very early pre-pro B cell (Fraction A) this rearrangement is practically null[2]. The rearrangement starts with the pro-B cell stage (Fraction B/C) where cells start to express the CD19 marker[4], while rearranging the D and J segments of the heavy chain gene. Afterwards, a V region connects with the DJ segment[9]. In addition, in this stage, transcriptions factors paired box

protein 5 (PAX5) and early B cell factor 1 (EBF1) are expressed and have an important role in B-lineage specification[10]. Importantly, *IG* gene rearrangements are mediated by recombinase-activating genes RAG1 and RAG2, known to be regulated by the immunoglobulin enhancer-binding (E2A) transcription factor[2,11].

Following the rearrangement of the μ-heavy chain genes, the large pre-B cell stage (Fraction C') arises with the association of the heavy chain to the surrogate light chain, composed by λ5 and VpreB, that allows the formation of pre-B cell receptor complex (pre-BCR). Assembling with accessory proteins such as Igα and Igβ is necessary for pre-BCR-mediated signaling contributing to further development of B cells[2,12]. That being said, if the heavy chains produced are effectively associated with the surrogate light chain, the pre-BCR signaling induces a proliferative burst of pre-B cells[2], the components of the pre-BCR complex are downregulated and immunoglobulin light chain genes are recombined[12].

Afterwards, the cells enter a non-proliferative stage and become small pre-B cells (Fraction D) where light chains are rearranged with the μ-heavy chains selected culminating in the first mature BCR[13], characterized by the expression of IgM immunoglobulin on the cell surface[9] of immature cells (Fraction E). These cells are now prepared to leave the bone marrow and enter the spleen, where they will further differentiate into mature functional B cells expressing a variety of receptors and different functions (Fraction F)[14].

IL-7 plays an important role in B cell development along with E2A, EBF, PAX5 transcription factors in the commitment of cells to B-lineage development and during *IG* rearrangement through modulation of RAG activity[15]. The IL-7/IL-7R axis in B lymphopoiesis is thus vital in proliferation, survival and differentiation[16], as further highlighted in the rest of the Introduction.

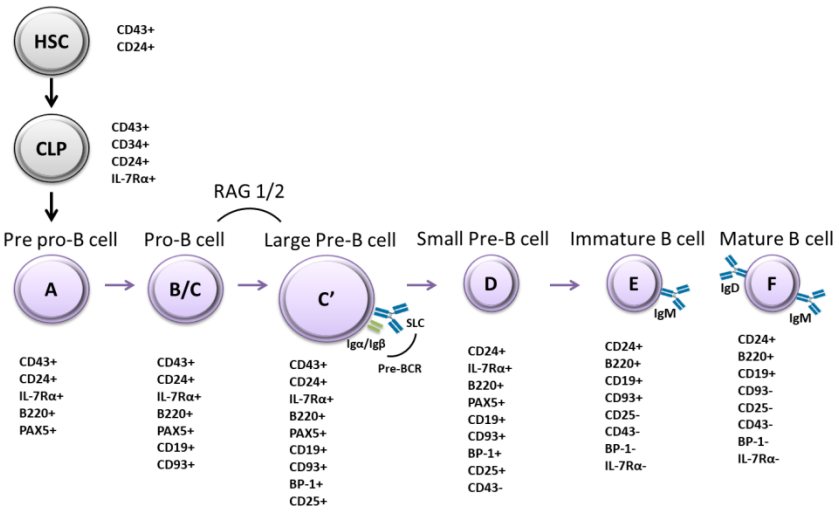


Figure 1 - Schematic representation of B cell developmental stages. B cell development initiates from a hematopoietic stem cell (HSC). B cell lineage starts with the expression of PAX5 in pre-pro-B cell, which then differentiates and expresses CD19 to become a pro-B cell. At this stage RAGs are expressed and promote *IG* gene rearrangements. Large pre-B cells express pre-BCR, and, after several rounds of proliferation, differentiate into small pre-B cells and subsequently into immature B cells that express an IgM molecule on the cell surface. SLC, surrogate light chain.

Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a hematological malignancy characterized by malignant transformation and abnormal proliferation of B or T cell precursors that invade the bone marrow and extramedullary sites. ALL is the most common cancer among pediatric patients and is the second most prevalent acute leukemia in adults. In the latter, the prognosis is considerably poorer. ALL has a peak of incidence around 2 to 5 years of age[17] and also in adulthood around the age of 50[18]. When the malignant transformation occurs, during B cell development, it gives rise to a specific type of ALL: B cell acute lymphoblastic leukemia (B-ALL)[18]. In the adult population, B-ALL comprises the majority of the cases, about 75% of ALL cases, while T-ALL comprises the remaining 25%[18]. In pediatric patients, B-ALL accounts for 85% of cases, whereas T-ALL comprises 15% of the cases[19]. Relatively to treatment, in pediatric patients, has made great progress, with 5-year overall survival rate from 85% to 90%, however, 20% of the patients still experience relapse[17], while in adults the long-term overall survival rate is about 30% to 40%[20].

B cell acute lymphoblastic leukemia (B-ALL)

B-ALL can be classified into early-pre-B, common B and pre-B-ALL, according to B cell differentiation markers[21]. Nevertheless, as more patients are studied and transcriptomics and genomics analyses increasingly performed in a routine fashion, other classifications, based on the expression of particular genetic aberrations and/or transcriptional profiles rather than immunophenotyping, gained increasing relevance[22]. An important and recently identified subtype is Philadelphia (Ph)-like ALL, lacking the hallmark BCR-ABL1 oncoprotein but with similar molecular features to Ph+ B-ALL[23–25]. Ph-like ALL is more prevalent in the adolescent and adult population[21]. Patients with Ph-like ALL exhibit poor prognosis and harbor a diverse range of genetic abnormalities, mainly divided in ABL-class and janus kinase-signal transducer and activator of transcription (JAK-STAT) alterations, but with a large fraction including genetic alterations in IKAROS zinc finger family protein 1 (IKZF1)[26]. Interestingly, *IL7R* insertions or deletions are also present[27]. As such, we have a particular interest in this subtype.

Genetic features in B-ALL

Several chromosomal aberrations and genetic alterations have been identified as being involved in the malignant leukemic transformation of B cells including numerical abnormalities, hiperdiploidy and hypodiploidy, being the latter less frequent (3%) but known to be a negative prognostic factor[28]. Chromosomal translocations such as those leading to the fusions breakpoint cluster region - abelson murine leukemia viral oncogene homolog 1 (BCR-ABL1) and ETS variant 6-Runt-related transcription factor 1 (ETV6-RUNX1) (which define important B-ALL subgroups), as well as deregulated tumor suppressor genes including cyclin-dependent kinase inhibitor 2A/2B (*CDKN2A*/*B)* and *TP53*, along with mutations affecting *IL7R*, *JAK2* and others have also been identified[19]. Importantly, lesions affecting transcriptions factors such as IKZF1, EBF1 and PAX5, leading to loss of function in B-ALL[29], constitute a hallmark of this malignancy, being present in more than 60% of the cases[30]. This includes a recently described mutation in PAX5 (P80R)[31]. Also, histone-lysine N-methyltransferase 2A (KMT2A) rearrangements are recurrent in children, being linked with poor outcome[28].

Symptoms and Prognosis

Patients often present symptoms non-related to the disease such as anemia, thrombocytopenia, and leukopenia and others present more B-leukemia-specific for instance fever, weight loss, fatigue and infection. When the cells invade other organs such as the spleen, lymph nodes and liver there is generally an increase in size, due to the accumulation of malignant cells, leading to splenomegaly, lymphadenopathy and hepatomegaly, respectively[18].

Patients can be classified into groups based on a range of biological, clinical and genetic features. For example, infants (less than 1 year) have more probability to develop aggressive disease, contrary to children from age 1 to 10[32], while older patients over the age of 60 present poor prognosis (10-15% long-term survival as compared to 30-40% for patients less than 60 years old[20]). White blood cell counts, immunophenotype as well as central nervous system (CNS) disease involvement are also high risk features, being the latter associated with the increased difficulty of chemotherapy penetrance. Additionally, presence of cytogenetic abnormalities can define favorable and unfavorable outcomes. For instance, high hyperdiploidy and ETV6/RUNX1 translocation are associated with the favorable outcome, while hypodiploidy, BCR-ABL fusion, MLL rearrangements and intrachromosomal amplification of chromosome 21 (iAMP21) can be associated with poor outcome[32]. Relatively to BCR-ABL fusions, Philadelphia chromosome prevalence in adults can range from 15-30%[33]. The prognostic factors that are more related with adverse prognosis in Ph+ ALL are the deletion of chromosome 9, the presence of +der(22) and trisomy 8[34]. Historically, Ph+ ALL patients are associated with very poor prognosis. However, introduction of tyrosine kinase inhibitors targeted therapeutics has improved treatment outcome[18]. Related to treatment, the response to initial therapy is also prognosis predictor, being complete remission linked with good outcome while induction failure and detection of minimum residual disease (MRD) at the end of first treatment phase induction are associated with low percentage of overall survival[32].

Treatment in B-ALL

Current therapies have a success rate of long-term survival of 80%-90% in children, consequently, being part of the category that have better prognosis. The treatment in adults is more challenging due to the low tolerance to standard chemotherapy, medical comorbidities and the higher probability to develop cytogenetic

unfavorable profile, for example, carrying the Ph chromosome[21]. As for the population in between these two main groups (adolescents and young adults) studies have revealed favorable outcomes in administering controlled pediatric-inspired chemotherapy protocols[35]. Overall, B-ALL chemotherapy treatment comprises 3 phases: induction, consolidation and long-term maintenance[18]. Firstly, induction therapy normally includes corticosteroids, vincristine and an anthracycline, afterwards, are given three series of methotrexate with leucovorin rescue and then L-asparaginase. After induction phase, high-risk disease eligible patients are selected to allogeneic stem cell transplantation (Allo-SCT), whilst others may go to consolidation and maintenance. Consolidation step recurs to similar agents as in the induction and in patients with involvement of CNS includes intrathecal therapy and/or radiation. Maintenance phase consists of using mercaptopurine and weekly oral methotrexate and it can last for 2 to 3 years[18,36].

IL-7/IL-7R- mediated signaling in normal hematopoiesis and leukemogenesis

IL-7/IL-7R signaling in hematopoiesis

Interleukin-7 (IL-7) is a cytokine, initially discovered in 1988 as growth factor of murine B cell precursors[37], essential for the development of T and B cells[11]. IL-7 is a member of the hematopoietin family, together with other interleukins such as IL-2, IL-3, IL-5, IL-9, IL-15, or stem cell factor[11]. It is produced by non-lymphoid cells localized in the thymic and liver stroma, bone marrow, spleen and kidney, fetal intestine, embryonic brain, keratinocytes along with other types of cells such as endothelial, fibroblasts and peripheral dendritic cells[38].

IL-7-mediated signaling requires a receptor complex, formed with the IL-7Rα chain, and the common cytokine gamma chain (γc-chain), shared with the receptors of several other cytokines (IL-2, IL-4, IL-9, IL-15, IL-21)[11]. The binding of IL-7 and heterodimerization of the IL-7 receptor (IL-7R) subunits activates a plethora of signaling pathways responsible for proliferation and survival[39], as described in further detail below.

The requirement of IL-7 in lymphopoiesis *in vivo* was first noticed when the use of IL-7 blocking antibodies in mice led to inhibitory effects on the development of B and T cells[40]. Later studies showed that the elimination of IL-7 or of its receptor in mice led to the loss of both T and B cells[29]. The absence of IL-7/IL-7R signaling resulted in arrest of B cell

development from the pro-B cell stage[41] and studies with *Il7* knock-out (*Il7*$^{-/-}$) mice displayed an interruption from the pro-B cell to the pre-B cell transition[42], while mice with knockdown of IL-7Rα expression the interruption occurs at earlier stage, at the pre-pro-B cell stage[43]. Nonetheless, the common lymphoid progenitors already show dysfunctional development in *γc*$^{-/-}$ and *Il7rα*$^{-/-}$ mouse models[44], suggesting an even earlier role. Interestingly, it is known that IL-7 requirements differ between humans and mice, B cell development seems little affected in human subjects with non-functional IL-7Rα or common γc chain[45]. However, *in vitro* experiments with human B cells, from adult human bone marrow suggest that IL-7 can still play a role in human B cell differentiation[46,47]. Thus, there are still unsolved issues concerning the exact physiological role of IL-7 in human B-lymphopoiesis.

Although the role of the expression of IL-7Rα in very early B-lineage lymphocytes may have not been completely elucidated, it is quite clear that upon differentiation the IL-7R is not expressed in mature B cells[48]. Of relevance for this thesis, the cooperation between IL-7R and pre-B cell receptor is crucial at critical stages during B-lymphopoiesis[49] highlighting the importance of a high degree of regulation in control of IL-7Rα expression, as it occurs in T cell development, where IL-7Rα expression is also under tight regulation (Figure 2). In early mouse B cell development, the transcription factor PU.1 is required to initiate the transcription of *Il7r* locus and in both human and mouse developing B cells the receptor is expressed until the cells differentiate to mature cells[29].

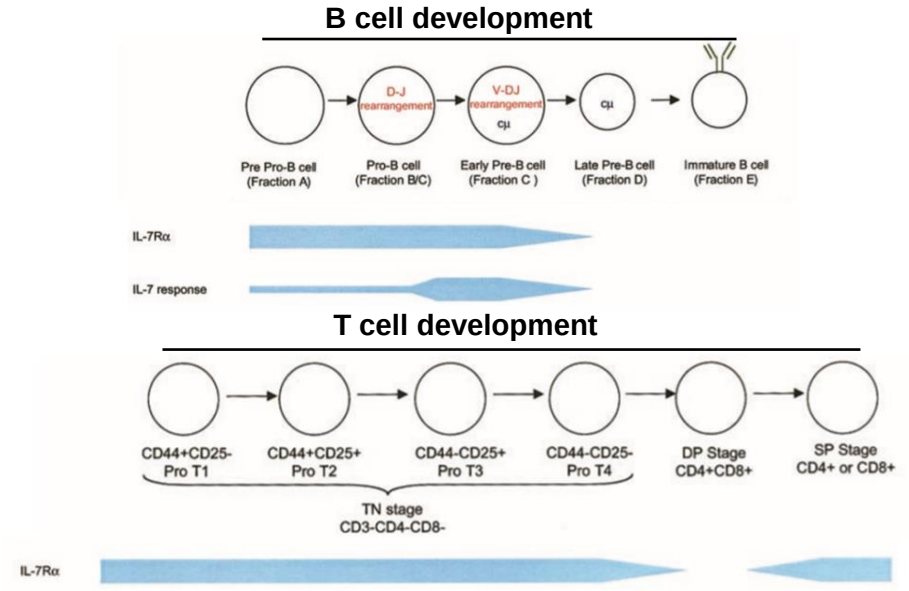


Figure 2 – IL-7R expression in B and T cell development. IL-7/IL-7R signaling is crucial to lymphopoiesis and acts differently in each lineage. In B cell development, IL-7R is expressed from the pre-pro-B cell stage and continues until maturation. In T cell development the receptor is expressed from the initial Pro T1 stage onwards, except at the double positive (DP) stage, where its transient downregulation is essential for proper T cell selection. Adapted from Fry *et al* 2002[11].

IL-7/IL-7R signaling initiates with the binding of IL-7 causing the heterodimerization of the two IL-7R subunits (i.e. IL-7Rα and γc), that results in a conformational change bringing JAK enzymes into close proximity and thereby phosphorylating the cytoplasmic tail of IL-7Rα and allowing a series of downstream intracellular phosphorylation events to occur through different signaling cascades: JAK/STAT, mitogen-activated protein kinase kinase/extracellular-signal-regulated kinase MEK/ERK, phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/Akt) and Src family tyrosine kinases - key pathways to promote gene transcription and cellular responses[29,39] (Figure 3). IL-7R and pre-BCR collaborate in the development of B cells and each receptor is responsible to activate specific signaling pathways, however, both receptors activate MEK/ERK and PI3K pathways[10].

PI3K signals have been stated to be crucial in B cell development[50], acting by phosphorylating phosphatidylinositol (4,5)-bisphosphate (PIP2) and leading to the generation of the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), that in its turn recruits v-akt murine thymoma viral oncogene homolog/protein kinase B

(Akt/PKB) to the plasma membrane, allowing the phosphorylation of Akt by phosphoinositide-dependent kinase-1 (PDK1) in Thr308 and by mammalian target of rapamycin mTOR complex-2 (mTORC2) in Ser473[10,51]. Activated Akt/PKB then negatively regulates apoptosis by targeting BAD protein, a member of Bcl-2 family, promotes cell survival and proliferation by phosphorylating of forkhead box class O (FOXO) family proteins, and regulates cell metabolism, and, again, viability, through phosphorylation of glycogen synthase-3 (GSK3)[52], amongst other targets.

MEK/ERK signals are relevant already in pro-B-cells where the stimulus of anti-Igβ antibodies and exposure to IL-7 were shown to activate this pathway[53]. ERK phosphorylates several targets in the cytoplasm and nucleus, including the p90 ribosomal kinase (RSK) that in turn phosphorylates cAMP response element binding (CREB). This transcription factor upregulates other transcription factors associated with pre-B cell proliferation, such as c-Myc, myocyte-specific enhancer factor-2c (MEF2C), MEF2D and interleukin enhancer binding factor-2 (ILF2). MEF2C is a target of the p38 mitogen activated protein kinase (MAPK) pathway, being required for mature B cell proliferation. ERK proteins can be involved in proliferation, inhibiting the activity of anti-proliferation agents such as Tob1 and B cell translocation gene (BTG), as well as survival, specifically in pre-B cells[10].

As referred above, the other most relevant IL-7R-dependent signaling pathway in B cell development is the JAK/STAT pathway, especially involving the activation of STAT5. Upon IL-7 binding and receptor dimerization, the tyrosine kinases JAK1 and 3[54] phosphorylate each other and become fully activated, leading to the transmission of intracellular signals by subsequently phosphorylating and thus activating the STAT family of transcription factors[55]. Activation and dimerization of STAT proteins are conducted by phosphorylation of a tyrosine residue localized in their C-terminal, transactivation domain[54], allowing for subsequent translocation to the nucleus, where STATs act as transcription factors controlling proliferation, differentiation and survival[55]. Particularly, it has been shown that STAT5 restores B cell development in *Il7r*$^{-/-}$ mice[56]. As noted already, JAK kinases are responsible for activating also other key signaling pathways, such as MEK/ERK or PI3K/Akt[57].

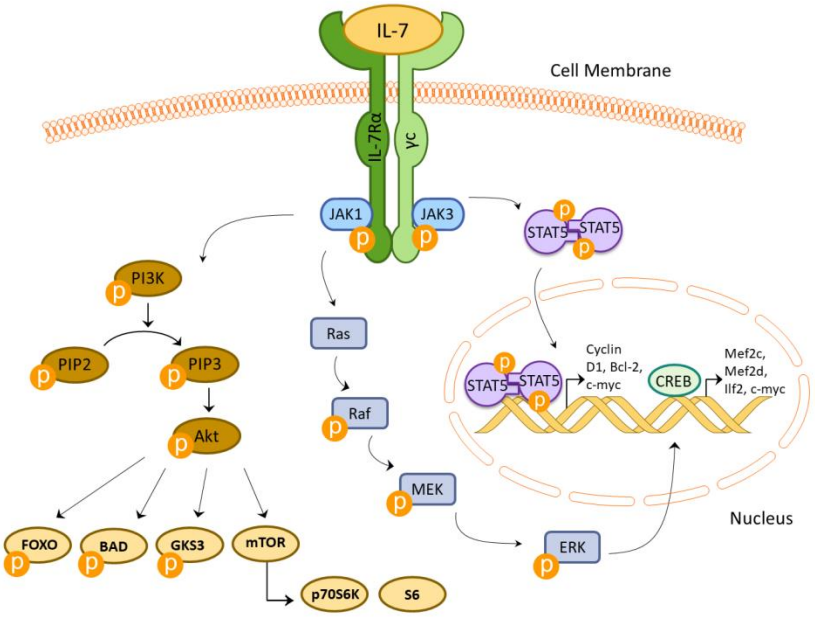


Figure 3 – Schematic summarized representation of the IL-7/IL-7R signaling. Upon binding of IL-7, the subunits IL-7Rα and γc dimerize and JAK1 and JAK3 proteins are phosphorylated and activate several pathways. The main pathways here represented are PI3K/Akt/mTOR, JAK/STAT and MEK/ERK that are essential in cell cycle progression, proliferation and cell viability.

IL-7R-mediated signaling pathways in leukemogenesis

The signaling pathways associated with IL-7 binding to IL-7R that were described above are known to be aberrantly active in leukemia. Given their importance in promoting proliferation, cell survival and metabolism, it is not surprising that abnormalities leading to the hyperactivation of these pathways should contribute to leukemogenesis. Indeed, the PI3K/Akt pathway[58], as well as its downstream target mTOR[59], have been found to be involved in T-ALL, and importantly PI3K/Akt is the dominant signaling pathway associated with IL-7 in T-ALL[60]. Moreover, STAT5 and STAT3 are considered important in the pathogenesis of lymphoid malignancies, including T-ALL[61,62]. Other studies showed that IL-7 induced upregulation of Bcl-2 that promoted leukemia progression in human T-ALL cells[63] and MEK/ERK cascade was found activated at relapse in pediatric ALL[64]. While IL-7/IL-7R signaling is widely characterized in T-ALL[65,66], PI3K/Akt pathway is also known to be aberrantly activated in B-ALL[67].

Overall, these findings suggest that the IL-7/IL-7R axis contributes to the progression and expansion of leukemia, although, the exact mechanisms by which IL-7/IL-7R contribute to leukemogenesis are not clear. For that reason, more studies are necessary to understand the processes underlying malignant transformation downstream from IL-7R signaling, particularly in the context of B cell development.

Gain-of-function *IL7R* mutation

In accordance with a role for these signaling axis in leukemogenesis, somatic gain-of-function mutations in *IL7R* (encoding IL-7Rα subunit) were found in pediatric B[68] and T acute lymphoblastic leukemia[39,66]. Our laboratory demonstrated exon 6 oncogenic gain-of-function *IL7R* mutations in around 10% of patients with T-ALL[39]. Other studies found that around 1% of patients with B-ALL also display mutations in exon 6, as well as in exon 5 (S185C), of *IL7R*[68]. Exon 6 gain-of-function mutations take place at the extracellular juxtamembrane-transmembrane interface and the majority of them result in an unpaired cysteine residue that is necessary for IL-7Rα homodimerization, and downstream constitutive signaling, independently of IL-7 or the γc subunit[39,68]. Cysteine-dependent signaling hyperactivation was further shown to be required for cell transformation[39,68].

Conditional knock-in mouse model

In order to investigate the putative role of *IL7R* gain-of-function mutations in leukemogenesis, several different experimental strategies have been attempted[69–71]. *In vitro*, we have shown that these mutations can be transforming[39], while *in vivo* experiments using transduced BM or thymic precursors provided only mixed evidence on the role of the mutation in ALL leukemogenesis. Although B and T cell leukemias developed in some instances[69,70] strategies used display caveats that can lead to misinterpretations. Indeed, these models include several artificial features, with important consequences on their capacity to mimic true leukemogenesis settings in patients. First, they rely on transplantation of transduced cells to recipient mice, which requires conditioning of the hosts and may interfere in leukemogenesis. Second, retroviral transduction does not allow for control of copy number or integration site. Last but not least, having the *IL7R* cDNA under the control of a strong promoter rather than of

endogenous regulatory elements will result in overexpression/ectopic expression of the receptor, which may have significant consequences in terms of IL-7R-signaling strength and downstream consequences. A good example of this is the fact that Yokoyama *et al* reported the development of mature "B-ALL"/lymphoma in their *in vivo* model of *IL7R* mutational activation[70], instead of bona fide B-ALL affecting B-cell precursors, as well as the development of myeloproliferative neoplasms – none of which have been reported in humans in association with *IL7R* mutation.

The Cre-LoxP system is a powerful tool that has been used to study molecular genetics through generation of tissue or cell-specific and development-regulated conditional mutants, where genes of interest can be modulated in a defined spatial and temporal context, avoiding resultant indirect effects[72]. Given the power of this method, our laboratory developed a conditional knock-in mouse mutated for IL-7Rα, in which Cre recombinase is under control of the promoter huCD2 and, most importantly, expression of the mutant IL-7Rα will still be under control of endogenous *Il7r* regulation (Figure 4). The huCD2 promoter is known to stimulate expression from the stage of common lymphoid progenitors, therefore we are able to target the consequences of such expression to the lymphocyte (CLP-onwards) lineage, thus to both B and T lymphocytes[72,73]. In this way, we ensure that the mutated receptor is expressed only in lymphoid cells and at physiological levels.

In detail, the mutation selected to characterize IL-7R impact on lymphopoiesis and leukemogenesis was previously identified in our laboratory in T-ALL patients, namely, c.731_732insTTGTCCCAC mutation that led to p.Thr244_Ile245insCysProThr alteration resulting in an insertion of a cysteine[39]. The mutation was further integrated into the genome of a C57BL/6J mouse strain, connoted IL-7RfxCys mouse. The exon 6 mutated sequence is inverted and integrated in the endogenous *Il7r* locus of the mouse where it is flanked by two lox sites, LoxP and Lox511, to avoid uncontrolled expression of the mutant *IL7Rα*. Since the mutation is flanked by lox sequences, Cre recombinase needs to be present in order to activate gene expression. Taking this into account, IL-7RfxCys mice with the flanked mutation were crossed with Cre-expressing mice, where Cre is under the control of the promoter CD2 (CD2CRE). Therefore, when the CD2 promoter is active it allows expression of the Cre recombinase, which in turn will drive the expression of the *IL7Rα* mutation. This knock-in is generated through a FLEX switch, Cre-mediated, recombination process[74], where the mutated exon 6 is reversed to its original orientation in order to be properly transcribed, while the wild type exon 6 is deleted, culminating in the

knock-in of the mutated sequence into the allele. The first cells expressing CD2 during hematopoietic development are the common lymphoid progenitors (CLP). As such these will be the first to recombine the *Il7r* locus leading to the expression of the mutated form of the receptor in all of their progeny, while normal IL-7R function will occur in all other cells that may naturally express the receptor.

This strategy is crucial to mimic a mutated IL-7R in normal lymphocyte maturation and in leukemia development, allowing a correct characterization of how much impact this mutation actually has in the absence of artefactual overexpression of the receptor. The knowledge generated should therefore be closer to the real pathophysiology of the disease downstream from *IL7R* mutational activation and, as such, it should constitute also an excellent tool to discover novel therapies.

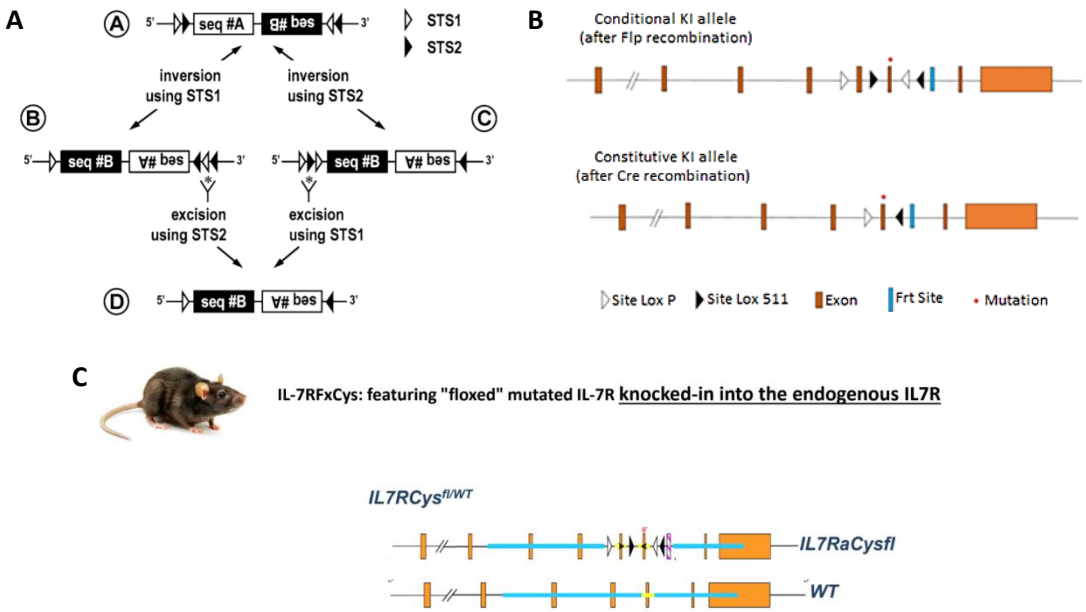


Figure 4 – Cre-LoxP conditional knock-in system A) Schematic of the FLEX switch system. Composition of Flex Switch mechanism by image order (A) a first specific target site 1(STS1) and a first specific target site 2 (STS2) and a A DNA sequence all in a given orientation, followed by a B DNA sequence and two others STS1 and STS2 with an inversed orientation when compared to the first sequences referred. Importantly, both STS1 and STS2 are heterotypes therefore there is no recombination between them. Then, B and C are Cre-mediated intermediate sequences, B is formed by an inversion mediated by recombinase STS2 and C is formed after inversion mediated by recombinase STS2. (D) Final product after Cre-mediated excision between homotypic STS2 and homotypic STS1. This reaction is irreversible. Adapted from Schnütgen *et al*., 2007[74]. **B)** Organization of the IL-7R knock-in allele before and after CRE recombination. **C)** Genotype of the heterozygous mice carrying the mutated allele. These mice were then crossed with huCD2CRE mice.

Homeobox genes and HOXA9

The *HOX* genes encode a family of homeodomain-containing transcription factors that are evolutionarily conserved between species[75] and were originally found in *Drosophila melanogaster* as regulators of trunk and tail development during embryogenesis. In mammals, these genes are divided in 4 clusters (A, B, C and D), located in 4 chromosomes[76] (Figure 5). *HOX* genes are very important in shaping animal morphology, more precisely, in specifying cell identity and positioning throughout embryonic development[77]. *HOX* genes are relevant in normal and aberrant hematopoiesis[78] being differently expressed in hematopoietic cells. For instance, HOXB8 and HOXA10 are expressed in myeloid cells, whereas HOXB3, HOXB4 and HOXA9 have high levels of expression in non-differentiated cells[76]. HOXA9 has been implicated in ALL and I will go into further detail below.

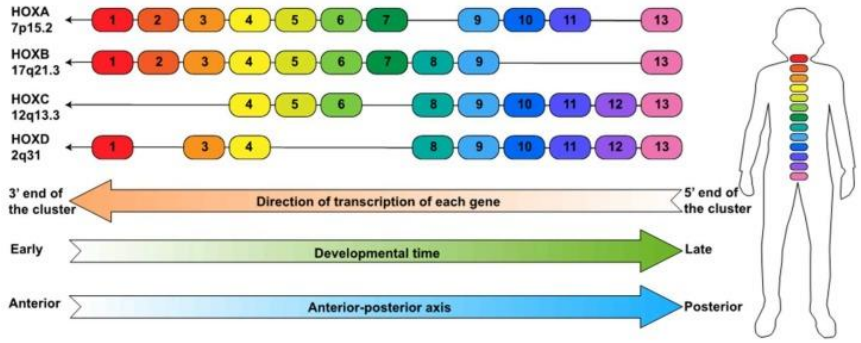


Figure 5 – Genomic organization of the *HOX* genes. The genes are divided in four clusters *HOXA*, *HOXB*, *HOXC*, and *HOXD* in which in total contain 39 genes and are expressed in certain body segments during different development phases. It is represented the direction of the transcription of each gene as well as the direction of the spatial and temporal line in which the *HOX* genes are expressed. From Luo, Z. *et al* 2019[79].

Although, regulation of expression of *HOX* genes is not well established, there is some evidence regarding post-transcriptional regulation by micro RNAs, miR-196[75] and miR-10[80] as well as epigenetic regulators. Polycomb group and trithorax group proteins are crucial to regulate gene expression, have a role in silencing and activating by binding to DNA and induce post-translational modification of histones, respectively[81]. Studies

showed that mutations in the genes belonging to these groups presented similar phenotypes to HOX mutants, suggesting that these genes may be involved in the regulation of *Hox* genes[82]. Moreover, it was found myogenic hypermethylation in regions of *HOX* genes[83] and also methylation of *HOXA* gene was revealed in normal and malignant tissues[84], while others studies suggest that histone (de)acetylation can be involved in the modulation of *HOX* gene transcriptional activity[85].

HOXA9 in hematopoiesis

HOXA9 is known to play a role in hematopoiesis (Figure 6), displaying higher expression in HSCs and being the *HOX* gene most expressed in CD34+ HSCs and early hematopoietic progenitors[76], being downregulated upon differentiation. Studies conducted with *Hoxa9* knock-out mice revealed that *Hoxa9* is necessary for lymphoid development since CLP or earlier phases[78], while in others demonstrated impaired hematopoiesis leading to reduced spleen and thymus size as well as decreased numbers of lymphocytes, granulocytes and committed progenitors[86]. Interestingly, *HOXA9* knockdown abolishes the differentiation of HSCs while preserving a pluripotency profile of the cells[87]. In addition to these findings, *Hoxa9*$^{-/-}$ HSCs present deficiencies in repopulating bone marrow in irradiated mice[88].

As HOXA9 is most highly expressed in the earlier precursors, it is not surprising that in a study, where a Cre-Lox model was developed in which *Hoxa* was conditionally deleted in a homozygous manner, the most notable finding was a decrease of the repopulation potential of *Hoxa*-deficient HSCs, a defect restored upon HOXA9 overexpression. At a molecular level, several genes were shown to be upregulated upon HOXA9 overexpression, such as CCAAT enhancer binding protein alpha *Cebpa*, integrin subunit alpha M *Itgam* and collagen type I alpha 1 chain *Col1a1*[89]. Focusing on the B-lineage, in (wild-type) WT mice, several *Hoxa* genes showed high expression. Mainly, *Hoxa7* and *Hoxa5*, along with *Hoxa9*, were still highly expressed in early B cell development (Fraction A), and their expression decreased progressively thereafter[90]. Using a similar conditional Cre-Lox *Hoxa* knock-out model, results showed a significant reduction of B cell populations, mainly at early stages, from A to D fractions, while stages E and F were not or little affected. Notably, when these floxed *Hoxa*-deleted mice were crossed with CD19-Cre mice, an increase of B cells of Fraction D was observed in these mice compared to the control[90].

Other experiments revealed that *Hoxa* genes may be crucial to maintain a specific pool of B cell progenitors, as aged *Hoxa*-deficient mice showed significant decrease of B cell progenitors in contrast to younger mice[91]. Curiously, in the younger *Hoxa9*$^{-/-}$ mice a delay in T cell development was apparent, as fetal thymuses presented a highly reduced size, whilst in adult mice the reduction was not significant[92,88]. Thus, *HOXA* genes (and *HOXA9* in particular) may participate in the fine-tuning of lymphocyte development.

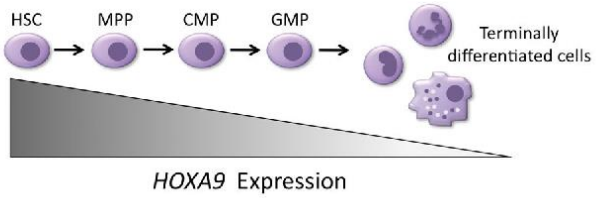


Figure 6 – Modulation of *HOXA9* expression in hematopoiesis. During hematopoiesis process *HOXA9* is highly expressed in hematopoietic stem cells and early progenitor cells and is downregulated upon differentiation. Adapted from Collins, C. *et al* 2016[96].

HOXA9 cofactors

HOX proteins can regulate in a positive and negative way transcription of targets through two domains, the homeodomain which refers to the DNA binding site, and hexapeptide that binds to cofactors[93]. Cofactors that belong to the three amino acid loop extension (TALE) proteins are the most relevant HOX-related cofactors including pre-B cell leukemia transcription factor (PBX) and myeloid ectopic insertion site (MEIS1)[76]. In human leukemia, it is often stated that PBX3 and MEIS1 are both overexpressed along with HOXA9[94,95]. Although HOXA9 can be deregulated through several genetic alterations, such as MLL1 translocations, NUP98 fusions and also translocations with HOXA9 itself[96], overexpression of HOXA9 seems to be the most important prognostic factor in acute leukemia[97,98,]. Moreover, in JAK3/HOXA9-driven T-ALL, STAT5 transcriptional activity was revealed to be significantly active upon HOXA9 expression, therefore, being indicated as a novel HOXA9 cofactor[99]. Transcription factor kruppel-like factor-4 (KLF4) may be also involved in HOXA9 activity as it is shown to be downregulated in HOXA9-mediated acute leukemia[100] and it is also demonstrated that KLF4 can interact with PBX1 and MEIS2 proteins[101].

HOXA9 in leukemia

Deregulated of *HOX* gene expression has been broadly characterized having a tumorigenic role, and it can act as proto-oncogene or tumor suppressor according to different cancers[102]. Recently, it has been discovered that mutations in IL-7R/JAK/STAT5 are frequently found within HOXA-positive T-ALL cases. In particular, *HOXA9* was found highly expressed in cases with JAK3 mutations[99]. Intriguingly, low levels of *HOXA9* in patients with B-ALL have been reported[22,103], suggesting that *HOXA* genes in general and *HOXA9* in particular may have tumor suppressor rather than oncogenic roles in at least some B-ALL subsets.

Objectives

IL-7/IL-7R signaling contributes to B and T cell development and its aberrant signaling is involved in leukemogenesis. Our group and others previously described gain-of-function mutations in *IL7Rα* in T- and B-ALL patients and this led to the need to investigate the impact of the mutations in lymphocyte development and their role in leukemogenesis. For this purpose, a mouse model that could mimic a mutant *IL7R*-driven leukemia was essential. As described in the Introduction, our laboratory developed a conditional knock-in mouse model where an *IL7Rα* gain-of-function mutation occurs in its correct genomic context, without overexpression, and induced upon CD2-controlled Cre recombination so that the expression of the mutation is restricted to the lymphoid lineage. In short, hematopoietic lineage cells from the common lymphoid stage onwards will express the mutated form of the receptor when expressing the IL-7R.

Our intent with this work is to characterize the consequences of such expression, both in lymphocyte development and in leukemogenesis, for the purpose of this thesis, focused in B-ALL. Further, in order to understand the molecular mechanisms involved, we also planned to use Next Generation Sequencing-based data (obtained from the characterization of the leukemias) to uncover putative contributors working with the IL-7R in the transformation process. These observations led us to a puzzling finding: downregulation of *Hoxa* genes, including *Hoxa9* - which are normally regarded as oncogenic - in leukemic cells. Due to its vital role in hematopoietic development, we thus set out to understand the role of HOXA9 in mutated IL-7Rα induced B-ALL.

Therefore, two main aims were defined:

1. Characterize the *in vivo* impact of gain of function mutant IL-7R signaling in B lymphocyte development and leukemogenesis.

2. Explore the role of *HOXA9* gene in gain-of-function mutant IL-7R driven leukemogenesis.

Material and Methods

Experimental animals used

As mentioned above, our laboratory developed a mouse model by crossing a mouse carrying the *IL7Rα* mutation with CD2CRE mouse[72] that expresses Cre recombinase under the control of CD2 promoter. This promoter will promote expression of Cre from the common lymphoid progenitor stage and it will allow the expression of the *IL7Rα* mutation in the following stages, therefore, in B and T cells. The resultant mice are hereafter referred to as huCD2Cre.IL7Rfxcys mice and particularly, two mice genotypes were used for the experiments: $CD2Cre^{pos}IL7R^{wt/fl}$, animals (experimental mutant *IL7R* heterozygous), $CD2Cre^{neg}IL7R^{wt/fl}$ (control animals expressing floxed allele in absence of Cre, thus WT). $CD2Cre^{pos}IL7R^{wt/wt}$ animals were also maintained, to monitor for any possible Cre-toxicity effects, which, as expected for this strain[104], were not detected. For transplantation assays were used Balb/c $Rag^{-/-}$ $\gamma c^{-/-}$ mice with B, T and NK cell immunodeficiency[105]. All the animal procedures were approved by institutional Animal Ethics committee of Instituto de Medicina Molecular and followed the guidelines for the use of laboratory animals by European commission and Portuguese authorities.

Mouse genotyping

Extracts of mice tails and toes were taken and placed into a 1.5ml eppendorfs. Firstly, the samples were lysate with 500µl of tail lysis buffer (Tris pH8 10mM, NaCl 100Mm, EDTA pH8 10mM and SDS 0.5%) and 10µl of proteinase K from *Tritirachium album* (20mg/ml) (Sigma-Aldrich) and left in a wet or dry bath for 3-4hours at 56ºC. After the digestion, the samples were centrifuged for 5 min at 13000rpm and the supernatant was collected and transferred to a 2ml eppendorf. It was added 1ml of isopropanol and the tubes were mixed without vortexing and were incubated overnight at -20ºC. Afterwards, the tubes were centrifuged for 25 min at 13000rpm, 4ºC and the supernatant was removed. Then, the samples were washed with 500µl of 70% ethanol, centrifuge for 15min at 13000rpm, 4ºC following the carefully removal of the supernatant and the samples were left to dry at air. Once dried, the DNA pellet was resuspended in 200µl of sterile Milli Q water and the tubes were left for 10 min in a wet or dry bath at 56ºC for the DNA pellet to go up and stored at -20ºC. For the genotyping, specific primers and different types of DNA polymerase were used, for the Cre recombinase genotyping was used a regular Taq DNA polymerase (Thermo Fisher Scientific) and the primers: CD2Cre-

Forward (AGATGCCAGGACATCAGGAACCTG) and CD2Cre-Reverse (ATCAGCCACACCAGACACAGAGATC). For the floxed *IL7Rα* mutation primers LBM-Foward (ATCTTTACTACTAGAAAGGAAGTGGGTCAG) and LBM-Reverse (GTGGGAAGAACATCAGCTACTTGAAATGTA) were used and also a different DNA polymerase, TaKaRa Taq enzyme (TaKaRa Bio), as suggested by the company that generated the animals (Cyagen). After preparing the amplification mix in the PCR reaction tubes, they were placed in a T100 ™ Thermal Cycler (BioRad) for the PCR assay with specific program of temperatures and times. After PCR assay, samples were run in a 1.5% agarose gel with Tris-Acetate-EDTA (TAE) buffer 1x and DNA bands were observed in Chemidoc XRS+ (BioRad) for analysis and genotyping the mice.

Mice leukemia characterization

Blood samples analysis

For the blood analysis, blood samples were collected in a 1.5ml tube from the facial vein of animals at weeks 4, 6, 8 and then every two weeks into a tube with Heparin 0.6mg/ml (Sigma-Aldrich). Red blood cells were lysed with 950µl of Red Blood Cell (RBC) Lysis Buffer 1x (eBioscience) and incubated for 10min at room temperature with and transferred to 5ml FACS tubes. Cells were washed with 3ml of phosphate-buffered saline (PBS) 1x and centrifuged at 1230rpm during 6min. The supernatant was discarded and the cell pellet was resuspended in 200µl of FACS Buffer (PBS 1x with 2% Fetal Bovine Serum (FBS)). The samples were then transferred into a 96-well round bottom plate, centrifuged at 2000rpm for 2min and the supernatant discarded to initiate the stainings. Then the cells were stained for 20 min on the dark/ice with two different mixes diluted in BD Horizon™ Brilliant Stain Buffer (BD Biosciences) that were previously prepared, mix 1 was composed by the following antibodies: CD4 FITC (BioLegend), IgM PE (BioLegend), Gr-1 PerCP (BioLegend), CD19 PeCy7 (eBioscience), CD8 APC (BioLegend), TCRβ APC-Cy7 (BioLegend), IgD BV-510 (BioLegend), CD45 BV-605 (BioLegend) and CD11b BV-711 (BioLegend). Mix 2 was composed by the following antibodies: CD24 FITC (BioLegend), IgM PE (BioLegend), BP-1 PeCy7 (BioLegend), CD93 APC (BioLegend), CD19 APC-Cy7 (BioLegend), CD127 BV-421 (BioLegend), IgD BV-510 (BioLegend), CD45 BV-605 (BioLegend) and B220 BV-711 (BioLegend). For the leukemic transduced cells (described later on) a different mix was prepared with the antibodies: IgM PE (BioLegend), BP-1 PeCy7 (BioLegend), CD19 APC (BioLegend), IgD BV-510

(BioLegend), CD45 BV-605 (BioLegend) and CD11b BV-711 (BioLegend). After staining, the samples were washed with 100µl of FACS Buffer and centrifuged for 2min at 2000rpm. Supernatant was discarded and cells resuspended in 100µl FACS Buffer and placed in 5ml FACS tubes containing 200µl of FACS Buffer. Finally, samples were analyzed using a BD LSRFortessa™ cell analyzer with FACSDiva software 6.2 (BD Biosciences). After acquiring, cells were analyzed using FlowJo software.

Leukemia characterization

After animals developed leukemia and presented signs of sickness such as paralysis and 20% loss of weight they were humanely sacrificed with pentobarbital injection (Euthasol) (Ecuphar) and the subsequent removal of critical organs. Spleen, bone marrow, liver, thymus and lymph nodes were collected and macerated with FACS Buffer into a cell suspension. Spleen and bone marrow cell suspension were centrifuged at 1230rpm for 6min, supernatant was discarded and was add to the pellet 4ml to bone marrow and 5ml to spleen of RBC lysis and left incubated for 6 min to lysate the red blood cells. FACS Buffer was added to dilute RBC lysis solution and stop the reaction and cells were centrifuged for 6min at 1230rpm. Supernatant was discarded and cells were resuspended in FACS Buffer and filtered through a 70µm cell strainer to remove the debris. Cells were then counted and divided in a 96-well round bottom plate for characterization. Then, cells were washed with 100µl of FACS Buffer, centrifuged at 2000rpm for 2min and supernatant was discarded. Leukemic cells were stained with three different mixes previously prepared, diluted in the same Brilliant Stain Buffer as the blood samples antibody mix, composed by mix 1: CD4 FITC (BioLegend), CD127 PE (BioLegend), Gr-1 PerCP (BioLegend), CD25 PeCy7 (BioLegend), IgM APC (BioLegend), TCRβ APC-Cy7 (BioLegend), CD19 BV-421 (BioLegend), IgD BV-510 (BioLegend), CD45 BV-605 (BioLegend) and CD11b BV-711 (BioLegend), mix 2 was equal to the mix 2 from the blood analysis and lastly, mix 3 was composed by: CD21 FITC (BioLegend), CD43 PE (BD Biosciences), CD45 PerCP (BioLegend), cKit PeCy7 (BioLegend), CD19 APC (BioLegend), CD5 APC-Cy7 (eBioscience), CD127 BV-421 (BioLegend), IgD BV-510 (BioLegend), IgM BV-605 (BioLegend) and B220 BV-711 (BioLegend). For the transduced leukemic cells recovered from the mice is the same mix as the blood samples. After 20min of staining with each antibodies mix, cells were washed with 100µl FACS Buffer and centrifuged at 2000rpm for 2min. The supernatant was discarded and cells were resuspended in in 100µl of FACS Buffer and placed in 5ml FACS tubes containing 200µl FACS Buffer. The samples were acquired using a BD LSRFortessa™ cell analyzer with

FACSDiva software 6.2 (BD Biosciences). After acquiring, cells were analyzed using FlowJo software.

Cell viability and proliferation assays

For cell viability, an Annexin V assay was performed with $0.5x10^6$ of cells in a 96-well round bottom plate. Then were washed with 100µl of Annexin binding buffer (PromoKine) and centrifuged at 2000rpm for 2min. Leukemic cells were stained for 15min room temperature/dark, with an antibody mix diluted in Annexin Binding Buffer composed by: CD45 FITC, 7-AAD PerCP (eBioscience), CD19 PeCy7, Annexin V APC (eBioscience) and IgD BV-510 (BioLegend). Regarding the transduced leukemic cells, were used few cells according to the limiting number of resultant cells from the sorting and the mix was composed by: IgM PE (BioLegend), 7-AAD PerCP (eBioscience), BP-1 PeCy7 (BioLegend), Annexin V APC (eBioscience) CD19 APC-Cy7 (BioLegend), IgD BV-510 (BioLegend) and CD45 BV-605 (BioLegend). In each annexin assay it was performed a staining in a well with an equal mix but without the Annexin V marker, being the control sample. After staining, cells were transferred to 5ml FACS tubes with 200µl of Annexin binding buffer and analyzed by flow cytometry, as described before.

Regarding proliferation assay, a Ki67 intracellular staining protocol was performed. The same criterion upon number of leukemic and transduced leukemic cells was used to transfer the cells to a 96-well round bottom plate. Cells were washed with FACS Buffer and centrifuged for 2min at 2000rpm. Supernatant was discarded and leukemic cells were stained for 20min on the ice/dark with a surface antibody mix diluted in Brilliant Stain Buffer with the following antibodies: CD45 FITC, IgD PerCP and CD19 PeCy7. For the transduced leukemic cells the surface mix was composed by: IgM PE, IgD PerCP, BP-1 PeCy7, CD19 APC-Cy7 and CD45 BV-605. After staining, the cells were washed with FACS Buffer and centrifuged for 2min at 2000rpm. Supernatant was discarded and cells were vortexed to dissociate the pellet and then fixed using 200µl of fixation buffer composed by ¼ of Fixation/Permeabilization concentrate (eBioscience) and ¾ of Fixation/Permeabilization diluent (eBioscience) and were incubated for 40 minutes at room temperature/dark. Next, cells were washed with 150µl of Permeabilization Buffer 1x (eBioscience) and centrifuged for 2min at 2000rpm. Supernatant was discarded and cells were stained differently: in one sample was pipeted Permeabilization Buffer 1x with no antibodies, other sample were stained with a mix composed by Fc Block (CD16/32) (BioLegend) and κ Isotype control Alexa Fluor® 647 (BioLegend) diluted in Permeabilization Buffer 1x. These last samples were the control. The other samples were

stained with the Ki67 marker with a mix composed by Ki67 APC (BioLegend) and Fc block diluted in Permeabilization Buffer 1x and incubate for 20min room temperature/dark. Then, the cells were washed with FACS Buffer, centrifuged for 2min at 2000rpm and the supernatant discarded. Cells were resuspended in 100µl of FACS Buffer, transferred to FACS tubes with 200µl of FACS Buffer and finally analyzed in Flow Cytometry.

Bacteria culture

Human HOXA9-MSCV-short plasmid was a gift from Corey Largman (Addgene plasmid # 20978; http://n2t.net/addgene:20978 ; RRID:Addgene_20978)[106] and MSCV-IRES-GFP plasmid (Addgene), both represented in the Figure 7, and pCL-Eco retrovirus packaging vector plasmid (Addgene) were grown overnight at 37ºC in plates of Luria-Bertani medium, LB (10g/L Tryptone (BD Biosciences), 5g/L Yeast Extract (BD Biosciences), 10g/L NaCl (Sigma-Aldrich)), supplemented with agar (15g/L) (Sigma-Aldrich) and 100µg/mL of ampicillin (Sigma-Aldrich). Upon overnight incubation at 37ºC, several isolated colonies were selected from each plate and pre-inoculated in LB medium supplemented with 100µg/mL ampicillin, overnight at 37ºC with orbital agitation of 220rpm.

The pre-inoculum was then inoculated in LB medium supplemented with 100µg/mL ampicillin, in bacteria to medium proportion of 1:100, for 6h at 37ºC with orbital agitation of 220rpm. To confirm the quality of the DNA, part of the pre-inoculum was used to performed DNA extraction using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according the manufacturer's instructions. DNA concentration was measured using spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific). To confirm the quality of the Human HOXA9-MSCV-short plasmid and the insert gene an enzimatic digestion was performed with SpeI (New England BioLabs) and SacII (Promega) enzymes, to confirm the pCL-Eco retrovirus packaging vector was used SacI (New England BioLabs) and the in MSCV-IRES-GFP plasmid, the AfeI enzyme (New England BioLabs) was used. The reaction mix with the different samples were put in a bath for 1h30 at 37ºC and the samples were run in a 1.5% agarose gel with Tris-Acetate-EDTA (TAE) buffer 1x and the plasmid DNA was revealed through Chemidoc XRS+ (BioRad).

After confirmation, one of the clones that were left growing overday (6h) was selected and left growing overnight at 37ºC with orbital agitation of 220rpm in LB medium supplemented with 150µg/mL of ampicillin (1:200 proportion of bacteria to medium). Then,

the inoculum were again supplemented with 100µg/mL of ampicillin and left in orbital agitation 220rpm during 2h, at 37ºC. Afterwards, the bacteria culture was centrifuged at 4ºC during 15 min, at 4000rpm and the pellet was stored at -20ºC. DNA extraction was performed following Genopure Plasmid Maxi Kit (Roche) following the manufacturer's instructions except in the step 4 since it was chose to clear the lysate by centrifugation at 4000rpm during 1h. DNA concentration was measured using a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific) and the DNA samples were storage at -20ºC.

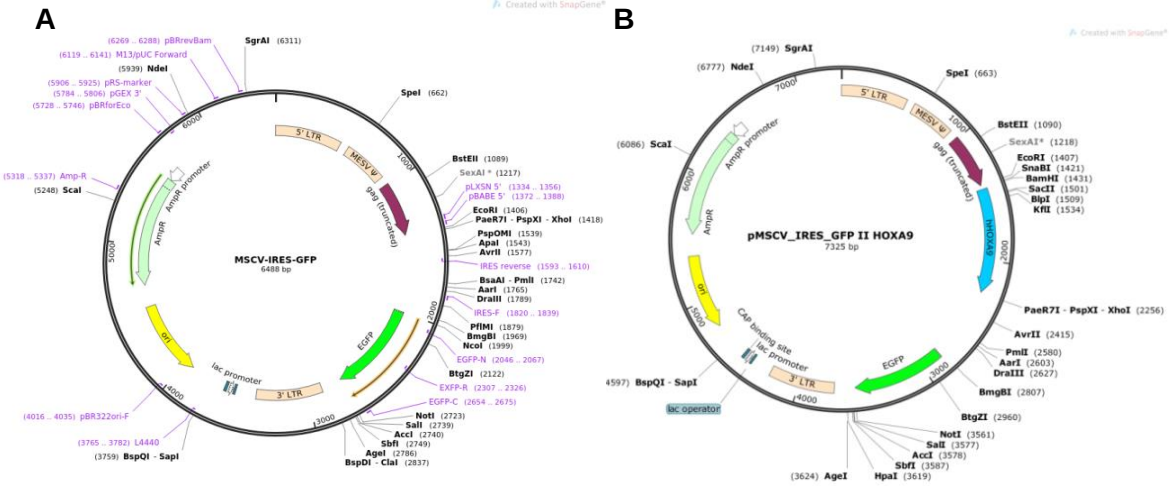


Figure 7 – MSCV-IRES-GFP and HOXA9-MSCV-short plasmid structure. A) Represents the MSCV-IRES-GFP plasmid and the image was acquired from Addgene website. **B)** HOXA9-MSCV-short plasmid is composed by vector MSCV-IRES-GFP and insert HOXA9 gene. HOXA9-MSCV-short plasmid was created with SnapGene software. Both plasmids have the reporter gene to codify a fluorescent protein GFP.

Cell culture

CD2Cre^{pos}IL7R$^{wt/fl}$ mice leukemic cells from the spleen were cultured in Iscove Modified Dulbecco Media (IMDM) GlutaMAX medium (Gibco) with 10% fetal bovine serum (FBS) (Biowest), 1% penicillin/streptomycin (Gibco), 1% Minimum Essential Medium Non-Essential Amino Acids (MEM-NEAA) (Gibco), 1% Sodium Pyruvate (Gibco), 0,1% Gentamicin (Gibco) and 2-Mercaptoethanol 1:1000 (Gibco) (referred as IMDM medium), at a density of 1x10^6 cells/mL at 37ºC in 5% CO^2 with 20ng/mL of IL-7 (Peprotech).

HEK293T (Human embryonic kidney 293T) cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose, pyruvate culture medium (Gibco), supplemented with 10% of FBS (Biowest), 1% of penicillin/streptomycin (Gibco) and 1% of L-glutamine (Gibco) (hereafter referred to as D10 medium). Trypsin-EDTA 1x (Gibco) was used to detach the cells, at 37ºC, and its activity was inactivated adding serum-supplemented medium.

Transfection – Retroviral production

Firstly, $2x10^6$ HEK293T cells were cultured in a 100mm dish and kept in culture in D10 medium for 24h. To initiate the transfection, the culture medium was changed for D10 medium without antibiotic. Then, lipofectamine™ 2000 (Invitrogen) was diluted 1:25 in Opti-MEM™ Reduced Serum Medium (Gibco) and incubated for 5 minutes at room temperature. A mix of 6,6µg of pCL-Eco DNA with 6,6µg of each DNA of interest, MSCV-IRES-GFP and HOXA9-MSCV-short was diluted in 600µl of Opti-MEM. The lipofectamine was added to both mixtures and left incubated for 30 minutes at room temperature. The final mixture (1200µl of final volume) was added dropwise to the cells plated with a confluence of 70-90% and incubated for 24h at 37ºC in 5% CO_2. Afterwards, the medium was changed to D10 again to keep the cells in culture. The first batch of viral supernatant was collected 48h after, carefully pipetting D10 medium afterwards, and 72h after transfection. Then, it was centrifuged at 1300rpm 5min, the viral supernatant was aliquoted, flash-frozen in liquid nitrogen and stored at -80ºC. The efficiency of transfection was assessed measuring GFP expression by flow cytometry analysis. The retrovirus produced containing HOXA9-MSCV-short plasmid is referred to as Hoxa9 virus and the retrovirus containing MSCV-IRES-GFP plasmid is hereafter referred to as Empty virus.

Transduction of the primary cells

Mice primary spleen leukemic cells were thawed either on the same day as transduction and left for approximately 8h or left for a few days to grow in culture in a density of $1x10^6$ cells/mL at 37ºC in 5% CO^2. Cells were first filtered through a 40 µm cell strainer and were centrifuge at 300g 7min and put at a density of $3x10^6$ cells/mL with 40ng/mL of IL-7. The cells were added first and then the retroviral supernatant in a 1:2 dilution to a 350ul of final volume in flat-bottom 96-well plate with a final concentration of 12 µg/ml polybrene (Sigma-Aldrich). The plates were then centrifuged at 32ºC during 90 min at 1500g. After spin infection, the supernatant (100µl) is removed and IMDM medium is added and each transduced cells from the same condition are collected. After a 300g 7

min centrifugation the cells were resuspended with IMDM medium and were incubated in 96-well plates round bottom at a density of $1x10^6$ cells/mL with 20ng/mL of IL-7, at 37ºC in 5% CO^2. Both retroviruses contain plasmids with a reporter gene that allows the expression of a fluorescent protein GFP when integrated in the genome and expressed by the host cell.

Sorting of transduced cells

Transduced cells were sorted 3 days after transduction and were first collected into a falcon tube and centrifuged for 6min at 1230rpm. Supernatant was discarded and cells were counted and washed with FACS Buffer and transferred to a 5ml FACS tube and centrifuged for 6min at 1230rpm with the supernatant discarded. Then were stained for 20min with a mix diluted in FACS Buffer composed by IgM PE (BioLegend), IgD PerCP (BioLegend), BP-1 PeCy7 (BioLegend) and CD19 APC-Cy7 (BioLegend). Cells were then washed with FACS Buffer and centrifuged for 6 min at 1230rpm. Supernatant was discarded and the cells were resuspended in IMDM medium if it was to put in culture after or FACS Buffer if it was to transfer to mice. Cells were then sorted using FACS Aria (BD Bioscience).

After sorting, cells were washed with PBS 1x, counted and centrifuged for 6min at 1230rpm, supernatant discarded and resuspended in PBS 1x to transfer to mice. For cell culture, cells were washed with IMDM medium and centrifuged during 6min at 1230rpm and the supernatant discarded. Were resuspended with IMDM medium and cultured at a density of $0.5x10^6$ cells/mL at 37ºC in 5% CO^2 with 20ng/mL of IL-7 in 96-well plate round bottom.

Treatment with epigenetic inhibitors

Leukemic spleen cells from #2674 mice were thawed with a following separation of live lymphocytes by Ficoll-Paque™ (GE Healthcare) (performed by a lab colleague). In a falcon with 25ml of cells in a pre-warmed IMDM medium pipet carefully 12.5ml of Ficoll and centrifuge for 20min at 2200rpm 18ºC with the brake turned off. Remove the mononuclear cell layer very carefully and transfer it to a falcon with pre-warmed IMDM medium, then centrifuge for 6min at 1230rpm and discard supernatant. Cells were cultured for 24h in IMDM medium at a density of $1x10^6$ cells/mL at 37ºC in 5% CO^2 with 20ng/mL of IL-7. Before the treatment, cells were centrifuged at 1230rpm during 6min and

counted and divided in a 96-well plate flat bottom at a density of $1x10^6$ cells/mL with 20ng/mL of IL-7. Histone deacetylase inhibitor, Suberoylanilide Hydroxamic Acid (SAHA) (Cayman Chemicals) was used in two different concentrations: 1 μM and 2.5 μM. Cells were treated and 8hours after were analyzed by Flow Cytometry and collected and stored as dry pellets at -80ºC. 5-Azacytidine was used as DNA methyltransferase inhibitor (Sigma-Aldrich) in three different concentrations: 25 nM, 250 nM, and 2.5 μM and cells were collected and stored as dry pellets at -80ºC and analyzed 48h and 72h after the treatment. In both experiments the control used was DMSO (Sigma-Aldrich) at the highest concentration used for the drug.

Quantitative Real-Time PCR analysis

The harvested and sorted cells were washed twice with PBS 1x (resuspend and centrifuge) at 4ºC 5 min 3200rpm and the dry pellet was stored at -80ºC. Afterwards, RNA extraction was executed following RNeasy Mini Kit from Qiagen and its quantification was performed using a NanoDrop 2000 then stored at -80ºC.

cDNA synthesis and RT-PCR

cDNA synthesis was performed according to manufacturer instructions using the SuperScript™ III First-Strand Synthesis SuperMix kit from Invitrogen following a pre-amplification with TaqMan™ PreAmp Master Mix Kit (Applied Biosystems) using manufacturer's instructions from Thermo Fisher Scientific. Both reactions were performed with a T100 ™ Thermal Cycler (BioRad). In order to quantify gene expression, a quantitative real-time PCR was performed with Single Tube TaqMan Gene Expression Assays (Applied Biosystems) following manufacturer's instructions (Thermo Fisher Scientific) using the Applied Biosystems™ 7500 Fast Real-Time PCR System. DNA probes used were *Hoxa9* (Assay ID: Mm00439364_m1), *P21* (Assay ID: Mm04205640_g1) and *Hprt* (Assay ID: Mm03024075_m1) from Taqman Thermo Fisher Scientific. Gene expression analysis from RT-PCR analysis was calculated with the comparative CT method $2^{-\Delta CT}$.

Statistical analysis

GraphPad Prism version 6.05 (GraphPad Software) was used to perform statistical analysis. Differences between groups were calculated using one-paired t test. Differences in the survival curves were calculated using logrank Mantel-Cox test. P-values lower than 0.05 were considered statistically significant.

Results

Mutant IL-7R expression from the CLP stage

As previously stated in the introduction, IL-7 and IL-7R possess a relevant function in hematopoiesis. For that reason, deregulation of their expression or activity, including as a consequence of *IL7R* gain-of-function mutations, can affect production of hematopoietic cells.

Developing of a mouse model that can mimic the functional expression of the IL-7 receptor in the correct stage during normal hematopoiesis was crucial to investigate its impact on lymphopoiesis. Given the studies reporting IL-7Rα importance from the stage of common lymphoid progenitors (CLP), it was important to have a model that allowed for the receptor to be physiologically expressed from that stage onwards. We thus developed a Cre-LoxP system-based conditional knock-in mouse model in which a line expressing Cre recombinase under the control of the promoter CD2 (CD2CRE) was crossed with a line that possesses a "floxed" exon 6 *IL7R* gain-of-function mutation, as described above. As also highlighted before, a key feature of this model was the ability to reproduce the expression in a physiological and controlled way in order to better properly evaluate the impact of a mutated *IL7R* in normal B cell development and its consequence for leukemogenesis without the confounding factors discussed in the Introduction.

Upon breeding, the offspring were genotyped for the presence of Cre recombinase and for the floxed *IL7R* mutation. As mentioned in the Methods section, two resultant genotypes were mainly used: $CD2Cre^{pos}IL7R^{wt/fl}$, animals (experimental animal, heterozygous for mutant *IL7R*), $CD2Cre^{neg}IL7R^{wt/fl}$ (control animals, expressing the floxed allele in the absence of Cre, thus being wild type). The different mice were maintained under regular monitoring through weighing, behavior evaluation, signs of disease and weekly bleeding with further analysis by flow cytometry.

CD2Cre^{pos}IL7R$^{wt/fl}$ animals display increased frequency of B-lineage cells

In order to explore the impact of the mutation, we analyzed weekly the blood of CD2Cre^{neg}IL7R$^{wt/fl}$ compared to CD2Cre^{pos}IL7R$^{wt/fl}$ animals. The blood samples taken from the mice were processed by first lysing the erythrocytes and further staining with an antibody mix that consist of several cell-surface markers for the different hematopoietic lineage subset of cells (see Methods). Expression of cell-specific markers on the surface allowed the identification of different subsets in the blood samples using flow cytometry and further analysis with FlowJo software. In the context of lymphoid development, we studied the percentage of B cells (expressing CD19) and T cells (expressing TCRβ) at 6, 12, 24, 52, 72 and 104 weeks of age. As shown in Figure 8, we found an increased fraction of CD19 positive cells, from early time points (6 weeks) and extending up to 2 years of age, while TCRβ positive cells were, on the contrary, found at lower fractions throughout time. Myeloid cells seemed largely unaffected. This aberrant phenotype was seen in all IL-7Rα mutant mice. These observations indicate that the IL-7Rα in mutated form has a functional impact in lymphocyte development and provide insights into the timeline of effect of the receptor.

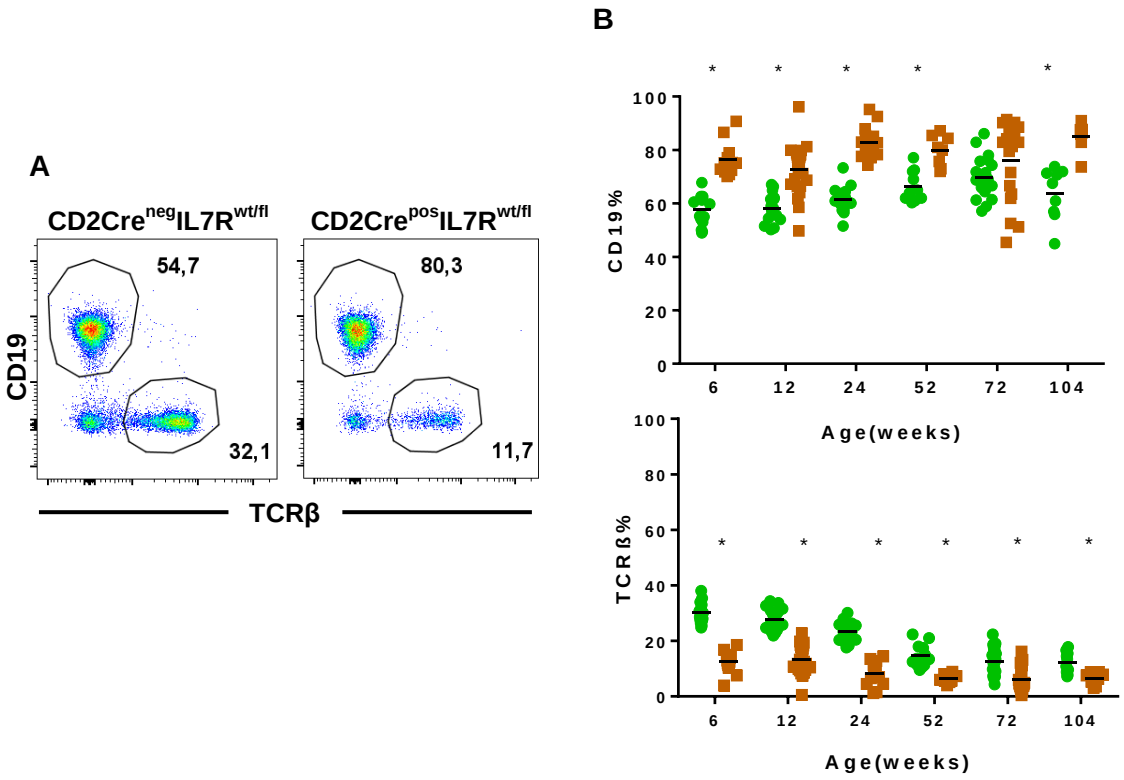


Figure 8 - FACS analysis of blood samples from CD2Cre^{pos}IL7R$^{wt/fl}$ and CD2Cre^{neg}IL7R$^{wt/fl}$ controls. A) Dot plots show analysis of a representative animal from control and CD2Cre^{pos}IL7R$^{wt/fl}$ groups at 12 weeks of age. Dot plots are gated in CD45^{+} cells and show the major lymphoid lineages subpopulations. **B)** Summarized data from blood analysis for the indicated ages in weeks (6, 12, 24, 52, 72 and 104 weeks) in the graphs shown on the right. Each dot represents one individual mouse. Note an increase of CD19^{+} cells in CD2Cre^{pos}IL7R$^{wt/fl}$ mice represented in brown when compared to controls, represented in green dots. P-value * <0.05

Increase in B lymphoid cells is due to the expansion of immature/precursor B-lineage cells

The increase in B cell frequency could be the result of a direct positive effect of the mutation on the B cell compartment, or alternatively, a negative effect on the T cell compartment. In order to solve this issue, we included further markers in the analysis, in order to evaluate direct impacts on the two lineages. As we were interested in investigating acute lymphoblastic leukemia-related phenotypes, we have thus evaluated the possible presence of abnormal levels of B-lineage precursors.

An additional antibody mix specific for B cells was prepared and the blood samples were stained. Besides CD19, markers used for deeper characterization were the surface immunoglobulins IgM and IgD and CD93, a B cell precursor marker. Analyses were performed at 6 and 12 weeks of age, comparing control and heterozygous *IL7R* mutated mice (Figure 9).This analysis revealed a clear increase in the fraction of precursor B lineage cells, seen as a relative increase of IgM-IgD- cells expressing CD93 (Figure 9). Similar analysis of frequency and cellularity in the bone marrow indicated that there is not only a relative increase in B cell precursors but also in absolute numbers in mutant *IL7R* animals as compared to controls (not shown).

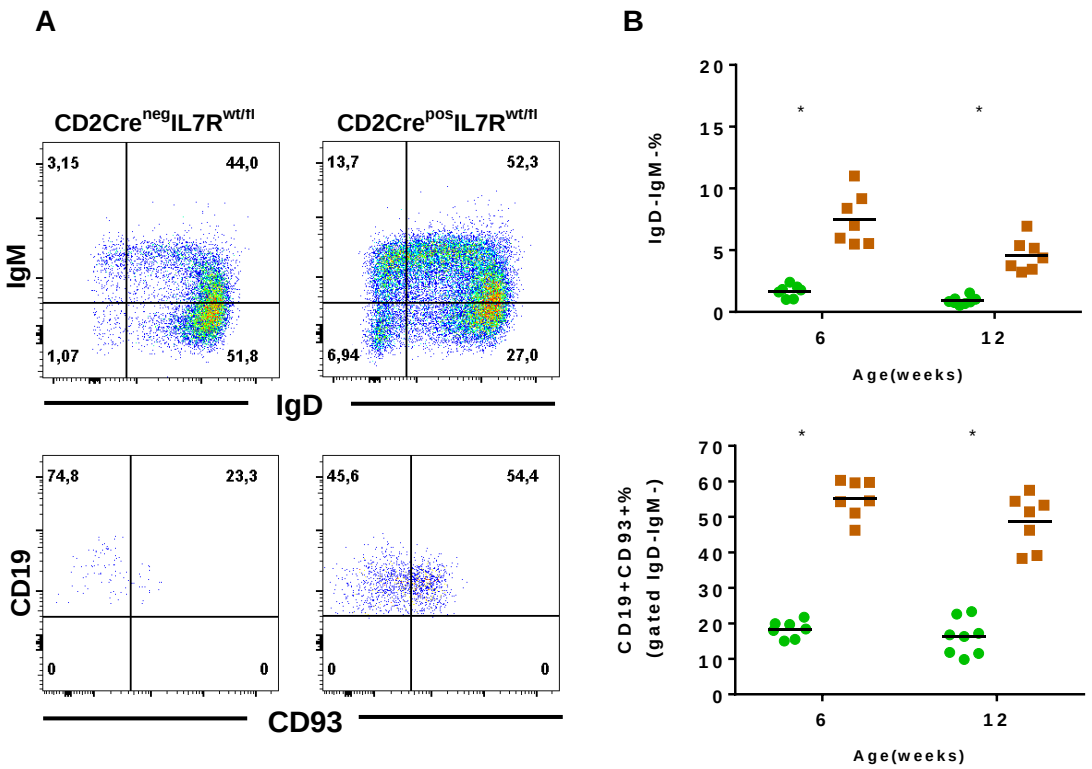


Figure 9 - B-lineage cells analysis from blood samples. A) Dot plots show analysis of representative controls and CD2Cre^{pos}IL7R$^{wt/fl}$ animals at 12 weeks of age. The top two dot plots are gated in CD19+ cells and show the percentage of double negative IgM-IgD- B cells while the bottom two are gated IgD-IgM- (from the gate above) and shows the B cells precursors. **B)** On the right is the graph that shows the data collected from the blood analysis at 6 and 12 weeks of age. Each dot represents a mouse, specifically, the green dots are control mice and brown dots represent the CD2Cre^{pos}IL7R$^{wt/fl}$ mice. Notably, there an increase of double negative IgM-IgD- and precursors B cells in CD2Cre^{pos}IL7R$^{wt/fl}$ when compared to controls. P-value * <0.05

CD2Cre^{pos}IL7R$^{wt/fl}$ animals develop B-ALL

As mentioned above, the increase in precursor CD19-positive cells described above was found in all CD2Cre^{pos}IL7R$^{wt/fl}$ animals (Figure 9) but with time we noticed that some of the animals presented dramatic increases in this fraction, as shown in a representative animal in Figure 10. Animals developed severe signs of disease such as hind leg paralysis and 20% loss of weight, reaching thus the established humane endpoints. We collected spleen, bone marrow, liver, lymph nodes and thymus and prepared cell suspensions for flow cytometry-based immunophenotyping. We used a mix of antibodies directed to B-cell precursor antigens in order to characterize the expanding cells and to further use in transfer experiments. In the latter experiments we were able to transfer disease (not shown), confirming its malignant nature. In parallel, we collected other organs and tissues (kidneys, liver, bone (humerus), CNS, heart, lungs, and also, a piece of the previously weighed spleen) to be preserved in formaldehyde for posterior histological analysis. Importantly, we also froze spleen and bone marrow cells to be used in future characterizations and/or experiments.

The leukemia-free survival curve, as indicated in Figure 11, demonstrates extremely high penetrance of disease (near 80%). As shown, none of the control animals developed B cell leukemia throughout the follow-up, even though as animals got near known lifespan limit (we stopped the experiment at 104 weeks) some control animals showed disease symptoms (such as ascites -swollen belly-, buphthalmos -enlarged eyeball- or general debilitated condition) without signs of leukemia, as seen in our immunophenotypic characterization. Thus, we concluded in those cases that a non-hematopoietic disease was the cause of death.

Deep immunophenotypic characterization of leukemic cells in the bone marrow and spleen confirmed their immature phenotype, as shown in three representative cases (Figure 12). While leukemias coincided in being CD19-positive and lacking IgM and IgD surface expression, they were heterogeneous insofar as other immaturity markers (e.g. BP-1, CD93) were concerned and also different expression of CD45 marker (Figure 12 and Table 1). Nevertheless, as summarized in Table 1, all samples displayed an immature phenotype, ranging from Hardy fraction B (early pro B) to D (small pre B), compatible with *bona fide* B-ALL.

Furthermore, proliferation and cell viability analyses showed that leukemic cells displayed high levels of the proliferation marker Ki67 in immature cells (Figure 13), and also high levels of 7AAD/AnnexinV-negative cells were also observed in spleen and bone marrow (Figure 13). Thus, animals expressing the gain-of-function *IL7R* mutation from the CLP stage onwards have a strong probability of developing B-cell leukemia. Overall, these observations provide clear evidence for the leukemogenic role of IL-7R mutational activation in developing B cells leading to the development of B-ALL.

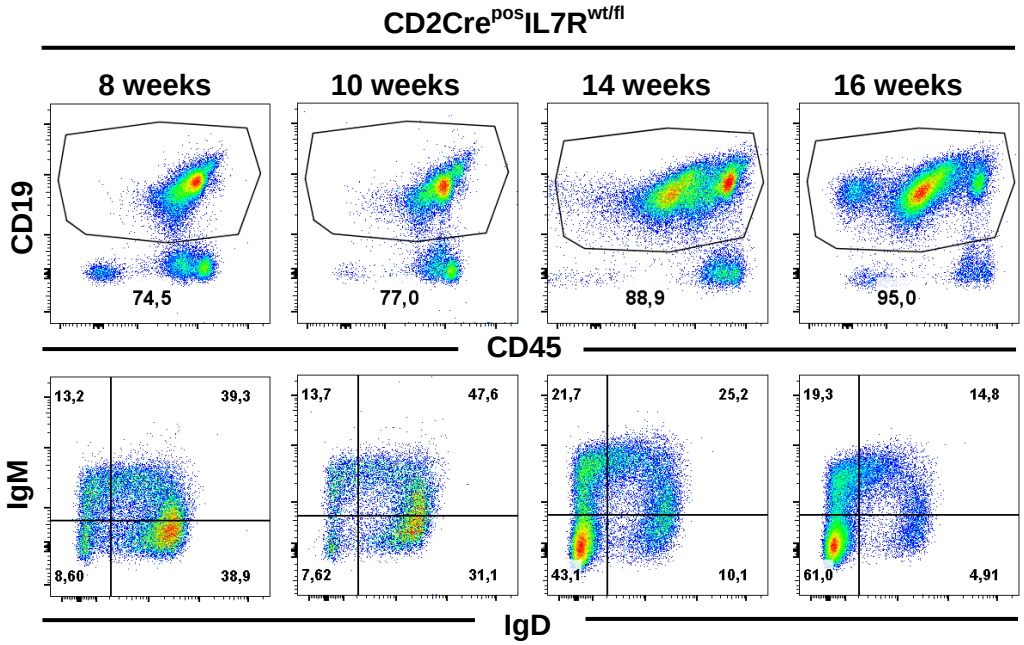


Figure 10 - FACS analysis of CD2Cre^{pos}IL7R$^{wt/fl}$ leukemic mice. Dot plots show a representative evolution of leukemia phenotype in blood samples. The top four plots are gated Lymphocytes and the bottom four are gated CD19+. Notably, increases of not only CD19+ but also double negative IgM-IgD- are observed.

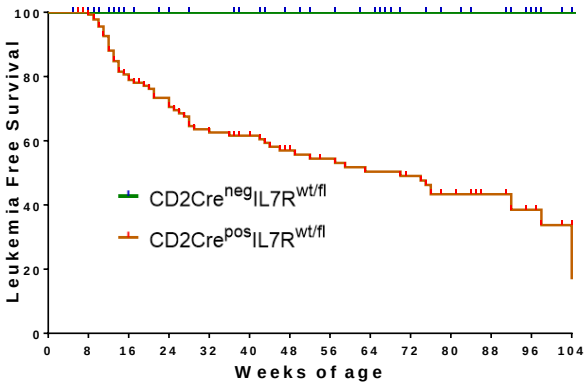


Figure 11 – Leukemia Free Survival Graph representing leukemia free survival curve of CD2Cre^{pos}IL7R$^{wt/fl}$ mice in orange and CD2Cre^{neg}IL7R$^{wt/fl}$ controls in green. P-value is < 0.0001.

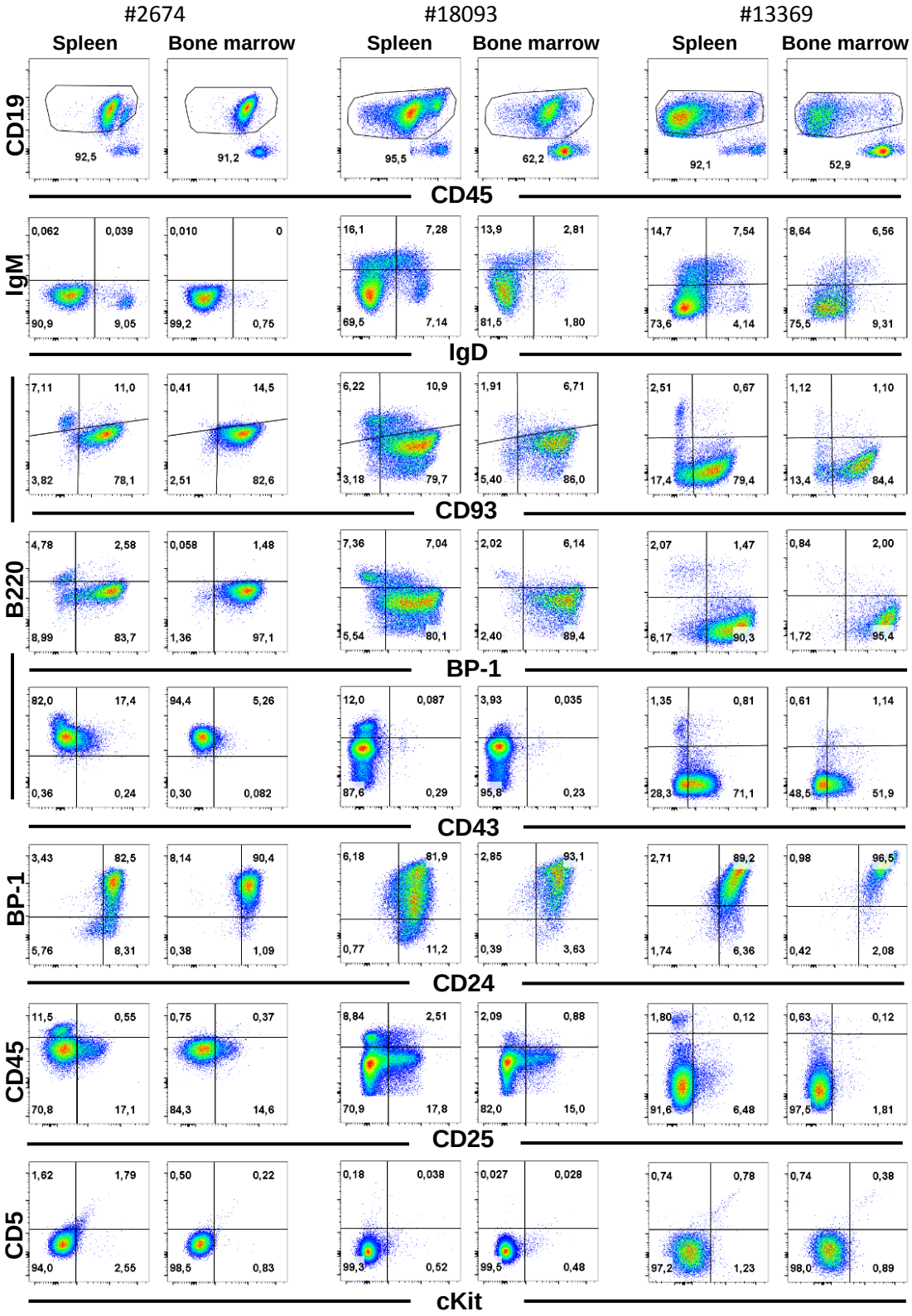


Figure 12 – FACS analysis of leukemic phenotype of CD2Cre^{pos}IL7R$^{wt/fl}$ mice. Representative dot plots of the organs spleen and bone marrow from three mice with the genotype CD2Cre^{pos}IL7R$^{wt/fl}$ that developed B cell leukemia. In a horizontal view, the first line of dot plots shows the gate CD19/CD45 and the remaining plots below are gated CD19+. Notably, the leukemias have a similar phenotype in the gate IgM/IgD being all double negative and also presenting precursor marker CD93+.

Table 1 - Overall characterization of leukemias developed by CD2^{pos}IL7R$^{wt/fl}$ mice by FACS. B cell specific stainings were used to characterize leukemia developed by CD2^{pos}IL7R$^{wt/fl}$ mice. A clear heterogeneity is observed among leukemia, although all share common features. Hardy's Classification is also indicated.

Leukemia (age on death)	CD19	CD45	B220	CD43 (S7)	IgM	IgD	CD93	BP-1	CD25	HSA (CD24)	cKit	Develop stage	Hardy Classification
Leukemia 1(56 days)	pos	low	neg	pos	neg	neg	pos	pos	neg	HSA	NA	Late pro-B	C
Leukemia 2 (70 days)	pos	int	int	pos	neg	neg	pos	neg	pos	pos	NA	Early Pro-B	B
Leukemia 3 (70 days)	pos	int	int	pos	neg	neg	pos	pos	pos	positive	pos	Early Pro-B	B
Leukemia 4 (80 days)	pos	int-low	neg	low-neg	neg	neg	pos	pos	low-neg	bright	low-neg	Small Pre-B	D
Leukemia 5 (93 days)	pos	int-low	neg	pos	neg	neg	pos	pos	neg-low	bright	neg-low	Late Pro-B	C
Leukemia 6 (114 days)	pos	int-low	NA	neg	neg	neg	pos	pos	neg-low	bright	neg	Small Pre-B	D
Leukemia 7 (179 days)	pos	int	int-low	pos	neg	neg	low	pos	pos	bright	neg	Large Pre-B	C'
Leukemia 8 (194 days)	pos	int-bright	int	neg	neg	neg	pos	pos	neg-low	bright	NA	Small pre-B	D
Leukemia 9 (301 days)	pos	int-low	int	pos	neg	neg	pos	pos	mostly neg	bright	neg	Large Pre-B	C'
Leukemia 10 (322 days)	pos	int-low	int-neg	pos	neg	neg	pos	pos	neg	pos	NA	Late Pro-B	C
Leukemia 11(341 days)	pos	int	int	neg	neg	neg	pos	pos	neg-low	bright	neg	Small Pre-B	D
Leukemia 12 (93 days)	pos	neg	neg	low	neg	neg	pos	pos	NA	bright	NA	Large to small Pre-B	C'
Leukemia 13 (187 days)	pos	int-low	neg	NA	neg	neg	pos	pos	neg	poa	NA	Pre-B	C to D
Leukemia 14 (65 days)	pos	low-int	int-low	neg	neg	neg	int-low	pos	mostly neg	pos	neg	Late Pro-B to Pre-B	C
Leukemia 15 (143 days)	pos	int- bright	int- bright	pos	neg	neg	pos	pos	pos	pos	neg	Pre B	C' to D

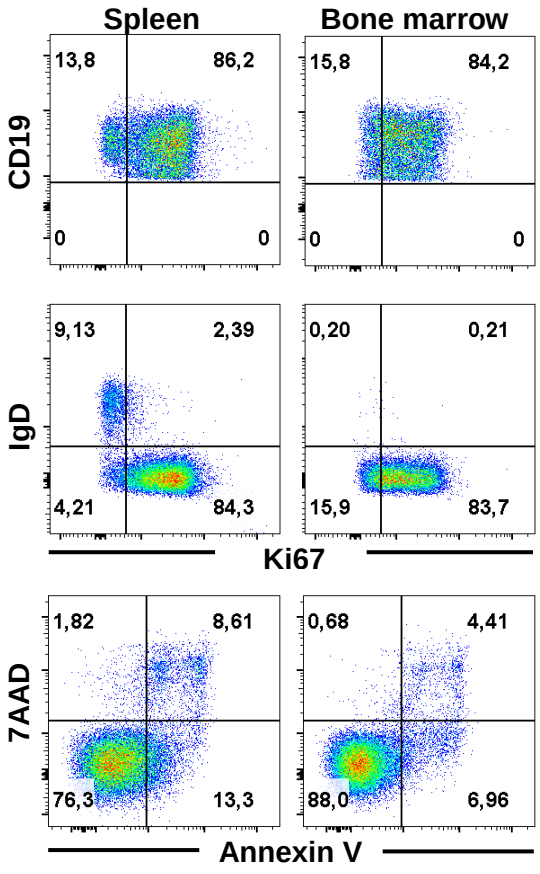


Figure 13 – Proliferation and cell viability assay in leukemic mice. The first four dot plot shows analysis of proliferation assay of leukemic mice spleen and bone marrow. The bottom plots are representative plots from cell viability assay in spleen and bone marrow. The dot plots are gated CD19+.

HOXA9 in CD2Cre^{pos}IL7R$^{wt/fl}$ mice leukemia

Our results demonstrating an impact of *IL7Rα* mutation in B cell development and leukemogenesis led to a need to understand the molecular events underneath the malignant transformation derived from *IL7Rα* mutation in order to fully understand the leukemogenic process and eventually discover novel targets for therapy.

In order to achieve this, we selected a number of bone marrow samples from control, pre-leukemic (animals expressing the mutant allele but without leukemia) and leukemic mice. Extracted mRNA from the sorted pre+proB cells from the bone marrow samples were sent for Next-Generation Sequencing in the laboratory of our collaborators (Charles Mullighan at the St Jude children's Hospital, USA). To our surprise, we found that *Hoxa* family genes, including *Hoxa9*, were significantly downregulated in the leukemic samples as compared to pre-leukemic samples and controls (Figure 14 and Table 2). Interestingly, pre-leukemic samples presented already clear downregulation of *Hoxa9*. With the exception of one case, all leukemias displayed even lower *Hoxa9* levels than pre-leukemic samples.

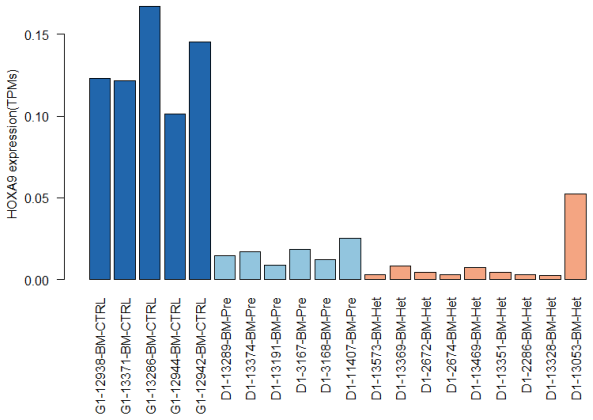


Figure 14 - Next-Generation Sequencing (RNA-sequencing) data of huCD2Cre.IL7Rfxcys mice. Analysis of RNA expression of the bone marrow pre pro-B cells from different mice. The dark blue bars represent different control mice with the genotype CD2Cre^{neg}IL7R$^{wt/fl}$, the light blue bars represent different pre-leukemic pre pro-B cells from CD2Cre^{pos}IL7R$^{wt/fl}$ mice and last, the orange color bars represent leukemic cells from CD2Cre^{pos}IL7R$^{wt/fl}$ mice. HOXA9 expression is higher in control mice, decreasing in pre-leukemic mice and presents even lower expression in leukemic mice cells, except for the #13053 mice leukemic cells.

Table 2 – Hoxa mRNA data from Next-Generation Sequencing (RNA-sequencing) of huCD2Cre.IL7Rfxcys mice. Fold change and adjusted p-value between expressions of mRNA from different Hoxa transcripts comparing control and pre-leukemic mice (the light blue columns) and control and heterozygous $CD2Cre^{pos}IL7R^{wt/fl}$ mice (the orange columns).

	Fold change	Adjusted P-value	Fold change	Adjusted P-value
Gene	BM_PrevsCTR_log2FC	BM_PrevsCTR_padj	BM_HetvsCTR_log2FC	BM_HetvsCTR_padj
The hox related				
Ctcf	0,057239758	0,938042018	-0,208008897	0,227549293
Klf4	**-1,042251983**	**0,874312545**	**-3,653852417**	**0,003307094**
Meis1	**-1,277960234**	**0,874312545**	**-4,239020762**	**0,000671141**
Hoxa1	-0,230863741	0,922793607	-0,232961471	0,612549681
Hoxa2	**-1,772143752**	**0,092747693**	**-1,591706126**	**0,001693832**
Hoxaas2	**-2,451621273**	**0,022608508**	**-2,330184136**	**0,000308662**
Hoxaas3	**-1,20834471**	**0,257426458**	**-2,210435723**	**4,17E-06**
Hoxa4	-0,890351376	0,874312545	**-3,831106192**	**1,36E-06**
Hoxa5	**-1,456814155**	**0,513572026**	**-2,197885261**	**0,000112509**
Hoxa6	**-2,923516536**	**0,000335756**	**-4,385942734**	**1,16E-08**
Hoxa7	**-2,43061476**	**0,036829593**	**-3,520994887**	**5,39E-06**
Hoxa9	**-2,689678481**	**0,207857889**	**-4,992829198**	**2,40E-06**
Hoxa10	-0,759427552	0,874312545	-0,578989926	0,143986284
Hoxa11os	0,191549948	0,874312545	0,371987575	0,143986284
Hoxa11	-0,272835671	0,912432038	-0,092398044	0,850050665
Hoxa13	-0,284822637	0,922827504	0,616357136	0,255875057

Overexpressing HOXA9 in CD2Cre^{pos}IL7R$^{wt/fl}$ mouse B-ALL

As reported in the Introduction, *HOXA* genes were found to be involved in IL-7R/JAK-driven leukemia, particularly in T-ALL[99]. In contrast, in B-ALL studies are discrepant, with some reporting *HOXA* gene upregulation[107] and others the opposite[22]. As such we next sought to clarify the functional relevance of our results from transcriptome analysis and determine whether *Hoxa9* downregulation may contribute to the development of *IL7R* mutated B cell leukemia.

In order to achieve this goal, different leukemic samples from our mouse B-ALL model were transduced with either a Hoxa9 virus (containing HOXA9-MSCV-short plasmid) or an Empty virus/control (containing MSCV-IRES-GFP plasmid). The transduced leukemic spleen cells were sorted for GFP expression, when needed, 3 days after transduction. For the *in vitro* experiments cells were maintained in culture with the conditions described in the Methods section.

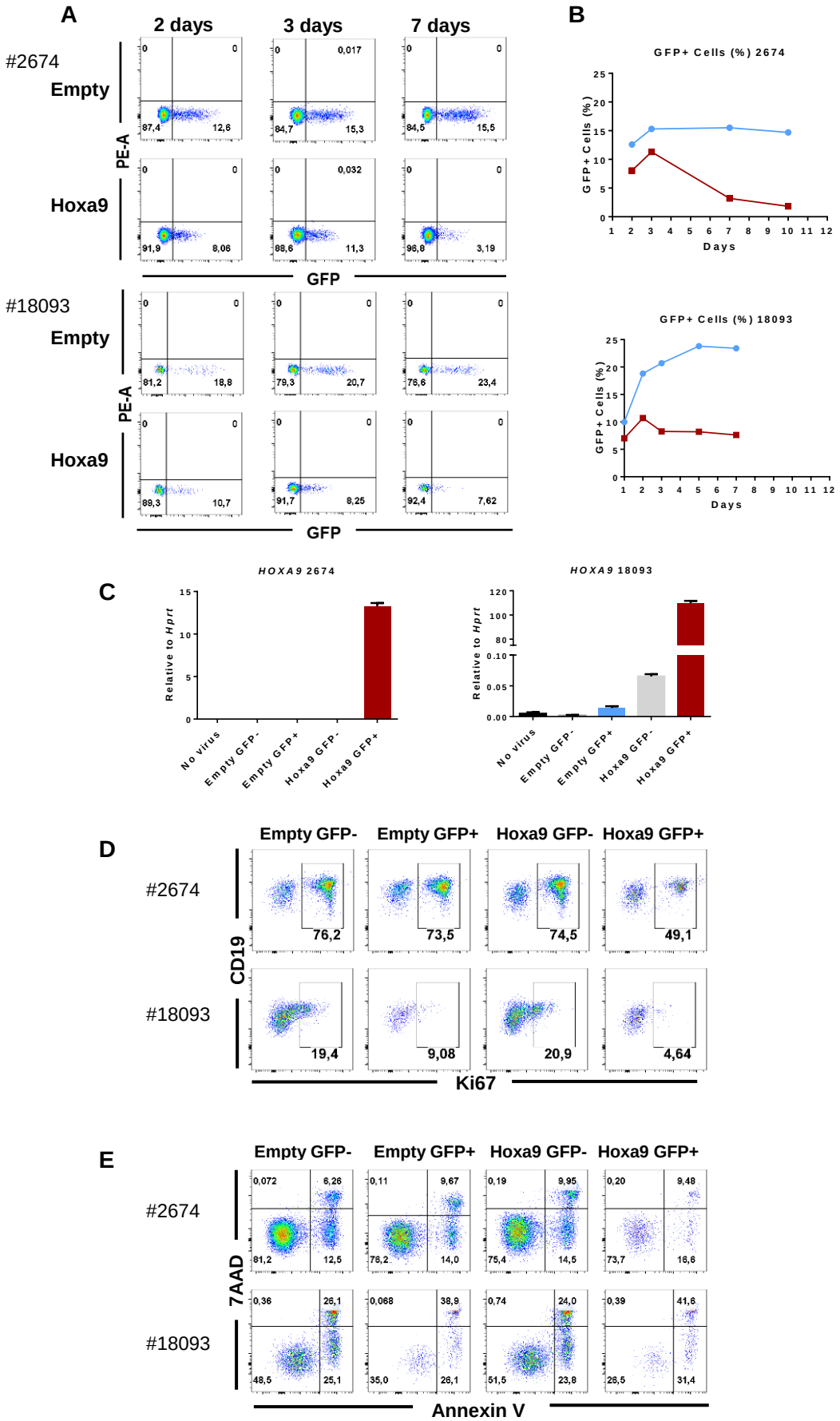
Proliferation differences in leukemic cells overexpressing HOXA9

We chose two leukemias that had similar phenotype for these studies, #2674 and #18093, both of which were CD19+CD45+, CD93+, BP-1+ and double negative IgM-IgD-. Spleen cells from these CD2Cre^{pos}IL7R$^{wt/fl}$ leukemias were transduced with Hoxa9 or Empty virus and were placed in culture, monitoring GFP expression by flow cytometry at different time points to evaluate the percentage of transduced cells. In both cases, cells transduced with Empty virus displayed higher frequency of GFP+ cells throughout time than those transduced with Hoxa9 virus (Figures 15A and 15B). These data suggest that cells overexpressing *HOXA9* have a growth disadvantage.

In order to confirm that GFP expression reflected overexpression of the *HOXA9* gene, we sorted the Hoxa9 virus- and Empty virus-transduced cells 3 days after transduction into GFP negative and positive fractions and evaluated *HOXA9* transcript levels by quantitative PCR. As expected, both qPCR graphs (Figure 15C) show a higher expression of *HOXA9* gene in the effectively cells transduced with Hoxa9 virus (Hoxa9 GFP+ cells) and very low or null expression of this gene in non-transduced leukemic cells.

In order to understand whether lower GFP expression levels throughout the culture time were the reflex of higher mortality or decreased proliferation rates, sorted GFP+ and GFP- cells were stained with a mix of antibodies for B cells and a marker of proliferation, Ki67. Transduced leukemic cells from the two animals were sorted, cultured in the conditions described in the Methods section in 96-well round bottom plates and analyzed 2 days after. As shown for sample #2674, Hoxa9 GFP+ cells displayed clearly lower levels of Ki67 as compared to Hoxa9 GFP- or Empty virus (irrespective of being GFP+ or GFP-) cells (Figure 15D). The other leukemia (#18093), appeared to be less proliferative in general but revealed a similar, although less obvious, tendency for HOXA9-expressing cells to display lower Ki67 expression (Figure 15D).

Next, to evaluate the impact of HOXA9 on cell viability, sorted cells were stained with a mix of B cell specific antibodies and 7AAD and Annexin V. In this analysis we observed very little differences in cells from leukemia sample #2674, suggesting that in this leukemia, most effects of *HOXA9* forced expression were due to a negative impact on proliferation, rather than on viability. In contrast, cells from leukemia #18093 seemed to display decreased viability upon *HOXA9* overexpression (Figure 15E). Taken together, results from these experiments demonstrate a negative impact of *HOXA9* overexpression in both leukemias tested, by affecting leukemia cell proliferation and/or viability.


A
2 days
3 days
7 days
#2674
Empty
Hoxa9
PE-A
GFP
0
0
87,4
12,6
0
0,017
84,7
15,3
0
0
84,5
15,5
0
0
91,9
8,06
0
0,032
88,6
11,3
0
0
96,8
3,19
#18093
Empty
Hoxa9
PE-A
GFP
0
0
81,2
18,8
0
0
79,3
20,7
0
0
76,6
23,4
0
0
89,3
10,7
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0
91,7
8,25
0
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92,4
7,62
B
GFP+ Cells (%) 2674
GFP+ Cells (%)
Days
GFP+ Cells (%) 18093
GFP+ Cells (%)
Days
C
HOXA9 2674
Relative to Hprt
No virus
Empty GFP-
Empty GFP+
Hoxa9 GFP-
Hoxa9 GFP+
HOXA9 18093
Relative to Hprt
No virus
Empty GFP-
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Hoxa9 GFP-
Hoxa9 GFP+
D
Empty GFP-
Empty GFP+
Hoxa9 GFP-
Hoxa9 GFP+
#2674
#18093
CD19
Ki67
76,2
73,5
74,5
49,1
19,4
9,08
20,9
4,64
E
Empty GFP-
Empty GFP+
Hoxa9 GFP-
Hoxa9 GFP+
#2674
#18093
7AAD
Annexin V
0,072
6,26
81,2
12,5
0,11
9,67
76,2
14,0
0,19
9,95
75,4
14,5
0,20
9,48
73,7
16,6
0,36
26,1
48,5
25,1
0,068
38,9
35,0
26,1
0,74
24,0
51,5
23,8
0,39
41,6
26,5
31,4

Figure 15 - FACS analysis of the CD2^{pos}IL7R$^{wt/fl}$ leukemic transduced cells *in vitro* and Hoxa9 expression quantification. A) Dot plots show analysis of the GFP expression on the transduced cells with Hoxa9 virus versus Empty virus that were put in culture after transduction, in three different timepoints 2, 3 and 7 days after the transduction. The dot plots are gated in live cells. The first set of plots is from 2674 CD2^{pos}IL7R$^{wt/fl}$ mice leukemic cells and the second from 18093. **B)** Scatter plot graphs show percentages of GFP positive cells in the cells transduced with the Empty virus (blue color) and with Hoxa9 virus (red color) in the different timepoints analyzed. In the 2674 leukemic cells it was analyzed at 2, 3, 7 and 10 days after transduction, while in the 18093 leukemic cells it was analyzed at 1, 2, 3, 5 and 7 days. **C)** Quantitative RT-PCR analysis of the sorted cells of each transduced leukemic cells to empty GFP- and GFP+ cells and Hoxa9 GFP- and GFP+ cells and non-transduced (no virus). The cells were sorted 3 days after each transduction. Expression of the Hoxa9 was evaluated using its specific Taqman probes. Results are shown as relative to *Hprt* levels in the same sample. Hoxa9 GFP positive cells shown higher expression of Hoxa9 DNA, as expected. Bars are average values with SD. **D)** Analysis of proliferation with the Ki67 marker, top plots shows analysis of the 2674 transduced cells and the bottom the 18093 transduced cells. Plots are gated CD19+CD45+. **E)** Cell viability assay of each transduced cells with Annexin V and 7AAD, top plots shows analysis of the 2674 transduced cells and the bottom the 18093 transduced cells.

In vivo impact of *HOXA9* overexpression in leukemic cells

In order to explore the impact of *HOXA9* overexpression in an *in vivo* environment we transduced #2674 spleen leukemic cells, sorted GFP+ cells 3 days post-transduction and transferred them *i.v.* into Balb/c $Rag^{-/-}$ γc $^{-/-}$mice. Sorted Hoxa9 GFP+ and Empty GFP+ cells (1.5 x 10^4 cells) were injected on each mouse (5 mice per group). Mice were weighed and blood samples were collected and analyzed 7 days after transfer (Figure 16A). We found a clear difference in the percentage of CD19+CD45+ cells in the blood of the mice that received Hoxa9 GFP+ cells with lower percentage than the Emtpy transduced cells. Within CD19+CD45+ cells, Hoxa9 virus transduced cells hosts presented halved the % of CD19+GFP+ cells comparing to Empty virus transduced cells hosts (Figure 16A).

Starting 10 days after transfer, host mice began to develop signs of leukemia, eventually reaching humane endpoint (20% weight loss, hind leg paralysis, and ruff fur appearance) and were humanely euthanized. Flow cytometry analysis of cells collected from the spleen showed similar infiltration in both groups of animals (Figure 16B). However, the percentage of GFP+ cells was evidently lower in the mice transplanted with HOXA9-transduced cells (Figure 16B). Moreover, animals receiving Hoxa9 virus transduced leukemic cells survived longer than the recipients of Empty virus-transduced cells (Figure 16C). These results demonstrate that *HOXA9* overexpression delays leukemia expansion in mutant IL7R-driven B-ALL.

As animals receiving the Hoxa9 virus transduced cells were also eventually developing leukemia, it was important to determine whether selection for lower HOXA9 expressing cells was occurring, thus leading to mortality driven by low- or non-HOXA9 expressing cells *in vivo*. In order to try and answer this question, we collected cells from the spleen of animals at the end of the experiment. We found that, while no changes in *HOXA9* expression occurred in the Empty virus transduced cells, progeny of Hoxa9 virus transduced cells recovered at endpoint of the experiment expressed considerably lower levels of HOXA9 than when transferred (Figure 16D). Interestingly, we also found that, within the GFP+ population, *HOXA9* levels were lower in recovered cells from the spleen than in the cells that were initially transferred (Figure 16D).

Overall, our data suggest that IL-7R mutant leukemic cells expressing higher levels of *HOXA9 in vivo* have a selective disadvantage and tend to be either eliminated or to downregulate *HOXA9*.

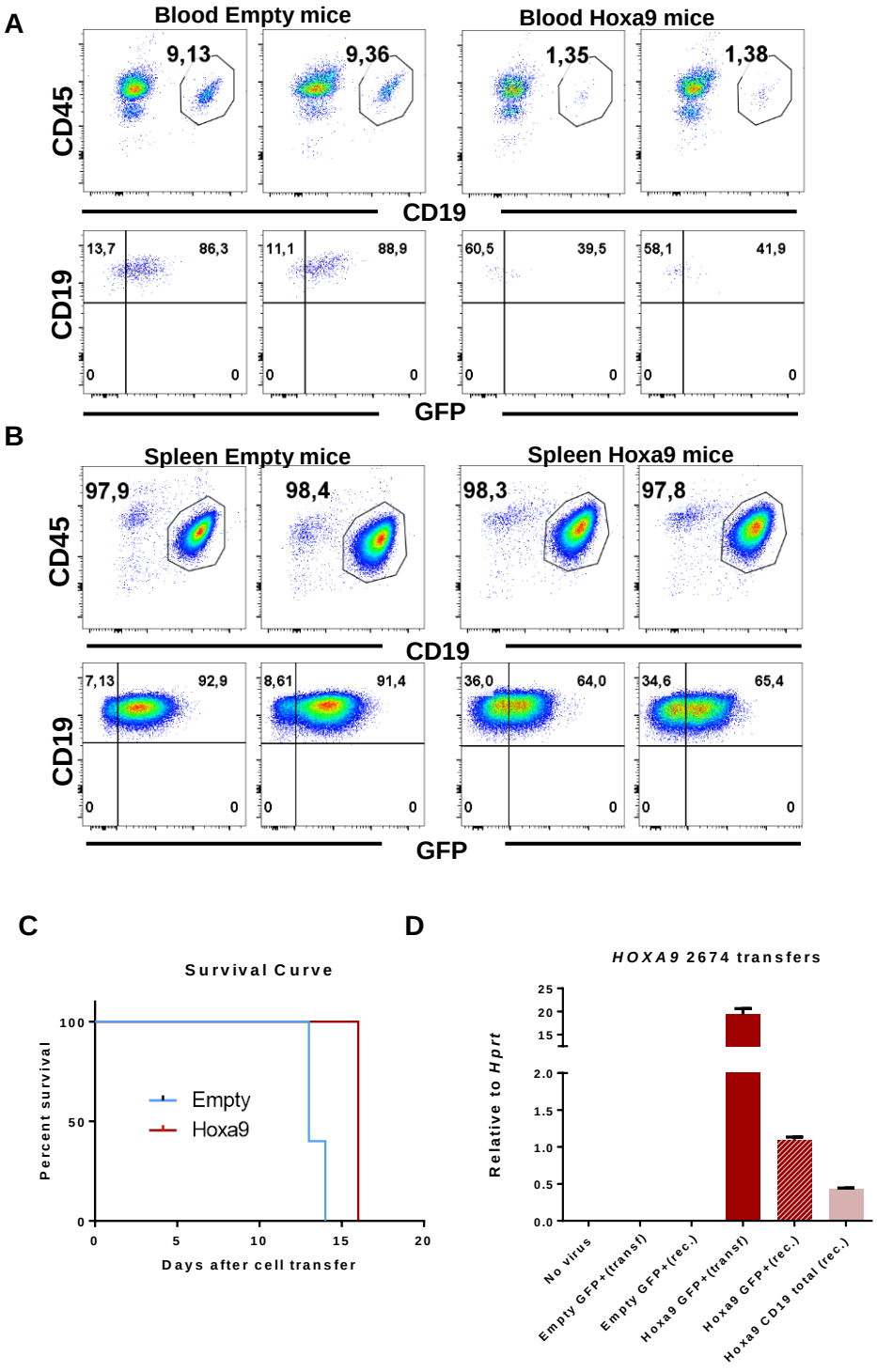

Figure 16 – Analysis of the mice injected with transduced cells. A) Dot plots show representative analysis of blood samples from two mice from each condition, taken 7 days after transfer of cells. **B)** Representative dot plots of spleen from 2 mice transferred with Empty virus transduced cells and Hoxa9 virus transduced cells. Note that these mice are the same chosen to represent analysis of blood samples (dot plots above). **C)** The 5 mice of each condition got sick in different timepoints and were sacrificed leading to a survival curve that shows that Hoxa9 mice were sacrificed later. P-value is 0.0023 **D)** Confirmation of Hoxa9 expression before transferring the cells and after, in the spleen of the mice. Results are shown as relative to *Hprt* levels in the same sample. Bars are average values with SD.

Epigenetic regulation of *Hoxa9*

As mentioned in the Introduction, there are several studies exploring the mechanistic features of regulation of the *HOX* genes. It was important to understand which mechanisms may be responsible for *Hoxa9* downregulation in CD2Cre^{pos}IL7R$^{wt/fl}$ mice B-ALL. In particular, we were interested in evaluating the possibility of epigenetic regulation of *Hoxa9*, thus we analyzed the modulation of *Hoxa9* expression upon activity of epigenetic inhibitors. We treated #2674 leukemia cells with a histone deacetylase inhibitor, SAHA (vorinostat), and DNA methyl transferase inhibitor, 5-Azacytidine, and analyzed the impact on *Hoxa9* expression. SAHA was used at 3 different concentrations (0 µM, 1 µM and 2.5 µM) and cultured cells were collected for qPCR analysis 8h later, whereas 5-Azacytidine was used at 25 nM, 250 nM, and 2.5 µM and cells were analyzed at 48h and 72h. Preliminary results with 5-Azacytidine did not indicate a clear modulation of *Hoxa9* levels (not shown). In contrast, treatment with SAHA led to HOXA9 upregulation at 1 µM, an effect that was no longer observed at 2.5 µM (Figure 17). P21, which is known to be upregulated by treatment with HDAC inhibitors, was used as positive control (Figure 17). Although these results are preliminary, they suggest that *Hoxa9* is downregulated in leukemia cells, at least in part, via histone-related epigenetic silencing.

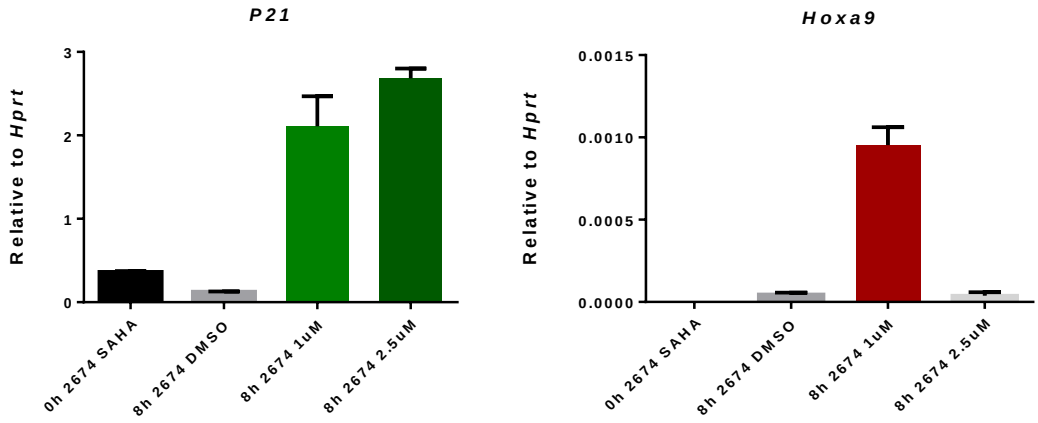

Figure 17 – Quantitative RT-PCR analysis of epigenetic regulators. The graphs represent the analysis of *P21* positive control gene and *Hoxa9* expression in the cells treated with SAHA, a histone deacetylase inhibitor, in different concentrations before treatment (0h) and after (8h). Results are shown as relative to *Hprt* levels in the same sample. Bars are average values with SD.

Discussion and future directions

ALL is an aggressive hematological malignancy that arises from transformation of B- or T- lymphoid progenitors[18]. Several subtypes of B-ALL are documented and although there have been major improvements in treatment outcome throughout the years, high risk B-ALL subtypes such as Ph-like leukemia, require more research to discover novel, more efficient therapies. Remarkably, in Ph-like B-ALL IL-7R insertions or deletions have been identified[27], including gain-of-function mutations[108] of the same kind we described in T-ALL[39].

Due to the relevance of IL-7 and its receptor in lymphopoiesis there are several studies characterizing this signaling axis and exploring the effects of IL-7/IL-7R modulation in lymphocyte development[29,41–47]. In leukemogenesis most of these studies relate to T-ALL and, in some cases, demonstrate that aberrant IL-7/IL-7R-mediated signaling can contribute to leukemia development and maintenance[60,63,65,66,69,70,109–111]. However, the mouse models generated to investigate the role of aberrant signaling IL-7/IL-7R display obvious caveats. For instance, in transgenic mice the expression is not physiologically controlled, being placed under a strong promoter[109,111], thus bypassing the stringent control of IL-7R expression that exists throughout lymphocyte development (Figure 2). Alternative strategies, relying on the transduction of thymocytes or hematopoietic precursors[69,70], additionally require conditioning of the host, therefore neglecting the relevant role of the microenvironment in disease progression, and are limited in the capacity to target the genetic alterations to specific lineages.

The Cre-LoxP system is a powerful tool that has been used to study molecular genetics. This system, as explained in the Introduction, allows the generation of tissue-specific and inducible knock-outs and knock-ins and thereby permits a precise control over the location and timing of gene expression. To circumvent the lack of specificity of the studies previously mentioned, in João Barata's laboratory we used the Cre-LoxP system to generate a mouse model by crossing IL-7Rfxcys mouse with CD2CRE, resulting in a line with conditional knock-in of mutated IL-7Rα in which Cre is under the control of the CD2 promoter. Since the CD2 promoter is expressed from the common lymphoid progenitor stage, subsequent stages of the B and T cell will be affected[72]. Recombination will occur when the Cre recombinase is active (i.e. in all cells that express CD2) but expression of the mutated IL-7Rα will only happen in cells that physiologically express the receptor (since the knock-in approach means the mutant *IL7Rα* will be regulated by the endogenous *Il7r* promoter and other regulatory elements -enhancers,

superenhancers, etc). This method is crucial to better mimic a mutated-*IL7R*-induced leukemia, allowing a correct characterization of this mutation's impact and its use in the discovery of molecular mechanisms that may lead to novel therapeutic avenues. This being said, two main aspects must be kept in mind when interpreting and extrapolating results from the model: first, when comparing to normal leukemogenesis the incidence of disease must be expected to be overestimated due to the extremely high fraction of precursors affected in the model (near >90% due to Cre efficiency) compared to real-life, where a mutation in *IL7R* should be a rare event happening in a single cell. Second, as IL-7R-mediated signals intervene from early on in development, including in B vs T lineage choices[44], precursors reaching further stages of development have already potentially been target of selection based on IL-7R mediated signals and thus do not mimic the mutation arising at a precise stage in development. Regarding this latter caveat, it is precisely the power of the model to use different Cre promoter lines to target the mutation appearance to specific developmental stages and the reason why we used the CD2Cre to study the impact of the mutation with a focus on the B cell lineage.

Firstly, we addressed the impact of the IL-7Rα mutation in normal lymphocyte development, by characterizing main lymphocyte subpopulations in progeny of the experimental crossings. We noticed an increase in the fraction of B cells in *IL7R* mutated mice in all the time points analyzed, in comparison to control mice (Figure 8). Although this could be due both to a decrease in T cell numbers or to an increase of B cell numbers, our deeper phenotypical characterization identified anomalies in the B cell compartment (Figure 9), suggesting that indeed an impact on B-lineage cells is dominant. Although this does not eliminate a role in T lineage cells, activation marker analysis on the T cells displayed no obvious impacts suggesting a limited impact. It can also occur that the mutation promotes B cell lineage commitment and thus decreases T cell percentage (Figure 8). In this regard it is interesting that the highest difference of the percentage of T cells between the control and the experimental mice is at 6 weeks, relatively to the later time points, suggesting the possibility of an early effect in promoting the development towards B cell lineage which is lost later on.

Since both B and T cells are originated from the same lymphoid progenitors and IL-7/IL-7R signaling is, as described in the Introduction, essential for both lineages, why do animals bearing the *IL7Rα* mutation present an increase in B cells? This suggests that there are other additional molecular events relevant in early T cell development and that

IL-7R mediated signals may be more important in more advanced developmental stages of the T cell lineage.

Studies in mouse lymphoid development suggest that T cell lineage commitment is not likely to be IL-7-dependent, being more dependent of Notch signaling[112], fact that could explain why the mutations in *IL7R* at the level of the common lymphoid progenitors (using the CD2 promoter) impact more on B cells. Additionally, in IL-7RCRE knock-in mice early T cells were found lacking the *Il-7r* mRNA, suggesting that earliest T cell development is perhaps not IL-7R dependent[113]. In addition to these findings, common lymphoid progenitors in *Il7* deficient mice showed impaired B-lineage potential as well as low levels of EBF and PAX5[114], known to be fundamental for B-lineage commitment. Overall, these observations suggest that *IL7Rα* has a more critical role for early B cell than T cell development and indicate that the CD2Cre driven model is best for the study of B-lineage impacts, our main focus. Studies directed to impacts on the T-lineage are ongoing in the laboratory using other Cre lines but were not the focus of this thesis.

After demonstrating that mutant *IL7R* affects normal lymphocyte development with an increase in B cells, the next step was to better characterize these cells using immature/precursor B cell lineage markers. An increased percentage of B cell precursors (CD93+) in the blood of IL-7Rmutated animals was found compared with control mice (Figure 9) suggesting to us that the mutant IL-7R impacts on the precursor B cells leading to their increase and extravasation from the bone marrow, in accordance with reports in IL-7 transgenic animals[115]. This was found in all animals expressing the mutation and thus represents a phenotypic consequence of the mutation. Importantly, some of these animals showed a pronounced increase over time of precursor IgM-IgD- cells, leading to disease and reaching humane endpoints (Figure 10 and 11). This disease could be characterized as a *bona fide* B-ALL, presenting all the hallmarks of leukemia, including the capacity to transfer the disease (not shown in this document, but obtained in the laboratory). Regarding the leukemia phenotype in Figure 12 and Table 1 it is observed a heterogeneous leukemic phenotype, even if presenting many common features, a result that mimics the one found in human disease as well. Overall these results support our hypothesis that mutant *IL7R* has a tumorigenic role in B-cell development.

Placing our results in context with previously reported studies, our observations show contrary results to studies in which *Il7* transgenic mice showed the development of B-lymphomas[109,111] although in another study the development of B cell leukemia[115] was found. Treanor and colleagues showed that thymocytes with constitutively active IL-7R

mutant receptors developed early T-ALL[69], whereas we found very few T cell abnormalities. The experimental strategy, however, is quite different, as the Treanor *et al* study relies not only in transforming already committed T cell precursors (Double Negative thymocytes) but also on the use of a favorable genetic background (*Arf*$^{-/-}$). More comparable are the observations by Yokoyama and colleagues[70] in a study where, in accordance to our results, the transforming potential of the mutant IL-7R starting from the common lymphoid progenitors was demonstrated. However, in this last study, animals developed mature "B-cell ALL"/lymphoma, while our model generated B-cell precursor ALL. Again, the experimental strategy used explains the differences: Yokoyama *et al* used the transduction of precursors rather than a knock-in strategy, thus forcing the expression of the mutated IL-7R also in mature B cells that physiologically do not express IL-7R. In accordance there are no descriptions of IL-7R mutations in human lymphoma, while in B-ALL, especially in the high risk subtype Ph-like B-ALL, IL-7Rα activating mutations were found[116]. Taking into account these considerations, our model makes an outstanding candidate to investigate the pathogenesis of gain-of-function mutant IL-7R-induced B-ALL with especial interest in high risk subtypes.

It is important to note that the less than 100% penetrance of leukemia and the time needed for development of the disease are indicatives of the probable occurrence of mandatory secondary genetic events in this setting. We can thus study our leukemias and compare them with pre-leukemic B cell precursors in order to uncover novel mechanistic aspects of mutant IL-7R-driven leukemia. This is the second topic of this work: understand the genetic features underneath the leukemogenic potential of IL-7Rα gain-of-function mutation. Transcriptome analysis was performed in the laboratory and it was discovered that in animals expressing the IL-7R mutation there is downregulation of several *Hoxa* genes, especially, *Hoxa9* (Figure 14 and Table 2).

Taking into consideration its vital role in hematopoiesis[86] and the known involvement of high levels of *HOXA9* in acute leukemia[97–99], it was intriguing that *HOXA9* levels were down in our mice B-ALLs. This fact is even more interesting when we realized that in at least some B-ALL cases *HOXA9* levels are also down[22,103]. We thus hypothesized that *HOXA9* overexpression in leukemic cells would counteract the leukemogenic role of aberrant IL-7/IL-7R signaling and impact on leukemic cell survival.

To overexpress the *HOXA9* gene we used viral transduction in primary leukemic cells from our model. Upon the transduction we analyzed by flow cytometry several time points and realized that the percentage of cells transduced with *HOXA9* decreased

throughout time, whereas the cells transduced with the Empty virus either increased or maintained a constant percentage (Figure 15A and B). This analysis was done using two different leukemic samples and the same tendency was observed. This suggests that HOXA9 may have a tumor suppressor role, either negatively impacting proliferation through repressing genes involved in cell cycle or promoting apoptosis by activating genes related to cell death. In order to further explore this effect, cell viability and proliferation assays on the sorted cells (GFP negative and GFP positive) were performed.

Ki67 analysis comparing cells transduced with the Empty virus (Empty GFP+) and Hoxa9 virus transduced GFP+ cells showed that *HOXA9* impairs proliferation (Figure 15D). Regarding the cell viability assays showed in Figure 15E, upon transduction with the Hoxa9 virus, only the cells from #18093 mice showed a decrease in the percentage of viable cells within the cells that were positively transduced with the *HOXA9* gene (Hoxa9 GFP positive cells). Important to mention that we had very few resultant sorted GFP+ cells comparing to GFP- cells due to the low transduction efficiency, thus we may need to repeat this analysis. Interestingly, these #18093 B-leukemia Hoxa9 virus GFP+ transduced cells presented higher expression of *HOXA9* upon transduction (Figure 15C) comparing to transduced leukemia #2674, suggesting a positive correlation between decreased viability and proliferation and the high expression of this transcription factor. Thus, upon IL-7/IL-7R aberrant signaling, *HOXA9* can possess a contrary effect from what is described so far in T-ALL. This constitutes preliminary evidence for the fact that *HOXA9* can act as a tumor suppressor and have anti-leukemic potential in B-ALL.

After analyzing the *in vitro* experiments above mentioned we explored the *HOXA9* mechanism *in vivo.* Equal numbers of Hoxa9 virus and Empty virus transduced sorted GFP positive cells were transferred to $Rag^{-/-}$ $\gamma c^{-/-}$ hosts. Blood samples collected from these mice (Figure 16A) showed that the anti-proliferative effect of *HOXA9* overexpression may be also occurring *in vivo*, since the leukemia burden observed at the same time point in mice that received the Hoxa9 virus transduced cells was weaker than from the ones that received the Empty virus transduced cells.

After sacrifice we noticed that cells found in organs of animals receiving Hoxa9 virus transduced cells still presented GFP positive cells in relative high expression (although lower than observed in Empty virus transduced cells) (Figure 16B), which can be translated to the differential survival of *HOXA9* overexpressing leukemia hosts since they survived longer than mice transferred with Empty virus transduced cells as it is

observed in the survival curve in Figure 16C, demonstrating a tumor suppressive effect of *HOXA9* overexpression in the proliferation of leukemic cells.

Our results contradict what is widely described so far in which HOXA9 overexpression induces leukemia progression. However, a few studies are in line with our observations. For instance, low levels of *HOXA9*, *HOXA10* and miR-196b were observed in B-ALL cases without MLL-translocation[103] and also *HOXA9* and *HOXA5* were found not to be expressed in ALL samples[117].

Importantly, it is still not fully understood how exactly the *HOXA9* gene is regulated but there are several studies exploring the activity of epigenetic regulators, such as histone deacetylase inhibitors[118], in the modulating *HOX* gene expression. For that reason and to try to unveil the *HOXA9* regulation mechanisms, we performed assays using a histone deacetylase inhibitor to investigate modulation of *Hoxa9* expression in our mice B-leukemias (Figure 17). In our results, cells treated with SAHA showed an increase of *Hoxa9* expression 8h after treatment with 1 µM of SAHA, along with an increase of the positive control gene, P21, when compared with cells that had not been treated with this inhibitor. This result is in accordance to what was expected, since the use of histone deacetylase inhibitors increases histone acetylation, resulting in DNA with a more open chromatin favoring transcription[119]. Our preliminary results suggest that HOXA9 may be downregulated, at least to some extent, upon epigenetic silencing in B-ALL cells. Evidently, we need to validate these results in further samples. Whether transcriptional repression or whether genes loss or other inactivating events may also occur is still an open question. Another aspect left to explore is the possibility of a role for IL-7R driven signals in this process. Indeed, the lower levels of HOXA9 throughout B cell development seem to correlate with the intervention of IL-7R-mediated signals.

Moreover, given the higher expression of *Hoxa9* in pre-leukemic cells when compared to the leukemia cells, a future experiment proposed is to use the opposite strategy, silencing the *Hoxa9* gene in those cells with the goal of accelerating the leukemia phenotype in order to reveal if *Hoxa9* is necessary to initiate leukemia transformation in pre-leukemic cells. This experiment may be difficult due to the fact that pre-leukemic cells may not have the sufficient capability to grow *in vitro*.

In addition, it would be interesting to evaluate the impact of *HOXA9* retroviral transduction in B-ALL patient samples that present downregulation of this gene in order to corroborate our findings in the mouse B-ALLs.

Overall, our mouse model induces B-ALL with many common features with human B-ALL, making it an excellent model to investigate the IL-7R related characteristics of this leukemia and to unveil novel targeted therapies with the purpose of improving the quality of life of the patients with minimized side effects and increased survival rates. As future directions it would be interesting to see what other molecular events (apart from Hoxa-mediated) collaborate in B-ALL. Also worth exploring will be the understanding of the B versus T lineage ALL conundrum: why do our animals present so little T cell phenotype? Other future and ongoing avenues arise from the analysis of a large number of leukemias in the model. We noticed a downregulation of the marker CD45 in the leukemias. It may be an interesting feature to explore. For instance, leukemia aggressiveness could be negatively correlated with the levels expression of CD45 marker on cell surface. Interestingly, the CD45 protein is a phosphatase capable of downmodulating the levels of IL-7R signals.

Regarding IL-7/IL-7R signaling molecular characteristics, our laboratory already performed western blot analysis to identify signaling landscapes and showed clear differences between the control and leukemic mice that demonstrated upregulation of IL-7R signaling in the leukemic cells. Relatively to the second topic of this work, it is also necessary to analyze the signaling pathways, through western blot, which are downstream from *HOXA9* overexpression. We hypothesize that HOXA9 downregulates IL-7R-mediated signaling.

Conclusion

Our results suggest that our mouse model can accurately represent a mutated *IL7R*-induced B-ALL - in contrast to other mouse models developed before. This opens doors to better understand the pathophysiology of the disease as well as IL-7/IL-7R signaling in leukemia context. This model can also help discover novel therapies regarding high risk B-ALL subtypes that are linked to IL-7R aberrant signaling. Notably, we also provide evidence that *HOXA9* (and possibly other *HOXA* genes) can possess a tumor suppressor role in B-ALL.

Overall, we hope the results presented are a first step into a better understanding of IL-7R-mediated B-ALL that may help shed light into novel therapeutic targets and strategies that will increase patients' quality of life and their life expectancy.

References

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